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ADVANCES IN GENETICS

VOLUME V

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Population Dynamics of Rodents and Other Small Mammals

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I. Introduction

Rodents have distinct advantages as materials for evaluation of certain factors that affect gene exchange in natural populations. Individuals can be easily marked in nature and easily recovered to give information about normal, daily movements, about dispersal movements, about population density and size, and about the life span under natural conditions. Principally because of the economic importance of these animals, the behavior in natural populations of rodents and other small mammals has been studied fairly extensively in the past 15 years. Much of the literature that has accumulated in this time has been primarily concerned with other aspects of population dynamics than those of interest to the student of population genetics. Many of these data are, nevertheless, of great value in estimating actual and potential gene dispersal in natural population units. An attempt is consequently made here to survey this literature and to extract from it the observations that are of interest to the population geneticist. To assist workers in fields other than that of rodent-population dynamics to evaluate the results reported by various workers, it has been deemed necessary, also, to review the methodology of this new and growing area of ecologic investigation.

Studies of social behavior and dynamics in natural populations are important, because the behavior of the individual in nature is a basic factor in gene exchange between parts of a species population. Any tendency for the individual to restrict its activities to a limited area of terrain will tend to restrict the spread of hereditary characters, although the spread will also be affected by such other factors as distance, ecologic barriers, and adverse selection. Any tendency for the individual to disperse from its point of origin before reproducing will facilitate gene dispersal. In any given species, the rate of gene exchange between segments of the species population is probably conditioned by all of these factors. The present discussion is concerned principally with such population phenomena as: (1) localization of the individual, (2) dispersal, (3) pattern of reproductive behavior, (4) life span in nature, and (5) pattern of distribution. All have a bearing on gene exchange and geographic differentiation within natural populations.

II. Methods

The behavior of natural populations of rodents has been studied principally by marking individuals for future recognition when the animals are recaptured. This permits censusing of the population and the measurement of individual movements. Large diurnal species can be

marked so that they can be observed and recognized without the necessity of recapture. This is a desirable technique, as there is little interference with the normal activity of the animals. Its application among rodents is limited, however, because most rodents are small in size and nocturnal in their activity. Various stains and dyes have been used to mark the fur of such mammals as squirrels, woodchucks, and rabbits with broad patterns of contrasting colors. Commercial fur dyes have given the greatest success in terms of permanence and "readability" (Fitzwater, 1943). The chief objection to this method is that, used on prey species, it will increase conspicuousness of the marked individuals and may lead to greater than normal mortality from predation. Colored, plastic ear tags have been used successfully for mammals such as rabbits (Trippensee, 1941), but their use is subject to the same criticism as the dye method. The literature on methods of marking mammals has been reviewed by Manville (1949a).

Methods for censusing small nocturnal rodents and for following their movements require frequent recapture and handling of the marked individuals. Two principal methods have been used. One involves the use of live traps, and the other utilizes nest boxes. The live-trapping method has been used extensively in rodent population studies during the past 15 years. The nest-box technique has had only limited application. In live trapping, the traps are set in a grid pattern over an extensive area. Continued trapping will result in the marking of the resident population, of immigrants, and of maturing, young individuals as they leave the nest. Repeated captures of an individual afford information about the size of the area over which that animal ranges. The live-trapping methods have been described by Chitty (1937), Burt (1940), Blair (1940a, 1941a) and others. One of several types of box traps used in this work has been described by Blair (1941b). The results of such live-trapping work: (1) provide estimates of population density, (2) show any changes in the age-class and sex-class structure of the population, (3) reveal the extent of the reproductive season and provide data for estimating the rate of reproduction, (4) indicate the life span in nature, (5) provide estimates of the size of the home range, and (6) show intra- and inter-specific social relations.

The greatest difficulties of the live-trapping method stem from the fact that both the environment and the activity pattern of the animals are altered considerably by the introduction of a large supply of exotic foods in the traps and by the fact that a considerable part of the normal activity time of the animals is spent in the confines of the traps. Chitty (1937) has pointed out several possible effects of live trapping on rodent populations.

Live trapping unquestionably affects survival and reproductive rates, and it may influence the movements of individuals. The rate of reproduction is affected by the frequent loss of the litter when parturition occurs in a trap or by the occasional loss of a litter in the nest through neglect when the female is in a trap on several successive nights. Reduced opportunity to meet a mate may have some effect, but there is no information on this point. The rate of survival is affected both adversely and favorably by live trapping, and these trends may counterbalance one another in part. Animals in traps are comparatively free from capture by predators, but a predator such as a snake or weasel may sometimes force its way into a trap. Some mortality in traps seems unavoidable, owing to accidents, forced entry by predators, attacks by ants, chilling, overheating, or even drowning during heavy rainfall.

Live trapping may affect the movements of mammals in several ways. The presence of an abundant supply of preferred foods in the traps theoretically might induce transient animals to settle in an area that was being trapped. Rodents also learn to follow the trap lines in search of food (Blair, 1951a). The most serious effect is the restriction on movements, because a mouse usually spends much of each night in a trap and because the probability is greatest that an individual will be caught in the traps nearest to its nest (see Hayne, 1949). Various modifications of technique have been devised to minimize this difficulty (see Blair, 1951a).

The use of nest boxes instead of traps avoids many of the difficulties encountered in live trapping. Nicholson (1941) developed a nest box for rodents and first used this method in studying populations of the semi-arboreal wood mouse (*Peromyscus leucopus*). Howard (1949) used nest boxes in a study of the non-arboreal prairie deer mouse (*P. maniculatus bairdii*). Artificial homes, or nest boxes, are placed in the field, and the mice frequently occupy these in preference to their own nests. This method is particularly useful for studying the amount of inbreeding in populations, because the young can be marked in the nest and while they are still with the parents. The chief disadvantage of the nest-box method is that the introduction of such boxes into an area increases the number of homes and consequently may influence population trends. Howard (1949) states: "Live-trapping indicated that every prairie deer-mouse frequented a nest box if one was available in its home range. Additional mice apparently were not attracted into any of the . . . areas where extra artificial homesites were situated. The presence of the artificial homesites modified some of the minor movements of the mice, particularly with regard to the boundaries of their daily home ranges."

III. Localization of the Individual

That rodents tend to remain in the same small area of terrain once sexual maturity has been reached is one important contribution of rodent-population studies to the problem of the distribution of genetic characters in natural populations. The individual rodent does not wander at random. The reproductive period of life is spent, instead, in an area of limited geographic extent. This behavior obviously tends to restrict the distribution of genetic characters through the geographic range of a population. Three allied phenomena, all bearing on the restriction of gene exchange in a population, have been described from rodent population studies. These are: (1) home-range behavior, (2) territorial behavior, and (3) homing "instinct."

1. *Home-Range Behavior*

The concept of a home range is an old one in vertebrate natural history, but the measurement of home-range size in small nocturnal mammals was not feasible until the development of live-trapping and marking methods. Seton (1909) used the term home range in the sense that "no wild animal roams at random over the country; each has a home region, even if it has not an actual home." Burt (1940) has defined the home range as "that area about its established home which is traversed by the animal in its normal activities of food gathering, mating, and caring for young. Occasional sallies outside the area, perhaps exploratory in nature, should not be considered as in part of the home range." A preferable definition, because of its greater simplicity and greater inclusiveness, would be that the home range is the area over which an individual travels in its normal, daily activities.

The measurement and precise definition of the home range are complicated by the statistical and methodological problems of expressing the sum of an animal's movements in areal units of measure. An estimate of home-range size can be made from the places of capture of a marked individual in live traps. The most distant traps in which the animal is taken determine the limits of the estimated range. The numerous complications encountered in estimating home-range size from such records have been discussed at length by Mohr (1947), Hayne (1949), and others. The principal difficulties are that the intensity of use of various parts of the home range may vary greatly. There may be much use of the area immediately surrounding the homesite, and decreased use away from the home (Southern, 1940; Blair, 1942; Linsdale, 1946; Fitch, 1948a; and others). A home range estimated from peripheral points of capture may contain areas that are seldom, if ever, used; areas of intensive use;

and other, infrequently visited areas. Hayne (1949) has stressed that the probability of capture of an animal in a trap decreases with increased distance from the "center of activity," and he makes the point that "even when dealing only with the trap-revealed range, it seems necessary to accept the edges of the apparent area as shading off on the basis of probability of capture and not as discrete lines of biological validity." This relationship between estimated and actual home range is generally recognized by present-day workers in the field.

The various methods of estimating home-range size from live-trapping data have been compared and criticized by Mohr (1947) and Hayne (1949). The lack of uniformity of treatment makes it difficult to compare the results from different workers, but it does not detract from the biologically important conclusion that a small mammal is usually restricted to a small area of terrain throughout its mature life. The most widely used method takes the maximum distance between points of capture as the diameter of a circle or the major axis of an ellipse, which is assumed to be the animal's home range. The chief objection to this method is that it assumes the home range to be essentially circular in pattern. This method has often been used with scanty data, and, in such a case, the maximum distance of capture may often more nearly approximate the radius than the diameter, even if the home range is nearly circular. Another method gives what Mohr (1947) calls a "minimum home range" estimate. In this case, the outermost points of capture are connected to form a polygon, and it is argued that the home range is known to be at least of this size. Such an estimate is admittedly incorrect unless the unlikely assumption is made that the outermost traps in which the animal is caught are always set at the exact limits of the area of activity. Another method that has been used by a few workers might be called the subjective method, for the investigator draws the limits of the home range where he thinks they ought to be and attempts to exclude from the home range traps in which the animal was not caught. Burt (1943) defends this method by saying that many home ranges probably are somewhat ameboid in outline. Major criticisms are that this method assumes that the animal will be caught throughout its area of activity in a limited period of trapping and that it is too subjective. Two other methods assume that, on a grid-trapping area, the limit of the home range lies, on the average, halfway between the outermost trap in which an animal was caught and the next adjacent trap in which it was not taken. In one application of this method, the animal is assumed to have ranged over each grid square in which it was caught and over each grid square in a line between squares in which it was taken (Haugen, 1942). This method frequently indicates a T-shaped,

L-shaped, or even U-shaped home range. The principal criticism is that this is another minimum estimate, for it assumes that, even in areas of uniform environment, the animal, for example, never crosses between the two tips of a U-shaped range. This method likewise assumes that the animal is caught throughout its home range in a limited period of trapping. Another application of the grid-square method gives a maximum estimate of home-range size. The outer limits of the estimated range are set by connecting points halfway between the outermost traps in which the animal was taken and the adjacent, more distant ones in which it was not caught to form a polygon. This method is entirely mechanical, and any two workers following the same rules and utilizing the same data will arrive at the same estimate of home-range size. This method has been criticized by Hayne (1949) as being too mechanical and as tending to exaggerate the size of the home range by including areas which may not be inhabited by the animal in question. This method has been defended by Blair (1951a). Its objectivity is strongly in its favor, and estimates of home-range size by other methods (tracks and location of home sites and refuges) indicate that it gives a fairly reliable estimate of the area ranged over.

2. *Size of Home Range*

Some estimate of the size of the home range is available for 39 species of rodents, representing 24 genera in 5 families. Home-range estimates are also available for 10 other species representing 7 genera in 4 orders of mammals.

a. *Cricetine Rodents.* The deer mouse (*Peromyscus maniculatus*) was estimated by Blair (1940b) to have a home range of 0.63 $\pm$ 0.04 acre in the case of males and 0.51 $\pm$ 0.04 acre for females in a bluegrass association in southern Michigan. The tendency for the mice to remain in the same area throughout their adult life was illustrated by a female that ranged over 0.49 acre in a month but had a known range of only 0.53 acre over a year. Howard (1949), in the same area, estimated that the deer mouse regularly travels over an area of 5 or 6 acres. He based his estimate on the combined results of nest-box observations and live trapping, by taking the distance of movement from a nest box of residence to the trap in which the mouse was caught as the radius of the home range. The assumption that the nest is usually near the center of the home range is probably not justified, as Howard himself pointed out. Blair's (1940b) estimate, on the other hand, is probably too small, as little was done to offset the tendency of the mice to be caught in the traps nearest to their homesites. The average home range of the deer mouse in bluegrass probably lies between the two estimates.

Murie and Murie (1931), trapping in a woodland population of deer mice in Wyoming, found that most mice were retrapped less than 100 yards from the point of release and concluded that the diameter of the home range was about 100 yards. Murie and Murie (1932) found that, after one year, 5 deer mice were living in the same home ranges they had occupied the previous summer. Storer, Evans, and Palmer (1944), in a forest population of deer mice in California, obtained a minimum home-range estimate of 0.24 acre for males and 0.21 acre for females. Their estimates were probably too low because of the method of plotting home ranges and because there was no manipulation of trapping technique to prevent most captures being in traps nearest to the homesites. Blair (1942), in a forest population of deer mice in northern Michigan, estimated home ranges of 1.39 ± 0.16 acres for females and 2.31 ± 0.27 acres for males. The largest home range of a male comprised 5.64 acres, and the largest for a female was 3.28 acres. The estimated home ranges for the males average significantly larger than for the females. That movement is not at random from the homesite was concluded from the fact that the grid squares in which the mice were most frequently caught were not in the centers of their home ranges.

Blair (1943a), in a deer mouse population of a mesquite desert in southern New Mexico, estimated the average home range of males at 4.66 ± 0.33 acres and the home range of females at 4.10 ± 0.39 acres. The largest home range of a male was 9.92 acres, and that of a female was 8.30 acres. There was no significant difference in the estimated size of the male and female ranges. Month to month variation in the size of the estimated home range was shown by a male that had a range of 3.53 acres in March, 3.22 in April, and 2.71 in May. Another male had monthly ranges of, respectively, 8.00, 5.76, and 5.27 acres, and a female had ranges of 5.43, 5.51, and 6.41 acres. Comparison of the different monthly records of movements of each of these individuals showed that there was some fluctuation in distance of movement, but that the animal remained in the same general area of terrain. From these studies of the deer mouse, it may be concluded that this species usually ranges over less than 5 acres and that the range of the species may be smaller in forest than in grassland habitats.

Beach mice (*Peromyscus polionotus*), studied by Blair (1951a) on Santa Rosa Island, Florida, ranged less widely in areas of dense vegetational cover than in areas of sparse cover, and they ranged less widely in the fall and early winter than they did in the spring. Mice living in the densely grassed, back-beach dunes had average home ranges of 1.97 ± 0.26 acres in fall-winter and 5.76 ± 0.74 in spring. Mice living in

the sparsely vegetated interior of the island had ranges of 6.38 ± 0.81 in fall-winter and 10.66 ± 1.46 in spring. Males and females did not differ significantly in size of home range. Tracking some of the mice in the sand tended to confirm the validity of the estimate of home-range size. Each home range contained an average of about 20 beach-mouse holes, of which an average of about 5 were entered by the mouse, while the others were used by mice with overlapping ranges. The holes utilized changed from time to time, which indicated that the location of a particular nest site has little to do with the location and permanence of a home range. The mice seemed to be attached to an area of familiar terrain rather than to a nest site. Movements within the home range appeared to be principally for feeding or food gathering. Other apparent motivations included exercise, sexual activity or play, and maintenance of nest and refuge holes. Activity of the mice was inhibited by half moonlight or more.

The wood mouse (*Peromyscus leucopus*) was studied by Burt (1940) in small oak-hickory wood lots in southern Michigan. Using the subjective method of plotting home ranges, he estimated the average home range of males to be 0.28 acre and that of females to be 0.21 acre. Nicholson (1941) studied the same species in the same region with nest boxes and found that, of 174 mice taken 2 or more times, 112 were in the same nest box each time, and 62 were in 2 or more boxes. Only 7 adults and 4 juveniles were retaken more than 200 feet away from the point of first capture. He believed that most of the movement when a mouse changes its place of residence seems to be local, that is, within the normal range of the individual. Blair (1940b), working in the same area, found that the wood mice ranged over both forest and grassland and reported an average home range of 0.81 ± 0.15 acre. These mice had about 25% of their range in the forest and about 75% in grassland. Burt's failure to recognize that the mice ranged out from the small wood lots into the grassland probably accounts in part for his small estimate of the home-range size. Blair (1943a), working in a mesquite desert in southern New Mexico, obtained home-range estimates for a few individual wood mice. Four males had an average range of 3.1 acres, with a maximum of 4.2 acres. Two females had ranges of, respectively, 3.5 and 5.5 acres. This suggests that the western, brush-inhabiting race of the wood mouse ranges more widely than does the northeastern, forest-inhabiting race.

The brush mouse (*Peromyscus boylii*) was studied by Storer, Evans, and Palmer (1944) in California. The estimated home range of males averaged 0.27 acre and that of females averaged 0.41 acre. These workers noted that there was much more of a tendency for the brush mice

to wander than for the deer mice to do so. Their estimates of home-range size are probably too low because of technique.

The pygmy mouse (*Baiomys taylori*) was studied by Stickel and Stickel (1949) in a prairie in southern Texas. The maximum distance of movement recorded for males was 43 feet, which was the minimum distance between traps. The maximum distance recorded for females was 86 feet. They concluded that it is probable that the normal *Baiomys* range in this habitat is less than 100 feet for both sexes. It seems doubtful that they recorded a complete home range or maximum distance of movement for any individual.

Two species of grasshopper mice (*Onychomys*), which are primarily insectivorous rodents closely related to *Peromyscus,* were studied by Blair (1943a) in mesquite desert in New Mexico. Four male *O. leucogaster* had an average home-range size of 5.8 acres, with a maximum of 8.0 acres. Two females had an average range of 1.8 acres, with a maximum of 2.1 acres, but both of these may have been incomplete. Four male *O. torridus* had an average home-range size of 7.8 acres, with a maximum of 12.4 acres. Five females had an average range of 5.9 acres, with a maximum of 7.8 acres. These insect-eating rodents had significantly larger ranges than did primarily seed-eating rodents in the same habitat.

Three species of cricetine mice (*Thomasomys sublineatus, Akodon arviculoides,* and *A. nigrita*) were trapped and marked along a line of traps in a second-growth Brazilian forest by Davis (1945). On the basis of the distance of capture along the line, Davis estimated that these species have home ranges with radii of less than 100 meters. The assumed relationship between distance of capture and radius of the home range, however, has not been established. The cotton rat (*Sigmodon hispidus*) was studied by Stickel and Stickel (1949) in a prairie in southern Texas. The maximum distance of capture of males was 279 feet, but it was only 94 feet for females. It was concluded that the diameter of the male home range was 200 feet or less and that the diameter was 100 feet or less for females. Erickson (1949) studied this same species in a Georgia marsh, where he found that most males were retaken within 200 feet of the original point of capture and most females were recaptured within 130 feet of it.

The movements of the Florida woodrat (*Neotoma floridana*) were studied by Lay and Baker (1938) along wooded stream banks in eastern Texas. The average distance traversed between traps by 8 rats was 108 feet. The shortest distance was 35 feet, and the longest was 285 feet. Erickson (1949) in Georgia reported that 2 immature rats of this species were retaken at distances of 100 feet. The dusky-footed packrat (*Neo-*

toma fuscipes) was reported by Vestal (1938) to bring cuttings of vegetation from at least as far away from their houses as 100 feet. For the same species, Linsdale and Tevis (1951) estimated that a rat is within 50 feet of its house nine-tenths of the time it is outside the house.

b. *Microtine Rodents.* The meadow vole (*Microtus pennsylvanicus*) was studied in New York by Hamilton (1937a), who estimated that the home range of an individual vole rarely encompasses more than $\frac{1}{15}$ acre. This estimate is questionable, because the traps were set only 3 to 5 yards apart, and there is no evidence of any attempt to make the captures at random. Blair (1940a) studied this species in southern Michigan. In dense bluegrass, males had an estimated home range of 0.28 $\pm$ 0.03 acre, while females had an average range of 0.19 $\pm$ 0.02 acre. In comparatively sparse bluegrass cover, the males had an estimated range of 0.50 $\pm$ 0.07 acre, and the females had an average range of 0.31 $\pm$ 0.02 acre. The estimated ranges of the males were significantly larger than those of the females in both types of cover. The ranges of the males were significantly larger in the sparse than in the dense cover. The estimates of home-range size are probably too small, because animals were included with as few as 2 captures. The largest range of a male was 1.11 acres, and the largest for a female was 0.45 acre. Gunderson (1950) studied this species in a "sedge mat" in Minnesota and obtained an estimate of 0.31 acre for females and 0.19 acre for males, but his estimates also are probably too small because of technique. Jenkins (1948), using the greatest distance between points of capture, estimated the average diameter of the home range of female *Microtus montanus* at 82 feet and that of males at 138 feet. The average diameter of the home range of female *M. longicaudus* was estimated as 161 feet and that of males as 194 feet.

The pine vole (*Pitymys pinetorum*) was studied by Burt (1940) in oak-hickory wood lots in southern Michigan. He estimated that the average home range was 38 yards in diameter and concluded that the normal home range of this species is about $\frac{1}{4}$ acre in extent. He also estimated that the home range of a female lemming vole (*Synaptomys cooperi*) had a diameter of 40 yards. The bank vole (*Clethrionomys glareolus*) was studied by Chitty (1937) in England. Most individuals moved less than 60 yards when the traps were moved about to prevent recapture in the same spot, and the maximum distance between captures was 300 yards. Evans (1942) studied this same species at the same place and concluded that 83% of 139 individuals were confined to home ranges of 60 yards or less, on the basis of maximum distances between captures. The greatest distance of movement was 300 yards. He also minimized the probability of recapture in the same place by moving the traps in a

regular pattern. A male red-backed vole (*Clethrionomys gapperi*) in northern Michigan was found by Blair (1941c) to have an estimated home range of 3.56 acres. A female had an estimated range of 0.57 acre, but this was possibly incomplete. Gunderson (1950) reported an estimated average range of 0.23 acre for 3 individuals of this species in Minnesota and quoted unpublished data of W. H. Marshall indicating an average range of 0.698 acre.

Errington (1939) found that muskrats (*Ondatra zibethica*) show varying degrees of attachment for their home ranges when their habitats go dry. He found that most remain where already established, although living routines must be changed, intraspecific strife increases, and predation may be heavy. Takos (1944) in Maine found evidence of small home ranges in the muskrat, for in 184 recaptures of 40 banded animals 50% of all recaptures were within 25 feet of the point of original capture. Seventy per cent of all recaptures were within a 100-foot radius of it. The maximum distance of capture was 570 feet for an adult male. Aldous (1947) in South Dakota found that in 250 recaptures of tagged muskrats only 15.2% of the animals showed movements of more than 31 rods (511.5) feet. The maximum distance of movement was 200 rods (3300 feet).

c. *Murine Rodents.* The long-tailed field mouse (*Apodemus sylvaticus*) in England was found by Chitty (1937) to make movements of up to 480 yards in the case of males and up to 200 yards in the case of females. Evans (1942) working with this same species in the same area found that 76% of 194 individuals observed in a period of 16 months were apparently confined to home ranges of 100 yards or less.

The house mouse (*Mus musculus*) in some environments has been reported to have the smallest average home range of any rodent that has been studied. Southern and Laurie (1946), working in England, reported that under "domestic" conditions the average home range was about 50 square feet. A small home range in buildings is also indicated for this species in Wisconsin by Young, Strecker, and Emlen (1950). The average distance between captures for males was 13 feet, and for females it was 11 feet. Mice in the building with the best "cover facilities" moved shorter average distances (11 feet) than did those in a building with less cover (16 feet). The reliability of these estimates must be evaluated in view of the fact that 64% of the mice retaken were recaptured only once, and 85% were recaptured no more than twice. King (1950), working with urban house mice in Kansas, found that 50% of the mice recaptured were taken at the original point of capture and that 71% of the recaptured mice appeared to remain within one building. He presented evidence to show that mice found to move more than 113

feet seemed to be seeking new homes. All these results indicate that the home ranges of commensal house mice are small.

Baker (1946), in an area of grassland, modified forest, and coconut groves on the island of Guam, found that 38.4% of the house mice were retaken at the point of original capture, 41.4% were taken at stations 5 to 25 yards apart, and 20.2% were taken more than 25 yards apart. The average, maximum distance of capture of males was 35.8 yards, and it was 29.8 yards for females. Erickson (1949), in a Georgia marsh, retrapped 7 house mice one or more times. He found that 3 were retaken at the place of original capture and the others were retaken at distances of, respectively, 200, 400, 600, and 2200 feet. Comparison of the results of studies in domestic and wild environments indicates that the home range is usually compressed in the case of commensal house mice.

The brown rat (*Rattus norvegicus*) in urban and farm-building environments in Maryland (Davis, Emlen, and Stokes, 1948) lived in definite areas of seldom more than 100 feet in diameter. Supporting evidence from Baltimore showed that 71% of recaptured males and 77% of recaptured females were retaken within 40 feet of the point of original capture. In farm buildings, 75.3% of the recaptured males and 84.0% of recaptured females were retaken within 40 feet of the point of original capture. Only 11.6% of the males and 10.0% of the females were caught in a different building from that in which they were first caught. Tracks of individual rats in fresh snow showed that the rats seldom moved more than 100 feet. Use of dyes in the food to color the feces and the distance of recovery of colored feces also indicated a range of about 100 feet in diameter. Wild populations of two species of rats (*Rattus mindanensis* and *R. exulans*) were studied on Guam by Baker (1946). He plotted average distance of capture and concluded that these rodents lived in small ranges of less than an acre in extent, but he believed that some males of both species ranged beyond the limits of his study area.

d. *Zapodid Rodents.* A jumping mouse (*Zapus hudsonius*) was studied by Blair (1940c) in southern Michigan grasslands and marsh. Males had estimated home ranges of 0.89 $\pm$ 0.11 acre, and females had estimated ranges of 0.92 $\pm$ 0.11 acre. The maximum range of a male was 2.59 acres, and for a female it was 2.50 acres. The mice disappeared in several cases only to appear again at a later date, which probably indicated less fixity of home range than has been found in most other small mammals. Gunderson (1950) worked with this same species in Minnesota and estimated that the ranges of both sexes averaged only 0.47 acre. He also noticed the tendency for the mice to disappear and later reappear

on his area. Quimby (1951) studied this species in Minnesota and estimated the home ranges of males at 2.70 ± 0.50 acres and those of females at 1.57 ± 0.22 acres. The difference between the sexes was significant at the 0.05 level. He believed that the home ranges were unstable and that the mice shifted ranges. Another jumping mouse (*Napaeozapus insignis*) was studied in a hardwood forest in northern Michigan by Blair (1941c). Eleven males had estimated ranges of 1.08 to 8.96 acres, and 12 females had ranges of 1.05 to 6.55 acres.

e. *Sciurid Rodents.* The California ground squirrel (*Citellus beecheyi*) was studied in central California by Evans and Holdenried (1943). Nearly 70% of the squirrels with adequate records were never trapped outside of areas whose greatest length was 150 yards, although some were occasionally taken several hundred yards from their normal ranges and others permanently transferred their ranges to different areas. These workers estimated the average home range of males at 0.36 acre and of females at 0.59 acre. Linsdale (1946), working with this species in central California, found that 84.2% of the animals were always retaken within 200 yards and 61.6% were always retaken within 100 yards. He concluded that most of the ordinary squirrel activity takes place within 100 feet of the burrow and that these squirrels rarely go outside an area 300 yards in diameter. Fitch (1948b) studied this species in California and observed that in nearly half of 1043 squirrels the maximum distances of movement were between 100 and 400 feet. He also plotted the ranges of a few individuals from "hundreds of hours of observation" of these diurnal animals and reported that the range is only 100 to 150 yards in greatest diameter and that most of the activity is restricted to an even smaller area in the central portion.

The Mexican ground squirrel (*Citellus mexicanus*) was studied by Edwards (1946) by direct observation of this diurnal species in western Texas. The area roamed over by a squirrel seldom exceeded 100 yards in diameter and it was usually no more than half of this. The long-eared chipmunk (*Eutamias quadrimaculatus*) was studied by Holdenried (1940) in California. The maximum distance between captures averaged 700 feet for males and 456 feet for females, and it was believed that the males range more widely than the females. Storer, Evans, and Palmer (1944) studied this same species in California and estimated the average home range of males at 2.2 acres and that of females at 1.2 acres. They, too, believed that the males range more widely than the females. The eastern chipmunk (*Tamias striatus*) was studied by Burt (1940) in southern Michigan. For 4 females, he estimated that the greatest diameter of the home range was, respectively, 67, 70, 85, and 100 yards. For 2 males, he estimated the greatest diameter of the home range at

100 and 125 yards. Blair (1941c) studied this same species in northern Michigan and estimated the average home range of males at 2.31 ± 0.30 acres and the average range of females at 2.15 ± 0.38 acres. The largest home range of a male comprised 4.71 acres, and the largest range of a female comprised 7.27 acres. There was no significant difference between the two sexes in home-range size.

The fox squirrel (*Sciurus niger*) was studied by Baumgartner (1943) in oak-hickory wood lots in Ohio. He found that 85.9% of the tagged squirrels were retaken at a greatest distance of less than 250 yards. The average distance traveled by 60 males was 154.3 yards, and that traveled by 69 females was 129.7 yards. The flying squirrel (*Glaucomys volans*), studied by Burt (1940) in southern Michigan, was found in some cases to include the entire area of a 3.72-acre wood lot in a single home range. Jordan (1948) studied this same species in the same area and found that the greatest distance between captures was 175 yards. He concluded that the cruising radius of these animals is small, usually covering about 160 yards.

f. *Heteromyid Rodents.* The Tulare kangaroo rat (*Dipodomys heermanni*) was studied by Tappe (1941) in California. He found that, of 49 animals recaptured from 1 to 10 times, most were taken within ¼ mile of the point of original capture and 1 was approximately ⅔ of a mile away. Fitch (1948a) studied the same species more intensively in California. Among 1270 individuals recaptured one or more times, 54.5% were always retaken within 100 feet, and 74.6% were always recovered within 200 feet. He concluded that most of the foraging range of an individual is probably in a familiar area 100 to 400 feet across. The Merriam kangaroo rat (*D. merriami*) and Ord kangaroo rat (*D. ordii*) were studied in southern New Mexico by Blair (1943a). The average home range of male Ord rats was estimated at 3.41 ± 0.25 acres, and that of females was estimated at 3.29 ± 0.47 acres. The differences between the sexes in home-range size were not significant. The largest home range of a male comprised 6.93 acres, and the largest of a female equaled 10.41 acres. The estimated average home range of male Merriam rats was 4.07 ± 0.24 acres, and the largest range of a male equaled 8.78 acres. There seemed to be a seasonal difference in the ranges of female Merriam rats. The estimated home range of females averaged 1.94 ± 0.22 acres in March, but in April and May it averaged 3.88 ± 0.51 acres. The largest home range of a female was 12.52 acres. York (1949) studied the Merriam kangaroo rat in Trans-Pecos Texas and estimated the average range of males at 6.89 acres and that of females at 4.26 acres. The average home range of the Merriam pocket mouse (*Perognathus merriami*) was estimated by this same worker at 1.88 acres for males

and 5.87 acres for females, but his numbers were too few to indicate whether or not the difference in home-range size between the sexes was significant. Another pocket mouse (*P. penicillatus*) was studied by Blair (1943a) in southern New Mexico. The estimated average home range of males was 2.72 ± 0.48 acres, and the largest equaled 5.54 acres. The average range of females was 1.09 ± 0.14 acres, and the largest was 1.43 acres.

g. *Lagomorphs.* The marsh rabbit (*Sylvilagus palustris*) was studied in Florida by Blair (1936), and 2 marked individuals were never caught more than 100 yards from the point where they were first trapped. One was shot after 6 months within 30 feet of where it was first trapped. When the traps were moved 200 yards, other individuals were trapped, but the marked ones were not taken. The eastern cottontail (*Sylvilagus floridanus*) was studied in Connecticut by Dalke and Sime (1938), where they estimated the range of males to average about 8.3 acres and the range of females to be about 3 acres. Allen (1939) studied this same species in southern Michigan and obtained an estimate of 3.62 acres for males in winter and 2.22 acres for females at the same time. He believed, however, that his estimated ranges were incomplete and that the rabbits actually ranged over 5 to 10 acres. Schwartz (1941), working with this same species in Missouri, estimated the average range of males at only 1.4 acres and that of females at only 1.2 acres. He attempted to explain the small ranges on the basis of a uniform distribution of food and cover. Haugen (1942) studied this species in southern Michigan and reported much larger home ranges than those estimated by any previous worker. He estimated the average home range of females at 22.5 acres in the breeding season and he believed that the males ranged over 100 or more acres. He estimated the average range of females in the non-breeding season at only 14.0 acres. His results indicate sex and seasonal differences in home-range size. In the desert cottontail (*S. audubonii*) of California, Ingles (1941) estimated that the home range of a male may be as much as 15 acres, since 3 were taken at stations 400 yards apart. He estimated that the home range of a female is often less than an acre. Fitch (1947) worked on the same species in California and found that the average "foraging diameter" for 134 individuals was 632 feet. He concluded that, if the distances between captures actually represent the extent of the foraging areas, home ranges of roughly 8 or 9 acres are indicated for both sexes, but he believed that in most cases the ranges were somewhat larger.

The European rabbit (*Oryctolagus cuniculus*) was studied by Southern (1940) in England by marking the ears with celluloid disks and watching the movements of individual rabbits with a telescope. This

method of observation was facilitated by the fact that these animals are colonial and live in rabbit "warrens" containing numerous individuals. Southern observed that the approximately 150 animals living in a warren of about ½ acre used a total feeding area of about 2 acres. About 1 acre of this was heavily grazed, and the rest was used for more occasional excursions.

h. *Insectivores.* The short-tailed shrew (*Blarina brevicauda*) was studied by Burt (1940) in oak-hickory wood lots in southern Michigan. Twelve individuals were recaptured at distances of from 55 to 360 feet, and the maximum distance of capture averaged 153 feet. Burt estimated the home range at about 50 yards in diameter and at an area of about 0.4 acre. Blair (1940d) studied this same species in bluegrass fields in southern Michigan. Thirteen males had estimated home ranges of from less than ¼ acre to 4.43 acres, and 13 females had estimated ranges of from less than ¼ acre to 0.88 acre. One female was living in June in the same home range in which she was first marked the preceding September. Blair (1941a), in a beech-maple forest in northern Michigan, found that a male of this species ranged over 1.39 acres, and 5 females had estimated ranges of from 0.56 to 1.46 acres. Three of the females had ranges that exceeded 1 acre.

i. *Carnivores.* The raccoon (*Procyon lotor*) was studied by Stuewer (1943) in southern Michigan. On the evidence from trapping and from tracking in snow, the diameter of the male home range was estimated at about 1 mile, and the average female range was estimated at 0.7 mile. Both sexes of the spotted skunk (*Spilogale interrupta*), studied by Crabb (1948) in Iowa, were found to range over less than 160 acres in winter. In the spring, males ranged over 2 to 4 square miles, and females ranged over no more than ¼ of a section.

j. *Marsupials.* The common opossum (*Didelphis virginiana*) was studied by Lay (1942) in eastern Texas. For 29 opossums, the average maximum distance between captures was 1460 feet, which was taken to indicate the diameter of a theoretical circular home range of 38.4 acres. In Brazil, Davis (1945) marked small mammals along a trap line and observed that females of the common opossum (*D. marsupialis*) and the wooly opossum (*Caluromys philander*) remained within a small area for a long period of time, but the males were seldom recaptured.

3. *Factors Affecting Home-Range Size*

Burt (1940) believed that the size of the home range is limited by "the animal's ability to travel and its necessity for food and protection." Blair (1940b) listed the factors that may influence the size of the home range as: (1) location of homesite or homesites, (2) available food, (3)

distribution of preferred cover, (4) location of temporary refuges, (5) relations to other individuals of the same sex and species, (6) relations to individuals of the opposite sex of the same species, (7) relations to individuals of other species, (8) weather, (9) microclimates. The accumulated information of the past few years now permits a more precise evaluation of the factors that affect home-range size.

a. *Sex.* Males appear to range more widely than females in most species of small mammals in which the size of the home range has been estimated. The females presumably range less widely than the males because of their greater attachment to the nest and young, but there is no direct evidence on this point. Smaller home ranges in females than in males have been reported in: *Peromyscus maniculatus* (Blair, 1942; Storer, Evans, and Palmer, 1944), *Sigmodon hispidus* (Erickson, 1949; Stickel, and Stickel, 1949), *Microtus pennsylvanicus* (Blair, 1940a), *M. montanus* and *M. longicaudus* (Jenkins, 1948), *Apodemus sylvaticus* (Chitty, 1937), *Eutamias quadrimaculatus* (Holdenried, 1940; Storer, Evans, and Palmer, 1944), *Sciurus niger* (Baumgartner, 1943), *Perognathus penicillatus* (Blair, 1943a), *Blarina brevicauda* (Blair, 1940d), *Didelphis marsupialis* and *Caluromys philander* (Davis, 1945), *Spilogale interrupta* (Crabb, 1948).

Larger home ranges for females than for males were reported for *Citellus beecheyi* by Evans and Holdenried (1943), but this was refuted by Fitch (1948b), who found in an extensive study that females ranged less widely than males. Larger home ranges for males than for females were reported for *Sylvilagus floridanus* by Dalke and Sime (1938), Allen (1939), and Haugen (1942). Schwartz (1941), on the other hand, reported no difference in this species, but his estimates of home-range size probably represented small fractions of the actual ranges. Ingles (1941), on the basis of few observations, believed that male *S. audubonii* range more widely than females, but Fitch (1947) found no sex difference in home-range size in this species. Little or no sex difference in home-range size has been found in the species of kangaroo rats that have been studied. These include: *Dipodomys ordii* and *D. merriami* (Blair, 1943a) and *D. heermanni* (Fitch, 1948a). York (1949), on few individual records, believed that male *D. merriami* range more widely than females.

b. *Age.* Young animals, as they first venture from the nest, apparently range over smaller areas than do the adults. This has been observed in *Sylvilagus floridanus* (Haugen, 1942), *Peromyscus maniculatus* (Blair, 1942), *Citellus beecheyi* (Evans, and Holdenried, 1943), *Citellus mexicanus* (Edwards, 1946), *Ondatra zibethica* (Takos, 1944), and *Sylvilagus audubonii* (Fitch, 1947). However, Howard (1949)

caught a male *P. maniculatus* and his 4 offspring together 300 feet from the nest box in which the young were recorded the previous day. Blair (1951a) found that the young occasionally traveled with adult *P. polionotus,* for young mice were sometimes caught with an adult in single-catch traps.

c. *Food Habits.* There is some indication that insect eaters tend to range more widely than vegetation eaters. Blair (1943a) found that insect-eating *Onychomys leucogaster* and *O. torridus* ranged more widely than did comparable-sized, vegetation-eating members of the related genus *Peromyscus* in the same habitat. The short-tailed shrew (*Blarina brevicauda*), which feeds primarily on insects and other invertebrates, ranges more widely than do vegetation-eating rodents in the same habitat (see Blair, 1940b, 1940d). The explanation for this difference may be that insect eaters must cover more ground than vegetation eaters in order to obtain comparable amounts of food.

d. *Food and Cover.* The density of the vegetational cover has been shown to affect the size of the home range in several species of small mammals. The area ranged over tends to be larger where the cover is sparse and smaller where the cover is comparatively dense. It is difficult to analyze separately the effects of protective cover and food supply on the movements of small mammals, as both are provided mostly by the vegetative complex in the case of most small mammals. Availability of food is probably more important than availability of cover, but this needs further investigation. Allen (1939) stated that in *Sylvilagus floridanus* the "most favorably located" rabbits had the smallest home ranges. Blair (1940a) reported that *Microtus pennsylvanicus* living in sparse bluegrass had significantly larger ranges than those living in dense bluegrass. Linduska (1942) found that *Peromyscus maniculatus* living in field-shocked corn in winter in southern Michigan "moved only sparingly from the shocks," whereas those in near-by wheat stubble moved 200 to 300 feet. He concluded that the greater food supply available in the corn shocks accounted for the smaller ranges of the mice living there. Blair (1943a) reported that *P. maniculatus* and *P. leucopus* living in comparatively sparse cover on a desert basin in New Mexico ranged more widely than the same species in more dense cover in southern Michigan. Schwartz (1941) believed that the uniform distribution of food and cover accounted for the remarkably small estimates of home-range size he obtained for *Sylvilagus floridanus* in Missouri. Davis, Emlen, and Stokes (1948), writing of *Rattus norvegicus,* stated: "The distance a rat regularly moves is probably largely dependent upon the relation between suitable harborage and food supply. If the two are close together the rats travel little, if they are far apart the rats travel

much." Fitch (1948b), writing of *Citellus beecheyi,* stated: "Occasionally some preferred food source induces a squirrel to make unusually long foraging expeditions from its home base, beyond the limits of the usual home range." The home ranges reported for *Mus musculus* living under domestic conditions (Southern and Laurie, 1946; Young, Strecker, and Emlen, 1950; King, 1950) are generally larger than those reported for feral house mice (Baker, 1946; Erickson, 1949), which presumably must get their food from less concentrated sources. Blair (1951a) reported that *Peromyscus polionotus* ranged less widely in densely grassed dunes than in areas of sparse vegetational cover on the same island.

e. *Mobility.* Highly mobile mammals such as bats undoubtedly range more widely in their feeding activities than do earth-bound rodents. I have seen Mexican freetail bats (*Tadarida mexicana*) leaving their roosting places in the evening and traveling distances that would indicate that the diameter of the home range is to be measured in miles rather than in feet. Among terrestrial rodents, there is some indication that the saltatorial jumping mice (*Zapus*) are less restricted to a circumscribed home range than are non-saltatorial rodents (Blair, 1940c, 1941c; Gunderson, 1950; Quimby, 1951). Saltatorial kangaroo rats (*Dipodomys*), however, ranged no more widely than did non-saltatorial rodents in the same habitat (Blair, 1943a).

f. *Size of Animal.* Large mammals tend to range more widely than small ones. By comparison with the few acres regularly traversed by mice and other small rodents, the raccoon may have a home range a mile in diameter (Stuewer, 1943), and male eastern cottontails may range over 100 or more acres (Haugen, 1942). Greater food requirements may account in part for the greater home ranges of large mammals, and greater mobility also may play a part. Present data are inadequate for testing the relationship between body size and size of home range where there is not great disparity in body size. In *Peromyscus,* the smallest form studied, *P. polionotus,* ranges at least as widely as any species that has been investigated in this group (see Blair, 1951a).

g. *Season of Year.* Blair (1943a) reported that in 2 species of kangaroo rats the females ranged less widely in March, at about the onset of the reproductive period, than they did in April and May. Blair (1951a) noted that in the beach mouse the average home ranges were significantly smaller in spring than they were in the fall.

h. *Size of Area.* Baumgartner (1943) reported that home ranges of fox squirrels living in small wood lots were smaller than those of squirrels living in larger ones.

i. *Homesite.* The location of a suitable nest site may be a controlling factor in the location of the home range if the animal is highly

specialized in its choice of a nest. The location of a hollow tree might determine the location of the home range of such tree-nesting forms as the wood mouse, raccoon, or fox squirrel. The location of the nest, however, does not necessarily determine the location of the home range. In the beach mouse, Blair (1951a) reported that the location of a particular nest site has little to do with the location or permanence of a home range, for the holes actually utilized in the home range may change from time to time, whereas the home range remains comparatively constant. Linsdale (1946) apparently observed similar behavior in the California ground squirrel, for he wrote, "Apparently the site of a nest is occupied for only a short period, though removal to a new site may not be far."

j. *Population Density.* There is no evidence that population density has any influence on the size of the individual home ranges. Southern and Laurie (1946) found that in house mice living under "domestic" conditions the range was not compressed by an increase of density, but that under such conditions the range was shared. Calhoun (1948), working with *Rattus norvegicus,* found that when additional individuals were introduced into a population already at about carrying capacity the residents remained in their previously existing home ranges. Young, Strecker, and Emlen (1950) reported that lowering the density of a population of house mice by poison did not affect the range of those mice that remained.

4. *Significance of the Home-Range Habit*

Restriction of normal activity to a small area of familiar terrain is of obvious survival value to the individual animal. The chance of an animal's escaping from a predator would appear to be greater in the home range, where the animal knows the location of every refuge hole and the location of protective cover, than in an area of unfamiliar terrain. Burt (1940) and Blair (1951a) have pointed out that dispersing individuals, seeking an area in which to establish residence, are probably more vulnerable to predation than established animals. Davis, Emlen, and Stokes (1948) also hold that intimate knowledge of the terrain of the home range is of survival value, and they suggest that the home-range habit may have originated through selection. According to their hypothesis, "the individuals which tended to remain in a home range would be less susceptible to predation and hence on the average would survive longer than individuals which did not tend to remain in a home range." There seems no question but that the activities of most small mammals are strongly conditioned by an awareness of danger. Fitch (1948b) in writing of the California ground squirrel states: "The tendency of each to explore new territory is limited by the tendency to

keep where the home burrow or other safe and familiar shelter is easily accessible. As the squirrel works out from these it becomes increasingly uneasy and alert." Blair (1943b) showed experimentally that activity of deer mice decreased significantly when the illumination was increased to the equivalent of one-half moonlight, and he reported similar observations on wild populations of rodents. Blair (1951a) reported similar behavior in the beach mouse. Burt (1940), working with the wood mouse, wrote that, "my general impression is that one is likely to have a better catch on a dark, rainy night than on a clear, moonlight night." Fitch (1947) noted that *Sylvilagus audubonii* tends to forage in the vicinity of cover during the day and range more widely at night, for individuals were caught at night in open grassland where they were never seen to venture in daylight. The awareness of danger and the ability to learn the terrain are probably important in limiting the size of the area over which an animal ranges.

The home-range habit has important effects on geographic variation within the species population in that it tends to retard dispersal of hereditary characters through the population. Free mixing of individuals throughout the species population would strongly inhibit the formation of locally adaptive gene complexes.

Attachment to the familiar terrain of the home range may be extremely strong. Errington (1939) noted that even when dried-out muskrat habitations no longer show evidence of occupancy, animals may still be living not far away in woodchuck holes, corn fields, etc., in a range that is patently familiar to them.

5. *Territoriality*

Territoriality is the defense of territory, usually against other members of the same species and sex. The simplest definition of territory is the one by Noble (1939) that "territory is any defended area." Defense of a breeding territory by the male is of widespread occurrence in birds (see Howard, 1920; Nice, 1941). Defense of territory during the reproductive season has been reported from several species of mammals. The evidence comes principally from the failure of the home ranges to overlap and from observations of intraspecific strife. In the former case, complete failure to overlap is taken to indicate that the territory and the home range are the same, while marginal overlap is taken to indicate that a smaller, protected area exists within the home range (see Burt, 1940). Burt (1940, 1943, 1949) believes that territoriality is of widespread importance to mammalian populations, for he writes (1943) that "nearly all who have critically studied the behavior of wild mammals have found this behavioristic trait inherent in the

species with which they worked." Actually, among 46 species of small mammals studied by various workers, evidence of territorial behavior has been reported for only 11, and for 3 of these other workers have denied that there was territorial behavior.

Squirrels appear to be more inclined to show territorial behavior than any other group of rodents. Gordon (1936) observed several species of squirrels at feeding stations and concluded that *Sciurus fremonti, S. douglasii,* and *Citellus lateralis* showed territorial behavior. He reported a "chase order" in *C. lateralis* and *Eutamias quadrivittatus,* involving a hierarchy of progressively dominant individuals. Burt (1940), working in southern Michigan, reported territorial behavior in *Tamias striatus* on the basis of an observed female that protected an area about 50 yards in radius but had a foraging range that extended 100 or more yards from the nest. Blair (1941b), working with the same species in northern Michigan, observed that in only one case was there overlap between the ranges of 8 breeding females, and chipmunks were often seen chasing others of their kind. Storer, Evans, and Palmer (1944) reported that female *Eutamias quadrimaculatus* apparently maintain separate ranges, with only slight overlap. Evans and Holdenried (1943) wrote that in *C. beecheyi* adult males appear more aggressive than other squirrels and they may maintain individual territories which they defend against invasion by other males. This was denied by Fitch (1948b), for in an intensive study of this species he found no evidence of territorialism. He reported that 5 or more males occupied the same burrow during the breeding season, while adjacent areas were unoccupied, and, although fights were frequent, there was no evident intent to exclude a rival from any definite territory. Edwards (1946), working with *C. mexicanus,* observed that these squirrels tolerated other squirrels in their "territory" (home range) but not in their burrows. There was a great deal of overlapping of home ranges, for he reported that on an area of about 50 acres, all acceptable to the squirrels, there was an estimated population of 20 squirrels, of which 18 lived in an area 100 yards in diameter. The evidence seems conclusive that much intraspecific strife is characteristic of many, if not most, sciurid populations, and there is less satisfactory evidence that in some species there is defense of territory.

Heteromyid rodents resemble sciurids in that there is much antagonism between individuals of the same species, but there is little evidence for defense of territory in this group. Blair (1943a) found that *Perognathus penicillatus* females appeared to have mutually exclusive ranges, while the ranges of female *Dipodomys ordii* and *D. merriami* broadly overlapped the ranges of others of the same species and sex,

although apparently suitable, unoccupied areas existed nearby. York (1949) confirmed these observations on *D. merriami* and found that *Perognathus merriami* behaved similarly. He observed that in both species there was overlap of range but not sharing of dens. Fitch (1948a) found that *D. heermanni* is inclined to be hostile toward members of the same species, but he felt that lack of "true territoriality" comparable to that of some birds is indicated by the fact that burrow systems of different individuals are connected by surface runways. The antagonism between individuals of the same species that characterizes the sciuromorph rodents is probably influenced by the almost universal habit of food storage in this group. Any intruder is apparently regarded as a potential robber of the food cache.

Territorial behavior does not seem to be of very general occurrence among myomorph rodents, although a few apparent cases have been reported. Burt (1940) deduced territorial behavior in *P. leucopus* females from the failure of their ranges to overlap broadly and concluded that the territory is a smaller, defended area within the home range. Blair (1940a) also found evidence of female territoriality in *Microtus pennsylvanicus,* for the home ranges showed little overlap. Storer, Evans, and Palmer (1944), working with *P. maniculatus* in California, obtained results that "suggested that the home range of any single adult tended to be separate from those of other adults of the same sex." Very different results were obtained for this same species in Michigan by Blair (1940b, 1942) and by Howard (1949), for neither found any evidence of territoriality. Howard found that: "In areas where sex ratios of adults were unequal, as many as 3 males lived with one female, and as many as 3 females lived with one male during the breeding season [in nest boxes]." Absence of territorial behavior in this species was also reported in New Mexico by Blair (1943a). Another intensively studied species that has failed to give any evidence of territorial behavior is *Mus musculus.* Young, Strecker, and Emlen (1950) found these mice highly gregarious, with numerous adults and the young of several litters occupying the same community nest. Scott (1944), working with this species in the laboratory, found no evidence of territoriality.

Haugen (1942) reported territorial behavior in females of *Sylvilagus floridanus* on the evidence of the failure of the home ranges to overlap. Lay (1942) reported that *Didelphis virginiana* showed no discernible evidence of territories. Stuewer (1943) found no indication that either sex of *Procyon lotor* displays any territorial behavior.

The theoretical importance of territoriality has been discussed briefly by Burt (1940, 1943, 1949), who holds that territorial behavior

ensures (1) populations at or below the optimum size in the preferred habitat, (2) dispersal of genes through the population, and (3) expansion of the species range, possibly into other types of habitats. The first of these is of unquestioned theoretical value to the species population. In the case of gene dispersal and range expansion, there is no evidence presently available to indicate any greater rate of gene flow in territorial than in non-territorial species or any greater tendency for them to expand their range. Burt's (1940) measurements of dispersal distances in young *Peromyscus leucopus,* a species which he regarded as showing territorial behavior, were no greater than the measurements of dispersal distances in the non-territorial *P. maniculatus* by Blair (1940b) and Howard (1949). The non-territorial *P. maniculatus* is also the only species of the genus for which there is evidence of a major range expansion in historic times (see Dice, 1931a; Hooper, 1942; Hamilton, 1950).

6. *Homing "Instinct"*

The tendency for an animal to return home after being released at a distance has been called homing instinct. If such a phenomenon exists, it should be of some importance in ensuring the return home of accidentally dispersed individuals, thereby acting to restrict gene flow in the species population. The data respecting homing instinct in rodents fall into two classes: (1) the numerous instances in which an animal was transported such a short distance that it was apparently released within the normal home range or within a somewhat larger area with which the animal was almost certainly familiar, (2) the few cases in which an animal returned from greater distances than it would be expected to know.

Return of an animal released within or near the home range calls for no behavior other than the previously discussed attachment of an individual to an area of terrain, or home range. Cases that evidently fall into this category are those reported for: *Peromyscus leucopus* (Johnson, 1926; Townsend, 1935; Burt, 1940; Stickel, 1949), *P. maniculatus* (Kendeigh, 1944), *Microtus pennsylvanicus* (Hamilton, 1937a), *Neotoma floridana* (Lay and Baker, 1938), *Zapus hudsonius* (Quimby, 1951), and *Ondatra zibethica* (Takos, 1944). Linsdale (1946) reported that *Citellus beecheyi* made returns of up to ¾ of a mile, but he believed that this species had little homing ability. Fitch (1947) reported 3 returns of *Sylvilagus audubonii* from distances of 3150 to 4400 feet, but 20 others that were moved about ¾ of a mile failed to return. His results suggest that the rabbits failed to return when moved beyond familiar terrain.

The return of animals from comparatively great distances can be interpreted as indicating that (1) the return was due to chance as the animal moved away from the point of release, or (2) the animal knew a much larger area of terrain than its normal home range, or (3) the animal had some unknown ability to navigate homeward through unfamiliar terrain. Murie and Murie (1931) found that in *Peromyscus maniculatus* 4 of 40 mice returned when moved 1 mile, 1 of 23 returned from 2 miles, and none of 15 returned from 4 miles. They preferred the hypothesis of a homing instinct. Chance alone seems inadequate to account for their results. The probability that an animal would start in the right direction is not prohibitive, but there must be added also the probability that it would travel far enough to reach the old home range and that it would escape predators while traveling through unfamiliar terrain. Familiarity with the terrain seems an equally tenable hypothesis to homing instinct to account for their results, particularly in view of the decrease in the rate of return with increased distance. It is possible that the returned animals had retained knowledge of the considerable areas normally covered during the dispersal phase of the life history, when, in this species, dispersal distances of up to ¾ of a mile have been reported (Blair, 1940b; Howard, 1949).

Hungerford and Wilder (1941) moved 15 *Sciurus carolinesis* distances of 3,000 to 14,800 feet a total of 24 times. They obtained 11 returns, of which 2 were from the maximum distance of 14,800 feet. Only one of the returning animals was a female, which returned a distance of 5,600 feet. Dispersal distances of up to 14 miles have been reported for a related species, *S. niger,* by Baumgartner (1943), which indicates that tree squirrels may know much larger areas than their home ranges. The returns of tree squirrels reported here, therefore, could easily have been from areas with which the animals were acquainted. There seems no evidence at hand that requires the hypothesis of any special sense whereby rodents can return home through unfamiliar terrain.

IV. Dispersal

The movement of young animals away from the nest before they, in turn, reproduce is the principal means by which dispersal of individuals (and hereditary characters) through the species population is accomplished. Dispersal movements also permit expansion of the geographic range of the species population and they may theoretically induce expansion of the ecologic range by leading to experimentation with previously unoccupied environments. Dispersal movements generally come at the onset of sexual maturity (Burt, 1940; Blair, 1940b; Southern, 1940;

Fitch, 1948a). This results in continuing dispersal through the reproductive season in species that produce several litters in a breeding season. In forms that produce a single litter per season, the dispersal movements may be concentrated in a comparatively short period. Evans and Holdenried (1943) reported that young *Citellus beecheyi* moved most often in August and September. Baumgartner (1943) noted that in *Sciurus niger* the dispersal movements in any one year seldom last more than 2 or 3 weeks, in August and September. In species that show marked, cyclic fluctuations in numbers, dispersal is possibly limited almost entirely to the periods when the population is building up to, or has reached, "peak" densities.

1. *Causes of Dispersal*

Dispersal movements of rodents may be attributed to one or both of two major causes: (1) an inherent tendency to disperse, stimulated by physiologic changes as the animal becomes sexually active, (2) population pressure, resulting from active and passive intraspecific competition. Both of these major factors may possibly enter into determining the time and distance of dispersal of an individual rodent. Howard (1949) argues that in *Peromyscus maniculatus* part of the stimulus for dispersal movements might be a direct response to a hormonal secretion by the reproductive organs, because many of these mice disperse when their reproductive organs are becoming functional for the first time. He found no evidence of dispersal before the approach of sexual maturity, and he found only one case of an extensive movement after maturity. His argument is strengthened by the fact that fall-born mice, which usually did not become sexually active until the following March, generally remained through the winter in family aggregations. That population pressures also influenced dispersal is indicated by the wide range of variation in distance of dispersal and by the fact that numerous individuals failed to disperse beyond the limits of the parental home range.

Drastic changes in population density (and pressure) may affect the behavior and local distribution of resident animals, and it is to be inferred that there would be major effects on young, unestablished individuals. Bole (1939) has written extensively on the problem of "drift" in making estimates of population density involving the removal of individuals from a quadrat. Population density of *Peromyscus maniculatus* was lowered by Blair (1940b) when 37 of 47 residents were removed from a 4.6-acre area in the midst of a uniform habitat. At the end of 2 weeks, the population had rebuilt to 25, of which 20 were sexually mature animals that moved from adjacent areas and 2 were young mice. An isolated 2.9-acre area inhabited by 1 pair of deer mice

was overpopulated when 22 adult males, 19 adult females, and 4 young females were released there. Trapping during the second week revealed only the resident pair, 2 liberated adult males and 3 females, and 5 juvenile mice. Ten of the mice that disappeared were retaken at distances of 825 to 1815 feet. Both of these experiments indicated movement from areas of high to areas of low population density. Stickel (1946) demonstrated similar behavior in *Peromyscus leucopus* when she marked the mice on a circular, 17-acre area before beginning to remove the mice from a central, 1-acre plot. As the animals were removed from the central plot, others were taken there from increasingly greater distances. Spencer (1941) ran snap-traps on a 5-acre plot for a period of 10 months to measure "drift" in rodent populations. His results strongly support the argument that there is a tendency for movement to be from areas of high to areas of low population density. Over the 10 months, 1254 individuals of 22 species of small mammals were taken from the 5 acres. Two species comprised the bulk of the records. Spencer reported that 174 *Neotoma albigula* originally resided on the plot or had the extreme edges of their ranges touching the plot, but during the 10 months he removed a total of 682 of these pack rats. An average density of 4 *Dipodomys merriami* per acre was estimated in the area, but he removed 251 individuals from the plot. The available evidence seems to indicate that dispersal is influenced by both an inherited tendency to move away from the place of birth and by population pressures.

2. *Distance of Dispersal*

The evidence for distances of dispersal in small mammals is fragmentary, with the best estimates coming from the few nest-box studies that have been made. Nicholson (1941), using nest boxes, reported movements of 200 to 1500 feet among 11 *Peromyscus leucopus*. The greatest movement was made by an adult male. Howard (1949), also using nest boxes, reported that among 155 young *P. maniculatus* of which the birthplace was known, 31% of the males and 15% of the females moved at least 550 feet. The maximum distance of movement was 3300 feet, but 119 of the mice bred within 500 feet of their birthplace. Howard's results suggest greater distances of dispersal in males than in females and they suggest a rather slow rate of dispersal in this species. Dispersal distances estimated from trapping records alone are rather unreliable, because one is never certain that the animals were first recorded at the birthplace. Burt (1940), working with *P. leucopus*, reported that 51 of 154 young mice moved distances of 100 to 900 yards from the place of original capture, and 103 remained within 100 yards of it. The average distance of movement of the mice that dispersed

more than 100 yards was 266 yards. Among 133 adult mice, only 7 moved distances of more than 200 yards. Burt also reported that 5 young and 3 adult *Tamias striatus* moved distances between 225 and 700 yards (average 370), and 1 *Pitymys pinetorum* moved 300 yards. Blair (1940b) reported the average of the dispersal movements of 6 young male *Peromyscus maniculatus* as 1334 feet (maximum, 3960) and the average for 4 young females as 453 feet (maximum, 1030). Blair (1943a) reported movements of 1200 to 2300 feet for the same species in New Mexico, of up to ½ mile for *Peromyscus leucopus*, and of 2000 to 2700 feet for *Dipodomys merriami* and *D. ordii*. Blair (1951a) reported that 14 young *Peromyscus polionotus* moved an average distance of 1415 ± 89 feet (minimum, 980; maximum, 1970).

Distance of dispersal, like size of home range, seems to be roughly proportional to body size. Errington and Errington (1937) and Errington (1939, 1944) found that young muskrats dispersed distances of from zero to 21 miles. Tracking showed that one individual covered 2800 yards in ½ day. Baumgartner (1943) reported that 6 fox squirrels moved distances of from 0.4 to 14 miles. Dalke and Sime (1938) recorded a movement of 2.4 miles by a young male *Sylvilagus floridanus* and movements of 1.1 and 1.4 miles by 2 young females. Schwartz (1941) reported movements of 1322 and 3542 feet by 5 young and 5 adults of the same species. Fitch (1947) found that one young *S. audubonii* moved 3300 feet. Southern (1940) recovered or observed 21 *Oryctolagus cuniculus* after they had moved from the warren in which they were born. Six moved less than 70 yards, 9 less than 140, 3 less than 210, and 3 less than 280. Stuewer (1943) found that 9 young male raccoons dispersed an average distance of 11.75 miles (minimum, 3.5; maximum, 27) and 5 young females moved an average of 8.25 miles (minimum, 4.5; maximum, 16).

3. *Rate of Dispersal*

The actual rate of dispersal is affected by so many variables that it is not presently possible to estimate reliably the rate of spread of a character through a population. Haldane (1948) has used the available data to attempt an estimate of the relationship between the intensity of selection, mean distance migrated per generation, and the slope of the cline in races of *Peromyscus polionotus*. He has had to make so many assumptions that, as he points out, the results may be wholly fallacious. He has assumed that there is only one generation annually, but the evidence now available indicates more than one per year for this species (Blair, 1951a). He also assumed for purposes of mathematical analysis that the population density is equal throughout the area occupied. This

species is actually very discontinuously distributed because of its preference for certain soil and vegetation types (Blair, 1950). Since rodent populations generally show a mosaic pattern of densities, with some environments avoided, others tolerated, and others preferred (Blair, 1951b), measurement of the rate of dispersal through unfavorable as well as favorable environments becomes necessary before we can estimate the theoretical rate of diffusion of a character through a widely distributed population. We do know that dispersal may occur through unfavorable environments, although presumably at a lower rate than through favorable ones. Blair (1940b) found that deer mice moved through habitats not normally used when there was an artificial increase in population pressure. Howard (1949) reported that on occasion young mice of this species will disperse through unfavorable habitats such as woods.

V. Pattern of Reproductive Behavior

The duration of the reproductive alliance between members of opposite sexes has a direct bearing on the potential strength of reproductive isolation between members of closely related species. Species that form more or less permanent pairs are less apt to exchange genes through hybridization than are those in which mating is promiscuous (Mayr, 1942). Little attention has been given to this important phase of rodent behavior. A few species have been shown to form comparatively permanent pairs, but the extent of such a social pattern in rodent species is unknown. Howard (1949) found that once two *Peromyscus maniculatus* had become mated, they remained mated for the duration of that breeding season. No mouse selected a new mate during one breeding season so long as its original mate remained alive. Blair (1951a) found evidence for similar behavior in *P. polionotus,* although some sexually active individuals gave no evidence of pairing. Burt's (1940) records for one individual *P. leucopus* suggest the possibility of similar behavior in this species. Linsdale (1946) believed that *Citellus beecheyi* forms temporary pairs by occupying common ground for "possibly as long as 2 weeks." Fitch (1948a) pointed out that Linsdale's argument lacked supporting evidence and argued from his own evidence that these squirrels are probably promiscuous. The solitary nesting habits of sciuromorph rodents apparently preclude any such pairing as that reported for the few species of myomorphs that have been studied.

VI. Life Span

The average life span of a species in nature, by comparison with the potential or physiological span, is indirectly indicative of the selection pressure on the species, but, of course, it does not reveal the selective agents. A high annual turnover within the population is evident in all the species of rodents and other small mammals that have been studied. The estimates grouped in Table 1 suggest that in a given population of these animals not more than 6% will usually survive as much as a year. These significantly are all small animals that constitute a major part of the food supply of predatory birds, reptiles, and mammals. There is additional convincing evidence to support the argument that in nature small mammals live out only a small fraction of their potential life span. Pearson (1945) kept *Blarina brevicauda* in captivity up to 33 months and through 3 breeding seasons, but he found that in nature only 6% survived from one summer to the next. Hacker and Pearson (1946) kept *Apodemus sylvaticus* in captivity up to 53 months, but in

TABLE 1

Reported Percentage of Population Surviving Through One Year in 14 Species of Shrews, Rodents, and Lagomorphs

Species	*Locality*	*Per cent surviving*	*Authority*
Blarina brevicauda	Pennsylvania	6	Pearson, 1945
Sylvilagus floridanus	Michigan	7	Allen, 1939
Sylvilagus audubonii	California	9 (1939) 38 (1940)	Fitch, 1947
Dipodomys heermanni	California	5	Fitch, 1948a
Eutamias minimus	Michigan	6	Manville, 1949b
Tamias striatus	Michigan	21	Manville, 1949b
Peromyscus maniculatus	Michigan	6	Blair, 1941d
	Michigan	14	Manville, 1949b
	Michigan	34	Manville, 1949b
Peromyscus leucopus	Michigan	4	Burt, 1940
	Pennsylvania	4	Pearson, 1945
Microtus longicaudus	California	2	Jenkins, 1948
Microtus montanus	California	—	Jenkins, 1948
Clethrionomys gapperi	Michigan	3	Manville, 1949b
Clethrionomys glareolus	England	1	Evans, 1942
Apodemus sylvaticus	England	1	Evans, 1942
Rattus norvegicus	Maryland	5	Davis, 1948

wild populations they estimated an approximate mean monthly survival rate of 0.876 which means that one would expect 7 out of 8 individuals to be alive at the end of a month. They concluded that in some years few mice, possibly none, survive from one winter season to the next. Blair (1948) found that in 3 species of rodents the average life span among individuals that lived until they left the nest was less than 5 months. The estimates were: 4.88 ± 0.20 months for *Peromyscus maniculatus,* 4.64 ± 0.21 months for *P. leucopus,* and 4.23 ± 0.22 months for *Microtus pennsylvanicus.* That these rodents lived only a fraction of their potential life span is indicated by the facts that *Peromyscus* regularly lives more than 3 years in captivity and *Microtus* has been kept alive more than a year. Dice (1933) reported individual *P. maniculatus* living to 7 and 8 years in captivity. Howard (1949) reported that in this same species only one-fifth of the mice born in nature reached sexual maturity. He found a seasonal difference in mortality rate, with only one-fourth of those born in spring, summer, and early fall surviving to 4 weeks, whereas one-fourth of those born after the middle of September survived to 21 to 25 weeks of age. Mortality of both young and adults was high during the spring months owing to increased predation and occasional late snow and sleet storms at a time when dispersal movements away from the place of birth were under way. Fitch (1948a) found that in *Dipodomys heermanni* 35.3% remained in the population no more than 1 month and an additional 34.2% remained no more than 6 months. Blair (1951a) reported that in a *Peromyscus polionotus* population only 19.5% survived as much as 4 months. Southern (1940) observed high mortality in *Oryctolagus cuniculus* in the stages soon after weaning. In one set of observations, all young had disappeared from the population in 9 weeks, but this could have been accounted for in part by dispersal. Bourlière (1951) quotes evidence of a similarly short life span for *Microtus guentheri* from Bodenheimer and for *Citellus pygmaeus* from Kalabuchov.

The only argument contrary to the considerable evidence that a small mammal rarely lives out its potential life span in nature comes from Hamilton (1937b, 1940). He apparently believed that *Microtus pennsylvanicus* "burns out" physiologically because of (1) attainment of sexual maturity at an uncommonly early age, (2) extreme prolificness, and (3) little cessation of activity in its search for food. In regard to *Sorex fumeus,* Hamilton (1940) reported that the life span is short, for all adults die of old age when 14 to 17 months old. Hamilton's arguments are not supported by any other workers.

Although such factors as unfavorable weather, disease, and parasites undoubtedly affect survival, particularly in special cases, predation

is probably the most important single cause of the short life span of small mammals. The list of vertebrate predators that feed principally or entirely on small mammals is too large and too well known to need repetition here. In addition, the behavior in natural populations indicates that small mammals generally live in constant fear of capture by predators (Hatfield, 1938; Burt, 1940; Fitch, 1947, 1948b; Blair, 1943b, 1951a). The rate of selection imposed on a species population in respect to the characters that may affect ability to remain undetected by, or to escape, predators is obviously related to the rate of predation on it.

VII. Pattern of Distribution

The potential diffusion of hereditary characters through a species population is affected not only by the tendency for the localization of individuals except during the dispersal phase of the life history but also by the pattern of distribution of the population. A species population of small mammals is not evenly distributed through the geographic range of the species. Dice (1931b) has called attention to the limitation of mammalian species to particular kinds of physical and biotic (vegetational) environments, and this phenomenon is well known to any field naturalist. The pattern of distribution of a species population consequently will reflect the pattern of distribution of more or less suitable habitats for that species. Where favorable habitats are frequently interrupted by unfavorable ones for the species, there will be a greater or lesser restriction on the exchange of individuals between the subpopulations inhabiting favorable environments. The theoretical effects of such a pattern of distribution on the differentiation of the local subpopulations have been discussed thoroughly by Wright (1931, 1943, 1946, 1948, 1949a, 1949b) and others, but only a minute beginning has been made toward accumulating the data necessary before Wright's calculations can be applied to natural populations of vertebrates. Only enough is known at present about the pattern of distribution of small mammals to indicate that the pattern is usually a complex one. Blair (1950, 1951b) has discussed the general features of the pattern of distribution of mammalian species and has pointed out that there may be considerable geographic variation in distribution pattern, with attendant effects on local differentiation. A single species, such as *Peromyscus leucopus*, may show areal continuity of distribution (in the sense of Wright, 1943) in one area, dendritic distribution in another, and may exist in small, isolated populations in a matrix of highly unfavorable environments in another. A complication of the dendritic pattern of distribution in

P. leucopus has been noted by Thornton and Al-Uthman (1952). In the Texas Panhandle, this species is largely restricted to cottonwood groves along the major streams, but, since the groves are not continuous along the flood plains, there is a more or less linear distribution of small, semi-isolated colonies of usually less than a dozen individuals each. In southern Michigan, this same species is largely limited to wood lots of oak and hickory that were left intact when the land was cleared for farming, although the mice do range out into the edge of adjacent grasslands. Dice (1937) has shown that there is measurable differentiation between populations of wood lots no more than 8 miles apart.

McCabe and Blanchard (1950) reported that 3 species of *Peromyscus* (*maniculatus, truei,* and *californicus*) in the region of San Francisco Bay, California, show essentially linear distribution along the edge of the chaparral, although the mice may be missing from the "edges" where the chaparral changes gradually to other vegetation types. These workers reported that no *Peromyscus* were taken in dense and continuous chaparral more than 40 yards from the edge. The deer mouse (*P. maniculatus*) is the most widely distributed rodent in North America, and it occupies many kinds of environments within its range. At any given locality, however, the preference for certain environmental conditions results in a mosaic pattern of distribution. In northern Michigan, where this mouse is a forest inhabitant, Manville (1949b) reported population densities in 8 forest types over 3 seasons. Densities were highest in white-birch forest, with an estimated 4.25 to 11.04 mice per acre. They were lowest in jack-pine forest and black-spruce swamp, with none to 0.85 per acre, and the other forest types had intermediate densities. This species is a grassland inhabitant in central North America. In southern Michigan, deer mice are largely limited to bluegrass fields, where their home ranges rarely reach the edge of adjacent forests (Blair, 1940b), although Howard (1949) has evidence that they occasionally disperse through oak-hickory forest. Along the eastern border of the central grasslands in Texas and Oklahoma, the grassland habitats of this species are interrupted by the intrusion of forests on favorable soils and along streams. In New Mexico, this species occurs commonly in the high mountains, where it is abundant in the dense montane forests (Dice, 1942) and in the desert basins. Dice reported that it is rarely encountered in the sabinal belt of the lower mountain slopes, which would indicate little gene exchange between the montane and basin populations. The distribution of this species in the Tularosa Basin of southern New Mexico has been described by Blair (1943c). The deer mice are mostly limited to scattered areas dominated by mesquite and to grassy washes. Most of this desert basin is occupied by atriplex and creosote bush,

which are avoided by these mice, so the suitable environments for deer mice occur as isolated pockets in a matrix of unsuitable ones. The effects of this pattern of distribution on adaptive color differentiation of the deer mouse have been reported by Blair (1947). There was no differentiation in the frequency of adaptive color genes at a distance of 4 miles in a linearly distributed population, but there was strong adaptive differentiation on differently colored soils at a distance of 18 miles. Populations 13 miles apart and separated by a wide expanse of unfavorable environment for deer mice showed strong, adaptive differentiation on differently colored soils. Populations 20 miles apart and with gene exchange inhibited by a wide barrier of inhospitable environment, but on similarly colored soils, showed no adaptive differentiation in the frequency of color genes. Presently available information about the pattern of distribution in *Peromyscus* indicates that the distribution of genetic characters in the species population is strongly affected by the complex distribution of each species in many more or less isolated populations of various sizes.

Little accurate information is available about the size of the subpopulations of a species. Cursory observation indicates that freely interbreeding units may consist of only a few individuals, as in the *Peromsycus leucopus* populations of cottonwood groves in the Texas Panhandle. In other cases, there may be no limitation but distance on the interchange of individuals in a population of millions of individuals, as in the case of the same species in the brushlands of southern Texas (Blair, 1951b). A few estimates have been made of the size of subpopulations of small mammals. Dice (1939) estimated that the *P. maniculatus* population of the Black Hills included between 1,000,000 and 5,000,000 individuals in the late summer of 1935. The entire population of the Black Hills might be considered a major subpopulation, as Dice believed that the population of the Black Hills is to a large extent isolated. It is, nevertheless, divided into smaller subpopulations, for Dice recognized 10 vegetation types with varying population densities of mice and including grassland, pasture, and cultivated land in which he reported that deer mice were extremely scarce. Most estimates of the size of interbreeding populations are for insular populations. Hatt (1928) estimated that an island in Lake Champlain with only 1100 square feet of terrain had a total mammalian population of only 2 adult and 5 juvenile *Microtus pennsylvanicus* at the time of study. Manville (1950) estimated that a 9.5-acre island in Lake Huron had a population of 2 adult female *Peromyscus maniculatus* and 25 *Microtus pennsylvanicus*, exclusive of young in the nest. Blair (1946) estimated the population of *P. polionotus* on Santa Rosa Island, Florida, at between 9270 and

17655 in the winter and spring of 1941-42. McCabe and Cowan (1945), in analyzing the remarkable, post-Pleistocene differentiation of *P. maniculatus* on the islands off the coast of British Columbia, estimated the populations of the smallest islands at less than 100 individuals. On larger islands, they estimated populations of from a few hundred to 3000 mice.

VIII. Summary

The behavior in natural populations of rodents and other small mammals, as revealed by live-trapping and nest-box studies, theoretically has important effects on gene exchange in these populations. Attachment of the individual to a limited area of familiar terrain, or home range, tends to restrict gene dispersal. Estimates of home-range size for 39 species of rodents and for 9 other species of small mammals, although difficult to compare because of differences in technique, do show the widespread occurrence of the home-range habit. Size of the home range is affected by such attributes as sex, age, mobility, body size, and food habits and by such ecologic factors as vegetative cover, size of area, and season of year.

Defense of territory against others of the same species, or at least strong antagonism between individuals, is common in sciuromorph rodents but rare in myomorphs. Various cases of homing by small mammals that had been moved from the point of capture can be explained by return from within the home range or return from an area with which the animal made some acquaintance during the dispersal phase of the life history.

Dispersal of young away from the place of birth at the approach of sexual maturity is the most important means of dispersing genes through the population. The tendency to disperse may be inherent and it may be stimulated by sex hormones, but population pressures probably have important effects on the distance of dispersal. The little information available indicates that distance of movement is roughly proportional to body size; it also indicates that there may be a sex differential in dispersal distance and that the distance of movement may be quite short in small rodents.

The formation of permanent mating pairs, a reproductive pattern that should make for strong restriction on hybridization of sympatric species, has been reported from a few species of myomorph rodents. The average life span of small, prey species of mammals in nature is only a small fraction of the potential life span, which is taken to indicate high selection pressure on these animals through predation and through other selective agents. The pattern of distribution of any species of

small mammal is usually complex, and there is frequent subdivision into numerous, partially isolated subpopulations. Freely interbreeding subpopulations may sometimes consist of less than a dozen individuals.

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The Genetics of Polymorphism in the Lepidoptera

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CONTENTS

I. Introduction

There are many striking examples of polymorphism in the Lepidoptera, yet little is known of their genetics. This is the more surprising since the few instances which have been subjected to adequate genetic analysis have proved of exceptional interest. Some of this work was undertaken at Oxford, and we hope to continue it and to enlarge its scope in the new Genetic Laboratories now being developed there. Thus it seems appropriate to review the genetics of polymorphism in these insects before the start of this more extensive program. I have twice before undertaken such surveys. The first of these was restricted to the Lepidoptera but, having been published in 1937, is largely out of date. The second, though more recent (1945), covered animals and plants as a whole, and consequently the space which could be devoted to any one Order was limited. It is not my intention now to catalogue all the work which has been done on this subject, however superficial, but rather to describe and discuss the more important results which have emerged from it.

Advances in our knowledge of the polymorphism of the Lepidoptera have been retarded for several reasons. Unfortunately these insects are, in general, slow breeding. Great numbers of the species have but one generation in the year, and more than four generations is quite excep-

tional. Moreover, the most numerous and remarkable of the polymorphic forms occur in the tropics. These are often difficult to breed and the material difficult to obtain in temperate climates, while facilities for the necessary researches in their own habitats have so far been poor. There can be no doubt also that the Lepidoptera have proved unattractive to some geneticists because they do not provide good cytological material, their chromosomes being small and numerous. Furthermore, in many instances, the polymorphism is restricted to one sex, a condition which greatly handicaps genetic work. These considerations have delayed the study of a situation which, analyzed by a combination of genetic and ecological methods, is exceptionally well fitted to unravel and demonstrate certain aspects of evolution.

I have defined polymorphism (Ford, 1940a) as the occurrence together in one habitat of two or more discontinuous forms of a species in such proportions that the rarest of them cannot be maintained merely by recurrent mutation. A few words are needed to expand and explain that definition. In the first place, it excludes the following types of variation: (1) geographic races, though, of course, the occurrence of polymorphism in one district, and its absence or different nature in another, may be an important attribute of them; (2) "continuous variation" under multifactorial (or environmental) control. In a given locality a butterfly or moth may have an intergrading range of varieties falling within a curve of normal distribution. This is no more polymorphism than is the variation to be observed in human height in a freely interbreeding community; (3) the segregation of rare recessives; and (4) the appearance of heterozygous forms, both of which are normally eliminated by selection and maintained in population only by mutation pressure.

On the other hand, polymorphism as here defined, includes two distinct conditions:

1. A permanent or *balanced polymorphism* is maintained by a balance of selective agencies which favor diversity and oppose uniformity. This is exemplified by the instances in which a heterozygote is at an advantage compared with either of its corresponding homozygotes and also by those mechanisms which ensure outbreeding, such as heterostyly in plants or sex itself. Here it is necessary to remark that, though clearly falling within the definition of polymorphism, it is not my intention to discuss sex determination in this article, for that constitutes a special aspect of the problem which requires, and is usually accorded, separate treatment (See the studies of Goldschmidt, 1933, on *Lymantria dispar* L.). Neither is its fundamental analysis at all suitably restricted to the Lepidoptera, or indeed to any other single group. In fact, the success

of Winge (1932), whose work has so greatly advanced our knowledge in this field, is due largely to his freedom from the restriction of material, for it was by passing, with rare facility, from fish to flowering plants that he provided an experimental demonstration of the evolution of the XX,XY chromosome mechanism.

2. A *transient polymorphism* involves only the temporary diversity which occurs while a previously disadvantageous gene spreads and displaces its allelomorph. This is usually due to a change in environment or to a species colonizing some new habitat.

II. Balanced Polymorphism

A balanced polymorphism necessitates the existence of some "switch mechanism" which shall maintain alternative forms within the same population. This is unusually achieved genetically by the segregation of a pair, or at most a few pairs, of genes. The environmental control of polymorphism, such as that determining the development of a fertilized egg into a queen or a worker in the honeybee, is rare. No instance of it is known in the Lepidoptera, though some combination of the two types is inherently probable, since environmental seasonal variation of an extreme kind occurs in the group. Indeed, in some species the temperature difference, or other agency responsible for modifying the color-pattern, works so accurately that intermediates between the forms characteristic of spring and summer or of the dry and wet seasons are very infrequent (e.g., *Araschnia levana* L., Europe; *Precis sesamus* Trim., East Africa: both Nymphalidae).

When the members of a pair of allelomorphs are of equal survival value, the rarer can displace the commoner only at an exceedingly slow rate. If derived from a single mutation, the number of individuals which possess it cannot greatly exceed the number of generations since its occurrence (Fisher, 1930a), while the mutation rate is too low materially to accelerate the process. That is to say, the period of time involved is so great that the balance of neutrality must almost inevitably be upset by changing conditions long before the process is complete. Fisher (1930b) has shown that to be effectively neutral this balance must be remarkably exact. In consequence, it must not only be short-lived but also seldom attained. Therefore, even if the less common of two allelomorphs occupies no more than one or two per cent of available loci, we can be almost certain that it must have reached that frequency because it has some selective advantage. We have here only to except very small populations (of a few hundred individuals or less) judged over a brief period of time; nor will the genetic "drift" which can occur

in such circumstances contribute anything of importance to evolution, since its effects will be overwhelmed by selection as soon as the numbers rise (Fisher and Ford, 1947, 1950; Sheppard, 1951, and in press).

If a gene spreads unchecked through a population, the polymorphism involved is of the transient type. It can be permanent only when an advantage decreases and is converted to a disadvantage as the form possessing it becomes relatively commoner. It is not to be expected that the point at which the opposing selective agents are balanced will remain constant in all conditions, genetic and environmental. Thus, even in the same locality, the frequency of polymorphic forms may sometimes vary from season to season or it may fluctuate in relation to population size, for a species is in a different ecological situation when abundant than when scarce. Such changes in the frequency of the genes controlling a balanced polymorphism, owing to variation in their equilibrium position, have been little investigated (see, however, p. 74). On the other hand, though geographical variation does not constitute polymorphism, it may greatly affect it: a subject on which we possess much more information. A species may be monomorphic in some parts of its range and polymorphic elsewhere, while the forms constituting the polymorphism may differ in kind or in frequency from one locality to another. These situations will all be illustrated by the examples described in this article.

1. *The Control of Balanced Polymorphism*

The nature of the opposing selective agencies which give rise to balanced polymorphism is sometimes evident. Any outbreeding mechanism, such as heterostyly or sex, clearly promotes diversity, and so does Batesian mimicry. In other circumstances, we have little or nothing to show what are the advantages that are opposed to one another.

If equilibrium is reached when one of the controlling genes is at a low frequency (occupying a few per cent of the loci), we have effectively a dimorphism, segregating on the basis of heterozygote and one homozygote (assuming that a single pair of allelomorphs is involved). The other homozygote will be very rare, so that there will probably be little opportunity for dominance-modification to take place. Consequently, the heterozygote will usually be semi-dominant.* There may sometimes be physiological reasons why it approaches the rarer homozygote in appearance, though the needs of the polymorphism will tend to keep it distinct from the commoner one. Thus a single dose of a gene may so far check pigment production that two doses cannot double the visual

* There is, unfortunately, a tendency to describe any distinguishable heterozygote as "dominant," whether it is identical with one of the homozygotes or not.

effect; similarly, in the opposite direction, if a given quantity of pigment approaches visual saturation, the result produced by doubling the amount may be negligible.

An important consequence automatically arises from the fact that in polymorphism we frequently have the situation, not otherwise encountered, that heterozygotes may be common and the species adjusted to their occurrence, while one of the corresponding homozygotes is a rarity which does not play an essential part in maintaining the balance of variation. Genetic research in many different organisms has clearly shown that one of the most frequent types of mutation is the recessive lethal. Yet here is a situation in which such lethals can accumulate in a region of chromatin close to the semi-dominant gene, since this will, to a preponderant extent, be present in the heterozygous condition. Thus, assuming equal viability of all genotypes, when one form of a polymorphism is due to a semi-dominant gene occupying two per cent of available loci, its homozygotes will be approximately two hundred times as rare. Evidently the lethals will only appear in double dose and be eliminated by selection in the occasional homozygous phase of the dominant (or semi-dominant) gene controlling the polymorphism, and when separated from that gene by crossing-over. The protection accorded to the lethals diminishes as their distance from the dominant gene increases. Consequently, the rare homozygotes in a polymorphism tend to become inviable, and the process is a cumulative one, since such inviability augments the shelter given to the lethals. Therefore, in F_2 segregations it is usual to obtain an approach to a 2:1 ratio when a 3:1 ratio is expected. Moreover, it is frequently found in breeding such polymorphic forms that the rare homozygotes are delicate, infertile, or nearly unobtainable, but that occasionally homozygous individuals approaching normal viability appear. These, of course, result from a crossover which has separated the gene from some of its associated lethals.

If the heterozygous form in a polymorphism occupies a considerable proportion of the population (say 18 to 50%), the "rarer" homozygote would be quite frequent (1 to 25%), all other conditions being normal. It may be that the advantages of the semi-dominant gene have been improved by selection, so that it has spread through the population from a lower to a higher frequency—from a condition, therefore, in which it accumulated lethals because of the rarity of its homozygote. This may ensure that the homozygote remains quite uncommon and but semiviable even when the proportion of heterozygotes is high. In these circumstances dominance may still not have become complete.

The control of polymorphism in the Lepidoptera can now be illustrated from examples.

The European moth *Sterrha aversata* L. (Geometridae) is of a pale brown color and dimorphic in both sexes. There is an abundant plain form, *remutata* L., and a rarer one, *aversata*, in which the wings are crossed by a broad greyish-brown band. This seems to occupy about 5% of the population. Hawkins (1937), confirmed by Bergmann (1938), found that the difference in unifactorial, with the unbanded form homozygous and the banded normally heterozygous. The small amount of breeding work which I have myself been able to conduct on this insect shows that the rare homozygotes are in general similar to the heterozygotes but that their banding is rather more extreme, being blacker. However, the two genotypes overlap one another in appearance and are not always distinguishable. Thus the banded form is nearly, but not quite, dominant. I obtained only three F_2 broods. Since these proved to be substantially homogeneous, they may be combined, and give a total of 88 unbanded and 208 banded forms. These latter fall below expectation, though the difference does not reach significance ($\chi^2_{(1)} = 3.5$). It is likely, however, that with larger numbers the deficiency of homozygotes would be clearly established.

Cleora repandata L. (Geometridae) is monomorphic in eastern Britain but dimorphic in the west, including the extreme south and north of the country, where, in addition to the normal unbanded *C. r. repandata*, there occurs an alternative black-banded form *C. conversaria* IIb. This is an incomplete dominant, the ground color of the heterozygotes being grey or brownish and that of the homozygotes white (Williams, 1950). Most of the bred families have been of the backcross type, and F_2 broods are small and few in number. These have segregated in their correct proportions with the exception of one which produced 8 *repandata* and 11 heterozygotes, but no homozygous *conversaria*, which suggests that this genotype is sometimes defective. Cockayne reports a large count of wild specimens in Ross, northwest Scotland, amounting to 480 *repandata*, 141 heterozygotes, and 5 homozygous *conversaria*. Considering the small numbers of the latter class, no importance can be attached to the slight excess of heterozygotes over expectation. This species has an extensive distribution in Continental Europe, and *conversaria* is widespread there, but exact details of its occurrence are lacking.

Angeronia prunaria L., a member of the Geometridae, found in Continental Europe and in Britain, is a dimorphic species with a commoner unbanded form (*prunaria*) and a rarer one in which the orange (male) or yellow (female) ground color is reduced to a central band bounded by dark pigment. This is *corylaria* Thun., which is almost a complete dominant; but the homozygotes are distinguishable owing to the absence of dark striations in the pale areas (Williams,

1946-47). It is not known whether they are relatively inviable. The width of the band in *corylaria* is very variable, and Williams has shown that it is much affected by the segregation of other genes. By means of selection, he has, on the one hand, been able to reduce it to a small spot in the center of the fore wings, and, on the other, greatly to extend it.

Ellopia fasciaria L. is widespread in Europe and monomorphic, with a reddish-brown ground color, over the greater part of its range, including Britain. In some districts (*e.g.*, southern Germany) a green phase, *prasinaria* Schiff., also occurs which, though rarer, is common enough to indicate a dimorphism. Heydemann (1943) has shown that this is dominant to the brown *E. f. fasciaria.*

Xanthorhoë ferrugata Cl. (Geometridae), found in Britain and Continental Europe, exists in two forms: the broad bands on the fore wings are purple or black in the commoner and red in the rarer. Their proportions seems to vary considerably in different localities but have never been accurately assessed. Doncaster (1907) pointed out that the extensive breeding work conducted by Prout (1906) on this species indicates that the red-banded phase is dominant. Occasionally intermediates are found, and it is likely that such variation is due to the segregation of other genes affecting the expression of the one chiefly responsible for controlling the dimorphism.

The species so far mentioned are dimorphic only, but we have some information on the genetics of those with multiple phases. *Dysstroma truncata* Hufn. includes a complex assemblage of forms. Some of these are subject to continuous variation, others are rare aberrations, but a number of polymorphic conditions can be recognized among them. Some of these have been studied by Groth (1935). The dark form *nigerrimata* Fuchs is a semi-dominant; *N* being less, and *NN* more extreme. The grey *perfuscata* Stephens is common in its heterozygous phase (*P*), but the homozygotes, which comprise the dark-banded variety *albonigrata* Lempke, are very seldom seen. The frequent variety with an orange central area on the fore wings, *rufescens* Ström., is nearly a complete dominant (*R*), and Groth was able to show that the homozygotes (*RR*) are less fertile than normal and deficient in numbers. *Russata* Hb. is the triple heterozygote *nnpprr*. This is an exceedingly abundant and widespread European (and British) species. Its three genes so far studied apparently assort independently. It must be admitted that the data are not complete enough to exclude a loose linkage, but the large number of chromosomes in the Lepidoptera makes this inherently unlikely.

Bovey (1941) has undertaken a detailed investigation of the geo-

graphical variation and genetics of *Zygaena ephialtes* L. (Zygaenidae). This is found throughout Europe, including southern Russia, but excluding the Scandinavian countries, Britain, and the Iberian Peninsula. It occurs in two principal forms:

1. *ephialtes.* This is a blackish insect with five or six spots on the fore wings and one or two on the hind. Red or yellow markings are reduced to a band on the body and the two basal spots on the fore wings, the other spots being white.

2. *peucedani* Esp. In this form, the red or yellow coloring is extended to all the fore-wing spots and occupies the basal area of the hind wings also (on which the hind-wing spots can still be seen by transmitted light).

Red coloring is a simple and complete dominant to yellow, and *peucedani* is dominant to *ephialtes* except that the heterozygotes are more variable than the dominant homozygotes. These two pairs of genes are not linked.

Peucedani is found almost exclusively in the West (France), and *ephialtes* in the South, but over large parts of Europe the two occur together. Red and yellow specimens may be intermingled in *ephialtes,* but in some districts (Cyprus) only the red are found and in others (the Italian peninsula, for instance) only the yellow. Consequently, it is clear that in certain areas either form or either color may have a selective advantage in nature. It is a matter of much interest, therefore, that in one combination the two genes interact in a disadvantageous way, for yellow *peucedani* are always rare and local; they are absent save in a few places in central Europe and are found there only where red and yellow *ephialtes* and red *peucedani* form a mixed population. This is a striking instance of the selective effect of factor interaction.

A rather similar situation involves the spotting of the hind wings. Two spots are dominant to one; the allelomorphs concerned are not linked with the two pairs already mentioned. *Ephialtes* is dimorphic for this character, but two-spotted *peucedani* are unknown in nature, though they can be produced by the appropriate crosses in the laboratory. On the other hand, variation in the spotting of the fore wings is multifactorial, for there is no clear segregation when the five- and six-spotted forms are crossed.

In the instances so far mentioned, polymorphism affects both sexes, but it is often limited to one of them by means of sex-controlled inheritance. As indeed is to be expected (p. 68), species with polymorphic females and monomorphic males are far commoner than the reverse. We may take first an example of the latter kind, and then consider female polymorphism.

Parasemia plantaginis L. (Arctiidae) is a black and yellow insect (sometimes with the addition of red pigments in the female, see below) but in the males, though not in the females, the yellow color may be replaced by white, *hospita* Shiff. This is due to a sex-controlled dominant (Suomalainen, 1938). It is probable that the homozygous dominants are at a disadvantage in nature, but they are not detectably deficient in segregating families. This dimorphism is evidently related to the ecology of the insect, as all polymorphisms must be, and in a striking manner, for, though *hospita* occurs here and there throughout the range of *plantaginis,* it is found only on mountains, where it occupies a proportion of the males which varies according to locality, being about 5 to 10% in the Lake district of northern England, but much greater on some mountains in Continental Europe. Schulte (1952) reports that all the males are of the *hospita* form in a locality near Abisko, Swedish Lapland.

This same species also provides an example of the reverse type of sex-control, for the specimens marked with red, *rufa* Tutt., are all females. They are absent throughout the greater part of the species' range, but 15 to 20% of the females are of this form in some districts of north-eastern Scotland. Pictet (1938) states that they are dominant to the normal yellow, but it is desirable to confirm this in more detail and with larger numbers.

The Palaearctic butterfly, *Argynnis paphia* L., is monomorphic in the male sex, which has a rich brown ground color on the upper side. The females, however, are dimorphic in some localities, though monomorphic in others. The commoner, and in many places the only, form (*paphia*) resembles the male in coloring but is slightly more dusky. In certain areas, a second phase of the female, with a dull blackish-green ground color is also found. This is *valezina* Esp. In the New Forest in England it constitutes about 5 to 15% of the female population. Elsewhere in that country it is mainly of sporadic occurrence and has the status of a rare variety. A similar situation exists in Continental Europe, where the females are dimorphic in some districts only. Goldschmidt and Fischer (1922) showed that *valezina* is a heterozygous form, autosomal and sex controlled, and that the homozygous "dominants" are usually absent, so that the F_2 generation segregates in a 2:1 ratio. Yet during the course of their work they encountered homozygotes that were relatively free from linked lethals, and consequently they were able to produce broods in which all the females were *valezina* and to obtain segregation in a 3:1 ratio. It is not clear whether *valezina* is completely dominant, but certainly the heterozygotes and homozygotes closely resemble one another.

The significance of these facts will be evident from what has already been said, but two further points remain to be mentioned in regard to the female dimorphism of *A. paphia*. It is reported that all the females are of the *valezina* form in some parts of the southeastern Palaearctic region. The statement may or may not be correct. It is of the type often made on rather slender evidence and without verification from sufficiently large samples. It seems to me unlikely, but by no means impossible. Assuming the truth of it, we must suppose that the advantage possessed by this form is so marked there that it quite outweighs the counterbalancing claims of the normal females. In these circumstances, *valezina* would spread until the potential homozygotes comprised so considerable a proportion of the population that there will be heavy selection in favor of those of them which become separated from their linked lethals by crossing-over, as in the breeding work. The further accumulation of lethals would then be prevented, as they would, at a high frequency, be exposed to and eliminated by selection.

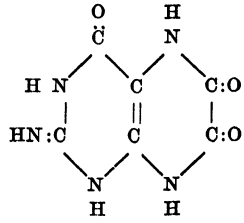
In addition, there is considerable though somewhat anecdotal evidence that this gene alters the habits as well as the appearance of the species. For the *valezina* females are generally thought to be the more shade-loving. *Argynnis paphia* is usually a woodland species, inhabiting sunny clearings and pathways, but experienced collectors believe that they are most likely to find *valezina* by hunting for it away from the normal form and within the shadow of the overhanging trees. I am, myself, strongly inclined to endorse this view, though I have no numerical data on the subject, which, in fact, might not be easy to obtain.

The genetics of habit, though obviously of importance, is a subject on which little information exists. For it is very difficult to study and our knowledge of it is restricted to those few instances in which genes having distinct physiological effects, by which their segregation may be followed, modify also the behavior of the organism. One possible example of this kind has just been cited, though it lacks confirmation; however, the work conducted respectively by Hovanitz on the butterfly *Colias chrysotheme* Esp. and by Sheppard on the moth *Panaxia dominula* L. provides precise data on the genetics of habit in the Lepidoptera.

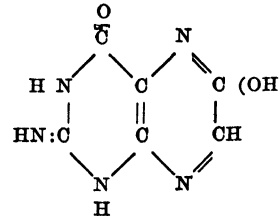
A dimorphism of a similar type is to be found in many species of the large genus *Colias* (Pieridae). In all these, the males are monomorphic, having an orange ground-color, while the females may have the same coloring as the male or else they may range from light yellow to pure white.

Unlike other families of butterflies, the white and yellow pigments of the Pieridae are uric acid compounds, though anthoxanthins are present in addition in some species of the subfamily Dismorphiinae

(Ford, 1941). The gene controlling the color-dimorphism of *Colias* converts the yellow xanthopterin to the white leucopterin (Ford, 1947).



Leucopterin



Xanthopterin

The pale females are nearly always the rarer, but their frequency varies from species to species, and between different seasons and localities. In *C. chrysotheme,* Hovanitz (1944c) has found occasional instances in which they are slightly the commoner form, but between 15 and 50% probably represents their usual frequency-distribution. In *C. croceus* Fourc., females of the pale phase rarely reach 40%, and 5 to 20% is more usual. In some of the other *Colias* species, the orange form is still more predominant and over 95% of the females are of this type.

In the few instances in which the genetics of this dimorphism have been studied, it has been shown that the pale females are, as expected, heterozygotes with dominance complete or nearly so (Hovanitz, 1944b & c). Indeed, the whole genetic situation conforms to the standard pattern for this type of polymorphism: that is to say, the phases are determined by a pair of autosomal but sex-controlled genes, and the rare (pale) homozygotes are difficult to obtain, owing to linkage with recessive lethals from which, however, during the course of breeding experiments, they have on a few occasions been freed by crossing-over. These facts have been established in *Colias chrysotheme, C. myrmidone* Esp., *C. croceus,* and *C. christina* Edw. (Gerould, 1923; Hovanitz, 1944c).

Hovanitz (1944b) shows that the difference between the two chief "races" of *Colias chrysotheme* (*philodice* Lat., and some others, yellow; *eurytheme* Bsd., orange) is multifactorial, the hybrids being generally fertile. These two "races" have different optimum diets as larvae, and the yellow can pass into a diapause in the middle of larval life while the orange cannot.

In *chrysotheme* the pale form is generally white, but in some other species (e.g., *C. croceus*) that phase includes also a considerable proportion of cream-colored and pale-yellow specimens. This minor variation within the ambit of the one major gene is certainly in part genetic, for the progeny of different females reared in identical conditions may be

markedly distinct, some of the families containing a much higher proportion of the light yellow specimens than others. That is to say, the pale phase is evidently slightly affected by the segregation of numerous other genes (which in this respect act as "modifiers" of it) against which the males and the normal females are buffered. The study of these modifiers is indeed hampered by that fact, since the constitution of a male parent cannot be assessed by inspection. Useful information could, however, be obtained by mating the same male to several pale females of distinct types; and in the Lepidoptera it is usual to find that a single male can pair successfully a number of times.

A very few white males of some of these *Colias* species have been reported, but we know nothing of their genetics and they may not all originate in the same way. They may be a form entirely distinct from the white females and controlled by a different major gene, or they may be due to a rare genetic or physiological condition which extends to the opposite sex the action of the sex-controlled gene for pale coloring.

Hovanitz (1948) has obtained valuable results by studying the ecology of the dimorphic females of *Colias chrysotheme*. He was able to supply clear evidence that the gene responsible for the color-phases affects also the habits of the butterfly, making the pale form relatively the more active during the early part of the day. If the times at which the specimens are caught are recorded, it becomes clear that the frequency of the white specimens is highest in the early morning and declines towards noon. It sometimes rises detectably also in the late afternoon.

It seemed probable that these daily changes in the activity of the color-phases are related to variations in such physical factors as solar radiation, temperature, and humidity. Hovanitz found that the curve of relative frequency of the orange and white females of *C. chrysotheme* neither accords with that for amount of solar radiation or for temperature alone. The fit is reasonably good in the morning but not in the afternoon. There is, however, a fairly close association between the total activity of the females (orange and white together) and a combination of temperature and solar radiation. That is to say, the activity is greatest as the conditions pass from high temperature and low solar radiation to low temperature and high solar radiation. The activity declines with an increasing tendency towards low temperature and low solar radiation, or towards high temperature and high solar radiation. Since, therefore, these factors evidently combine to influence the total activity of the females, it is probable that they are important in controlling the relative behavior of the two phases. More extensive data may thus de-

termine the exact environmental agencies which interact with the genetic dimorphism to modify the habits of this insect. In the localities where Hovanitz worked, relative humidity is so near to being inversely proportional to temperature that it was not possible to distinguish its influence on the behavior of the two forms of the female.

It appears that both of them increase in activity as the morning advances, but that the white phase does so less than the orange, so altering the proportions in which the two are to be caught. Thus, the white females tend to lengthen the period but reduce the degree of the insect's activity, whereas the yellow ones tend to concentrate it towards midday. The optimum balance between these effects will depend upon many factors in the ecology of the organism, but in this instance we can at least apprehend something of the opposing advantages which maintain a permanent dimorphism. Clearly we should not expect them to reach equilibrium at the same value in widely different environments. Hovanitz also has studied this aspect of the problem. He finds that the white females are relatively commoner in northern than in southern populations (1944a, 1944c) and at higher than at lower elevations (1945). Thus, such a dimorphism may be important in adjusting a population to different parts of its range. In this connection it is worth noting that Hovanitz (1944b) has shown that the genes controlling it affect not only color and activity but also time of emergence. On the average, the pale females of *Colias chrysotheme* appear in advance of the orange in each brood. This seems to indicate that they may have the more rapid developmental rate, a property which may be at a premium in some of the cooler localities.

The color dimorphism of female *Colias* is so similar in its effect on pigmentation, and probably in its genetic control, in the many species in which it occurs that it may well be associated also with differences in imaginal activity in all of them. Of this, however, we have no more than an indication, not amounting to proof, in one other species, the European *C. croceus*. Having heard personally from Hovanitz of his illuminating results in advance of their publication, Dowdeswell and I collected that species in southern England during 1947, recording the numbers of orange and pale females caught at different times of day. We were primarily engaged in other researches and were on our way to work on an uninhabited island, so that we could afford little time for such collecting. Moreover, though that season was one of the few in England in which *C. croceus* is common enough for such observations to be possible, it was far rarer than is *C. chrysotheme* in the Californian localities where Hovanitz worked. As a consequence, our numbers proved insufficient. Combining captures prior to 11 A.M. and after 5:30

P.M., we obtained in all 44 females: 28 orange and 16 pale. We had less time available between those hours, but during the period from noon to 3:30 we caught 24 females: 20 orange and 4 pale. The difference, 19.8 ± 10.5%, approaches but does not reach formal significance. Since, however, the departure from equality is in the same direction as that found by Hovanitz, it is likely that larger numbers would establish a similar dimorphism in activity between the two color phases of the female *C. croceus* (Dowdeswell and Ford, 1948, we purposely delayed publishing until the relevant paper by Hovanitz had appeared).

A further example of a gene affecting the habits of an insect is provided by the work of Sheppard (1952) on the moth *Panaxia dominula* L. (pp. 72-75), for he has shown that the *medionigra* gene influences the mating preferences in this species. Pairings prove to be significantly more frequent between unlike than between like forms in all three genotypes: a situation itself capable of maintaining a polymorphism.

2. *Mimetic Polymorphism*

As already indicated, the interplay of different habits between the phases of a polymorphism throws some light on the maintenance of diversity in the species concerned and helps us to understand why one of the controlling allelomorphs does not displace the other. Batesian mimicry, however, demonstrates more clearly one of the ways in which a balance of opposing advantages may be maintained permanently. Here we have a situation in which a relatively palatable form, the mimic, gains some protection from predators by copying a relatively distasteful or dangerous one, the model, which advertises its qualities by means of warning coloration.

As the numbers of a mimetic form increase relative to their model,* owing to the advantage which they gain from resembling it, so that advantage wanes and is finally converted to a disadvantage. This point is reached when a given conspicuous pattern tends to be associated with something unpalatable rather than edible. Thus, any particular mimic must remain at a low density compared with its model while, on the other hand, a mimetic species can escape from the impasse so created, and increase its density, by having polymorphic forms some or all of which copy different models. Their proportions will then be adjusted until they all possess an equal advantage. Therefore, we may expect Batesian mimics usually to be polymorphic, and that is what we find.

Why they are not always so, it is impossible to say; doubtless each instance would need to be judged on its own merits. One can only re-

* Such an increase may of course be due to a change in the frequency of the forms of a polymorphic mimic rather than to an increase in its absolute numbers.

mark that the evolution of living organisms has by no means always been able to achieve optimum results and that, as is well known, a general survey shows it to have been far more often a failure than a success. In such forms as the Sesiidae among moths, monomorphic species of the genus *Sesia* have achieved a striking general resemblance to hornets and wasps, involving extreme transformation of the superficial characters of the Lepidoptera. Perhaps it has not been possible to maintain so great a change within the sphere of action of one or two major genes which can act as a switch in determining alternative forms. However this may be, it is sufficient for our purpose to note that Batesian mimics are very frequently polymorphic, while Müllerian mimics are not.

Müllerian mimicry is worth brief mention for the contrast which it supplies in this respect. It involves a resemblance between a number of relatively protected species which, by their uniformity of appearance, reduce the depredations made by predators when gaining experience, since a single lesson of distastefulness is shared in varying proportion by the whole group. In such circumstances, diversity is evidently opposed, and we should expect these insects to be monomorphic, as indeed the facts confirm. It may be remarked that the resemblance of Lepidoptera to inanimate objects (such as, leaves, bark, or lichen) clearly places no check upon numerical increase (as that to another animal is bound to do), and consequently does not lead to polymorphism, since such abundance as threatens the usefulness of this type of resemblance can hardly be conceived.

Batesian (but not Müllerian)mimicry thus tends to produce polymorphism. Yet there is some evidence that it is associated with some relative inviability or infertility of the homozygous dominants. This would have been thought surprising at one time, as it might seem irrelevant to the situation responsible for the diversity, which is purely an ecological one. From what has already been said in this article, however, it will be apparent that, even so, the genetic barrier here mentioned may actually be expected to arise from it.

The African Nymphaline butterfly *Hypolimnas misippus* L. has monomorphic males, the appearance of which is characteristic of the group to which it belongs, while the females are utterly different and resemble *Danaus chrysippus* L., a species feeding on *Asclepias,* and shown on many occasions to be distasteful. Moreover, they are dimorphic. *H. misippus misippus* and *H. m. inaria* Cr. mimic, respectively, *D. chrysippus,* with white markings in the apical area of the fore wings, and *D. c. dorippus* Klug. in which these white marks are absent. It has long been known that both female forms of *H. misippus* are maintained

by a single pair of autosomal and sex-controlled genes (Carpenter & Ford, 1933). *H. m. inaria* is a homozygote, and *H. m. misippus* is normally heterozygous. It is usually described as a "dominant" to *inaria*, and that is doubtless true in a broad sense, though I question if the evidence exists to determine whether or not the dominance is complete. Nearly all the bred families so far obtained are derived from females fertilized in nature (and it must be remembered that even when the cross is made in captivity the genotype of the male cannot be determined by inspection). A number of these have segregated in a 1:1 ratio, but four families are available which appear to be derived from a mating between two heterozygotes. These may be summarized as shown in Table 1.

TABLE 1.

F_2 Segregation in Four Broods of *Danaus chrysippus*

misippus	*inaria*	*Total*		χ^2	
38	18	56		1.5	
12	7	19		1.6	4.5
19	8	27		.3	
30	14	44		1.1	
99	47	146	→	4.0	

The results show a close approximation to a 2:1 instead of a 3:1 ratio. The difference, which exceeds formal significance ($\chi^2_{(1)} = 4.0$) is, with fair probability, real. But we are faced with the difficulty that, while each of the three larger families differs significantly from a 1:1 ratio, the smallest does not, and consequently it may be a deviation from this rather than from 3:1. The four families, however, are exceedingly homogeneous (for the heterogeneity test $\chi^2_{(3)} = 0.5$). Consequently there is reasonable ground for combining them for a total, and the departure of this (99:47) from equality is very significant ($\chi^2_{(1)} = 18.6$).

The genetic control of multiple polymorphism can best be exemplified by the oriental *Papilio polytes* L. The males are monomorphic, the females polymorphic, and three forms of them have been studied genetically (Fryer, 1913). One of these, *cyrus*, Fab., is non-mimetic and resembles the males. Another (*polytes*) is a good mimic of the abundant *Polydorus artistolochiae* Fab., while the model for the third (*romulus* Cr.) is *Polydorus hector* L. Where both models are found, as in Ceylon, so are all three phases of the females; where *hector* is absent, as at Hongkong, so also is the *romulus* female of *P. polytes*.

A single sex-controlled gene *P*, dominant in effect, converts *cyrus* into *polytes*, and a second gene *R*, also sex-controlled and dominant, converts *polytes* into *romulus* but is without effect unless *P* is present. Thus, not only is an individual of the constitution *pprr* phenotypically *cyrus*, but so are the *ppR-* genotypes. *Polytes* is genetically *P-rr*, and *romulus* is *P-R-*.

It will be seen, therefore, that *polytes* females could be monomorphic (*cyrus* only), dimorphic (*cyrus* and *polytes*), and trimorphic (*cyrus*, *polytes*, and *romulus*). Evidently they could not easily sustain a di morphism including only *cyrus* and *romulus* and excluding *polytes*. Since, however, the distribution of *P. hector* is far more restricted than that of *P. aristolochiae* and falls wholly within it, a *cyrus*, *romulus* dimorphism is not required. On the other hand, the range of *P. aristolochiae* everywhere extends also beyond that of *P. polytes*, so that the monomorphic condition in which the females of the latter species are *cyrus* only does not seem to exist, though there is nothing in the genetic mechanism to exclude it.

In his extensive work on this butterfly, Fryer encountered many sterile matings. Fisher (1927), who subjected his data to detailed analysis, showed that the simplest explanation of these lies in a reduced fertility of the homozygous dominants. Thus, in both *Hypolimnas misippus* and *Papilio polytes*, the only mimetic Lepidoptera for which appropriate evidence seems available, there is reason to believe that a genetic sterility mechanism is included within, and has arisen from, a polymorphism which depends wholly upon the ecology of the species concerned.

Taking *Hypolimnas misippus* and *Papilio polytes* as examples, the control of polymorphism in a number of other mimetic or probably mimetic butterflies can briefly be compared and contrasted with them.

Both sexes of the African *Hypolimnas dubius* Beauv. are mimetic and dimorphic, the two phases being exceedingly dissimilar from one another. On the west coast, one form, *dubius*, copies *Amauris psyttalea damoclides* Stand., while the other, *anthedon* Dubl., is a striking mimic of *Amauris niavius niavius* L. These have been selected to resemble other models on the east coast, where those available in the west are not found. The black-and-white *dubius* is converted into *mima* Trim., which has a similar, though not identical, pattern but has a yellow area at the base of the hind wings in mimicry of *Amauris albimaculata* Btl. and *A. echeria* Stoll. *Anthedon*, on the other hand, retains the same model but follows its geographical race, *A. niavius dominicanus* Trim., by extending the white basal patch on the hind wings and is then known as *wahlbergi* Wallenger. Unfortunately, information on the genetics of this dimorphism is slight and imperfect, though it should be easy to obtain

since the sexes are similar, while the combination of mimetic dimorphism and geographical variation would make the work especially rewarding. It is clear that a simple genetic switch-mechanism, involving one pair of allelomorphs, operates in both regions, but their dominance-relations have not been properly established. Lamborne (1912a) bred large families from two female *anthedon* which had been fertilized by unknown males; one consisted of *dubius* only and the other of *anthedon* only. This is consistent with the view that *anthedon* is recessive and that the homozygous dominants, though almost certainly rarer than expected, are by no means absent. On the other hand, Platt (1914), working in the east, found two *H. d. wahlbergi* in copula, from which he raised 104 *mima* and 94 *wahlbergi*. Apart from the peculiar ratio, this, taken at its face value, indicates that *mima* is recessive. However, it would at least be very strange if the corresponding forms had reversed their dominance on the two coasts, especially as there is no complete geographical isolation between them. It is probable that Platt witnessed an event not infrequent in butterflies, but perhaps rarer in moths—the second pairing of a fertile female. This often seems to be ineffectual unless she has used up her store of sperm towards the end of her life, but the large size of this brood does not endorse the view that such had happened here and we may reasonably assume that the male which Platt saw was not the one which had impregnated the female, which must have been a (heterozygous) *mima*. This is really proved by the fact that the segregation is a clear 1:1 ratio instead of 3:1, or probably an approach to a 2:1 ratio, which should have been obtained were the two *wahlbergi* heterozygotes and the observed male the genuine parent. The deviation, even from a 2:1 ratio, is very significant ($\chi^2_{(1)} = 17.8$). Thus it is almost certain that *anthedon* and *wahlbergi*, which correspond to one another, are both recessives.

All the models in this instance belong to the Danaidae, a family well known to be distasteful (Aurivillius, 1910). The mimicry involves both color and pattern; but there is good evidence that it extends also to habits, though this is not of the accurate quantitative type obtained by Hovantiz for *Colias chrysotheme*, a work which has not yet been paralleled in the polymorphism of the Lepidoptera. Leigh (1906) and Platt (1914), both naturalists of extensive experience in South Africa, have independently discussed this subject. Combining their information, in which they corroborate one another on the points which they discuss in common, we find the following: *wahlbergi* is sun-loving and has a floating flight very like its model; it rests on the upper sides of leaves and emerges from the pupa in the morning. *Mima* tends to take short and more rapid flights and to settle much in shady places, where it rests

on the undersides of the leaves. It emerges from the pupa in the afternoon. It is interesting to note that when Leigh wrote it it had only recently been suggested that these are two forms of a single species (a fact which is, of course, now fully established). However, he, as an experienced naturalist, was so much impressed by the distinctions between them that he strongly maintained that *mima* and *wahlbergi* must be specifically distinct.

The genetics of the North American *Papilio glaucus* L. are much in need of study. In this species, the males are everywhere monomorphic, being yellow with black markings. The females, however, are monomorphic and malelike (the form *turnus* L.) in the nothern part of its range, but dimorphic from about New Jersey southward to Florida, where, in addition, a second female form (*glaucus*) also occurs. This is a blackish insect, thought to be mimetic of *Battus philenor* L. So far, the results of breeding from this species appear to be imperfectly recorded. However, it has frequently been reported that *glaucus* females produce either their own form alone or else broods composed both of *glaucus* and *turnus*. As Cockayne (1932) points out, it is probable that we have here a dimorphism based upon a single pair of sex-controlled genes with *turnus* the recessive. If so, the situation is similar to that found in *Hypolimnas misippus*, and we should expect to see a deficiency of the homozygous dominants.

Further information on the more complex polymorphisms involving mimicry is scanty. However, the genetics of *Papilio memnon* L. were rather thoroughly investigated by Jacobson (1909) and were subsequently analyzed by several geneticists; what is certainly the correct interpretation of them was formulated by Baur (1911).

The males of this insect are monomorphic and non-mimetic, and in Java, where Jacobson worked, a number of female forms, some mimetic and some non-mimetic, are found. However, he investigated only three of them. The commonest of these, *laomedon* Cr., somewhat resembles the monomorphic males. A sex-controlled gene (*I*) dominant in effect, converts this to *isarcha* Seitz.* These two female forms are non-mimetic and neither of them, nor the males, have tails. Yet a second sex-controlled gene (*A*) converts *isarcha*, but not *laomedon*, to the tailed mimic, *achates* Sulzer, which copies *Poydorus coon* F. Thus, the genetics of *laomedon*, *isarcha*, and *achates* in *P. memnon* correspond respectively with those of *cyrus*, *polytes*, and *romulus* in *Papilio polytes*. Yet we know less of the situation in *P. memnon*, since it has not been established whether the homozygous dominants are detectably deficient compared

* It is incorrectly described as *agenor* L. by Jacobson. Cockayne (1932) points out that this does not occur in Java and that the form intended is probably *isarcha*.

with the other two genotypes. The males and all the females of *P. polytes* are tailed, but it is noteworthy that in a tailless species like *P. memnon* the gene *A* (interacting with *I*) produces, in *achates,* a form which copies not only the color-pattern, but also the tails of its model, *P. coon.*

Probably the most complex instance of mimicry, and of polymorphism, in the Lepidoptera is provided by *Papilio dardanus* Brown, which occurs throughout the Ethiopian region but not elsewhere. Extensive breeding experiments have been conducted on this species, and I have collected the results and analyzed them in some detail (Ford, 1936). Yet in spite of all that has been done, our knowledge of its genetics is slight. This is due to several causes: the work has been haphazard or ill-planned; most of the data relate to the offspring of females which had mated before capture and later generations resulting from pairings arranged in the laboratory have seldom been obtained; also, the males are always monomorphic, the genes concerned being sex-controlled and expressed in the female only, and this, as always, is a considerable barrier to genetic investigation.

Papilio dardanus forms eight subspecies, all of which interbreed and merge into one another at their boundaries, except for the Madagascan ssp. *meriones* Felder. The males are always tailed and, as already indicated, monomorphic. The females, however, comprise an extraordinary assemblage of polymorphic forms. In a few areas they are tailed, malelike, and non-mimetic. Generally, however, they are mimetic, entirely different from the males, and, like their models, tailless. A very few tailed mimics are also known (in ssp. *antinorii* Oberthür from Abyssinia and Eritrea, though in this race the females are generally male-like). Unfortunately, we know nothing of the genetics of these, nor of their relation to the normal tailless mimics with some of which they otherwise correspond. There are also several female forms which, though without tails and very different from the males, are non-mimetic. But some of them may be rare varieties, maintained by mutation and eliminated by selection and not polymorphic at all.

On the west coast of Africa nearly all the females are of the form *hippocoon* F. and they mimic *Amauris niavius niavius.* A single gene converts this into the heterozygous form *dianysos* Dbl., which is rare and non-mimetic. In the east, *cenea* Stoll. which mimics *Amauris echeria* and *albimaculata* is dominant both to *trophonius* Dbl., which copies *Danaus chrysippus,* and to *hippocoonides Haase.* This latter is an adjustment of the western *hippocoon* to copy the eastern form, *dominicanus,* of *A. niavius;* just as we have seen *Hypolimnas dubius* modifies its western and eastern mimics, *anthedon* and *wahlbergi,* to correspond also

with the two geographical races of that same model. A single factor converts *hippocoonides*, but not *cenea*, into *trophonius*, but its dominance relationships are uncertain. Thus, in general terms, these three forms are controlled in the same way as the three forms both of *Papilio polytes* and *P. memnon* which have been analyzed genetically. *Hippocoonides* is recessive to the dominant (or semi-dominant) and non-mimetic "*proto-salaami*," of which *poultoni* Ford is a genetic modification. Only vague indications here and there have been obtained in regard to the genetics of the many other female forms of *P. dardanus*.

The African butterfly, *Pseudacraea eurytus* L. (Nymphalidae), is a remarkable and highly complex mimic. Unfortunately nothing is known of its genetics, though it would be a very suitable species to study, since in a number of the forms the sexes are similar. Consequently, if carried out on these, the work would not be impeded by the difficulty that the genotype of the male cannot be detected by inspection. It should, however, be emphasized that it would be better policy to undertake a thorough genetic analysis of one of the simpler instances first, such as *Hypolimnas dubius* so well provides, before attempting one of these very complex examples.

3. *The Evolution of Mimetic Polymorphism*

In mimetic polymorphism we are faced with two sets of facts which are at first sight contradictory. The forms may show detailed resemblances to their respective models in such characters as color, pattern, shape, and habits. Yet they are controlled by simple genetic switch-mechanisms, involving only a pair of allelomorphs for a dimorphism, the differences between which must have arisen suddenly by mutation. Now it would be difficult to imagine that species have had to await the fortuitous occurrence of a gene giving the correct set of resemblances to a butterfly belonging to a different family had this occurred only once or twice. That they have had to do so in the numerous instances involved in polymorphic mimicry is beyond belief.

It has long seemed that this paradox is to be resolved in the manner originally suggested by Fisher (1927), that the *effects* of the switch genes have been modified since their first appearance by selection acting on the gene-complex. This view has been considerably developed subsequently (e.g., Carpenter & Ford, 1933; Fisher, 1930a; Ford, 1937, 1945). Moreover, a number of experimental tests have now been conducted which demonstrate the possibility of altering the effects of the genes by selection in this way (e.g. Morgan, 1929, eyeless in *Drosophila;* Fisher, 1935, 1938; Ford, 1940b; Fisher and Holt, 1943).

Furthermore, genetic variability within the ambit of a particular

gene has repeatedly been encountered in these mimetic polymorphisms, and this provides precisely the opportunity for the selective adjustment of the mimicry which is here envisaged. For example, rare intermediates between the two forms determined by a pair of allelomorphs, one heterozygous and the other recessive, are well known to collectors. But in several instances it has been possible to breed from them and to show that they are, in fact, genetically determined, and, therefore, susceptible to selection, and not environmental intermediates. Thus Lamborne (1912b) captured a female of *Hypolimnas dubius* whose pattern was transitional towards the recessive *anthedon*. From it he bred 4 *dubius* also transitional towards *anthedon* and 3 normal *anthedon*. Leigh (1904) obtained a female *Hypolimnas misippus* intermediate between *misippus* and the recessive *inaria*. Of the 8 females which he bred from it, 4 were *misippus*, 3 were *inaria*, and 1 was an intermediate. Many examples of this kind in *Papilio dardanus*, some involving large families, are given by Ford (1936).

Now genetic variability is an equilibrium condition between mutation and recombination tending to increase it, and selection tending to diminish it. Consequently, if selection is reduced, the variability should increase if its existence has been essential in establishing the polymorphism concerned, while a rare and non-mimetic mutant should be more variable than an established mimic when both are controlled by single genes. The uniformity of each of the mimetic phases of a species is indeed striking, even though they may differ from one another to an extreme degree; it is in great contrast to the notably unstable patterns of rare varieties (e.g., the non-mimetic and highly variable form *dionysos* of *Papilio dardanus*, compared with the mimetic females of that species in the same area; Ford, 1936).

The important fact that mimetic forms maintained by single genes are variable when their mimicry becomes ineffective is shown by two extensive pieces of random collecting within the range of *Papilio dardanus* in Africa. In this work every butterfly seen was caught, as far as possible. It was carried out at Entebbe, where models of *P. dardanus* are common, and in the mountainous region near Nairobi, where they are rare. The numbers of female *P. dardanus* and their models found among the vast mass of specimens obtained at each place are given in Table 2.

The data show that at Nairobi the models are 73 times rarer, relative to their mimics, while imperfectly developed forms of the mimics are 8 times commoner, than at Entebbe. A similar type of association between the variability of a mimic and the frequency of its models has

TABLE 2

Frequency of Imperfect Mimics, Relative to Models, in Female *Papilio dardanus*

Locality	*Models* (totals)	*P. dardanus* (♀) *mimics*	
		Totals	% imperfect
Entebbe	1,949	111	4
Nairobi	32	133	32

been demonstrated by Carpenter (1913) in his study of *Pseudacraea eurytus* on Bugalla Island and the mainland of Africa.

It is therefore concluded that when any mutation chances to give an unprotected species some slight resemblance to a protected one, the mutant gene not only spreads relative to its allelomorph, owing to the advantage which it confers, but its effects are gradually improved by selection operating on the gene-complex, just as are cryptic resemblance to leaves or lichen. With Batesian mimicry, however, the original mutant may remain as a switch gene controlling alternative forms, the need for which has already been explained, since selection can still work upon the variability which modifies its expression. That is to say, when two mimics are controlled by a pair of allelomorphs, one of these must have mutated spontaneously from the other, yet such spontaneous mutation is consistent with the gradual improvement of the mimicry by means of natural selection.

Punnett (1915) had realized the evolutionary difficulty involved by the fact that complex mimetic resemblances may be controlled by a single pair of allelomorphs. He had attempted to explain this on the basis of parallel mutation, assuming that the same gene, evoking the same set of effects, had mutated both in mimic and model. On further study, the impossibility of such a suggestion is evident, for the resemblances involved are entirely superficial, such as deceive the eye and no more, and they are generally brought about by quite different means in the species concerned. For example, the Australian wasp, *Abispa ephippium* F., which is brown with a transverse black abdominal band, is mimicked by two longicorn beetles. In one of them, *Tragocerus formosus* Pascoe, the pattern is copied on the elytra, but these are short in the other species *Esthesis ferrugineus* Mackay, and the mimetic coloring is on the exposed abdomen (Carpenter, 1946). Also, the only thing which the red marks of *Papilio polytes romulus* have in common with those of their model *Polydorus hector* is their "redness," for the pigments producing them are chemically different, as almost universally

throughout these two great genera between which so much mimicry exists (Ford, 1944). Since it is impossible to appeal to resemblances which are only superficial as evidence of genic identity, the explanation of mimicry advanced by Punnett fails. Moreover, it is not competent to account for other essential features of the situation: for example, the variation of mimics outside the range of their models.

A modified form of Punnett's theory has, however, been advanced by Goldschmidt (1945). As I believe his account contains numerous errors both of fact and of theory, it is necessary briefly to discuss it.

Apparently his view is that the color-patterns and shapes of butterflies, though they belong to different Families, are so circumscribed as to permit only phototypic changes of a definite type such that the mutation of different genes, having different effects, will sometimes bring about approximately the same appearance. Thus the correspondence between a mimic and model, involving the many characters responsible for mimicry (the existence of which as a protective agency Goldschmidt does not question) is, on his view, due to the mutation of a single gene. This he says (p. 215) "we may call parallel mutation as seen from the angle of the phenotype." Such a concept leads him to certain wide conclusions in essential conflict with those of Fisher and myself: that mimetic resemblances have arisen at a single step by mutation, without general selective adjustment, and that the differences between the geographic races of mimics (and models) must be due to multiple allelomorphs at the same locus. Some criticism of the foundation on which these conjectures rest is now desirable.

It is unfortunately necessary to point out that in his article Dr. Goldschmidt has failed to read with sufficient care passages which he has criticized. He accuses me of regarding a segregating family of 8:5 as a significant departure from a 3:1 ratio, which it obviously is not, when in fact I made it perfectly clear that I was discussing a situation in which the recessives formed the larger class, so that the comparison is not 8:5, but 5:8, against 3:1. If Dr. Goldschmidt reworks his calculation, he will find that such a departure from expectation will be obtained by chance less than once in five hundred times. His misquotation is somewhat surprising, since I actually gave the χ^2 involved (Ford, 1936, p. 449). I should like to take this opportunity of saying that Dr. Goldschmidt twice refers to me as a former student of Prof. R. A. Fisher's, which I never was, with the implication that I am, therefore the more ready to accept his views.

At the outset, it is clear that Goldschmidt has not appreciated the nature of balanced polymorphism. He actually says (p. 163): "In some instances there are found non-mimetic and mimetic females. One would

expect the non-mimetic females to disappear." Of course not: as already explained, the polymorphic forms, whether mimetic or non-mimetic, must be balanced at such frequencies that they all receive equal protection. The advantages of the non-mimetics will be in other directions, such as those which maintain the many instances of polymorphism (already described) in the species which are wholly non-mimetic. Were the mimetic forms to displace the non-mimetic in those forms in which both occur, the advantage which they derived would decline until converted to a disadvantage.

Goldschmidt is here appealing to an original mutation having a big effect, one which produces the full mimetic characters at a single step. In the first place, he fails to see that a slight resemblance, subsequently to be improved, can be of any advantage when it initially occurs. The point of view which requires a limen of advantage before selection can become operative is, of course, well known to mathematical evolutionists and long ago disproved. Thus Fisher (1930a) shows that even a 1% selective advantage "may well have to occur 50 times, but scarcely in mature individuals as many as 250 times, before it establishes itself in a sufficient number of individuals for its future prospects to be secure." When we consider the probable number of specimens comprising a butterfly species, it may well be that a given gene, with average mutation rate, may be presented to the population every ten generations or so. The significance of small selective advantages is entirely missed in Goldschmidt's criticism.

On the other hand, he fails to appreciate the difficulty of using genes with big initial effects which greatly influence the physiology and genetic adjustment of the organism. For, since it is far easier to damage than to improve a highly organized system, such as the body of any animal, mutations will usually be disadvantageous, and the chances of their action so fitting in with the physiology of the organism as to promote harmonious working become less, the greater their efforts may be. Thus Goldschmidt says: "Such frequent types of variation found in nature as melanism, leucism, xanthism, rutilism, are known to have occurred in laboratory conditions as ordinary mutants with the maximum effect accomplished in one step. Therefore, nothing else is required in nature either." But something else most certainly is required, for such laboratory mutants have almost always proved at so heavy a physiological disadvantage compared with the normal that this is readily evident in genetic work (the poligenes, which have small cumulative effects, excepted). Almost all the laboratory mutants of *Drosophila melanogaster* are below normal viability, nor are they on theoretical grounds likely to reach it. Consequently, the mutants with big effects,

to which Goldschmidt appeals, will generally be unusable. Thus his remark that the fortuitous occurrences of a suitable mutant, provided the proper developmental system is present, has as much chance to happen as the initial mutation with slight effects required by the selection theory is not correct. The words "the proper developmental system" deserve attention, for since it is not contemplated that the same set of genes (or physiology) occur in mimic as in model, the environmental background provided by the two species is distinct. Moreover, Goldschmidt is mistaken in thinking (p. 163) that variation in the expression of a gene must be produced only by specific modifiers of that gene which, as he says, "involves the assumption that modifiers of all kinds of changes are always available for selection." On the contrary, genes interact with one another and have multiple effects, so producing a variable gene-complex against the background of which each must act (Ford, 1949). It is upon the genetic variability of the gene-complex that the selection here envisaged operates. When the effects of the "eyeless" gene in *Drosophila,* of the gene for crest in poultry, of that for *lutea* in *Abraxas grossulariata,* or of the gene responsible for var. *cory-laria* in *Angeronia prunaria,* are effectively modified by experimental selection, as they have in fact been, we are certainly not to suppose that this has taken place because specific modifiers for all these were appropriately waiting for use.

It is to be noticed that Fisher (1935) was able to alter by selection the effect of the "crest" gene in poultry so as to cause it to produce cerebral hernia, of which there is no sign in the normal crested animals. This is to be contrasted with Goldschmidt's statement that the modifiers so far studied are unlike those postulated for mimicry because they have simple quantitative effects only.

Goldschmidt is impressed by the fact that mimicry is often, though by no means always, sexually controlled to the female, but not the reverse. This indeed is to be expected on a consideration of the ecology of these forms. The female requires special protection while exposed and vulnerable during egg laying. On the other hand, since in butterflies sight plays some part in sexual stimulation during courtship, there often will be a tendency to retain the same pattern throughout the male sex.

It has already been pointed out that the expressions of the switch genes responsible for mimetic (and other) polymorphisms are genetically variable. This provides the type of variation on which selection has operated gradually to perfect mimicry. Goldschmidt, adopting the view that the mimicry has arisen complete by (his form of) parallel mutation, has to find an alternative explanation for this effect. This he does in

two directions. (1) He favors the idea that it may be the result of a change in dominance, remarking "selection for dominance does not have anything to do with selection for pattern modifiers." If one postulates, as Goldschmidt does, that the pattern has not been attained by selection, one is of course forced to make that statement. But the variation upon which selection for dominance operates is precisely of the same type as that upon which it is suggested that selection acts to adjust the effects of the switch genes, since it flows from the variability due to recombination within the gene-complex. (2) Goldschmidt suggests that intermediates may also be "the product of incomplete sex-control on a genetic basis. The genetic basis may be a mutant shifting the process of sex-controlled inheritance more or less beyond the threshold or it may be a multiple allele of the pattern factor which happens to entail a less perfectly working sex-control." Yet both sexes are represented among the intermediates obtained by Leigh from an intermediate female. Moreover, such variation is well known without any apparent connection with sex. For instance, in *Angeronia prunaria,* in which the effect of the *corylaria* gene can be modified in either direction (more, or less, extreme) by selection. But *corylaria* occurs in both sexes, and these are equally modifiable to magnify or diminish the action of the gene.

Goldschmidt dismisses the differences in habit controlled by a single pair of allelomorphs, as in *Hypolimnas dubius,* as not yet fully established. They would put a further strain upon the concept of parallel mutation in which genes are supposed to have effects of similar type, though producing them by different means, because of the limited repertoire of basic patterns in the models and mimics concerned. Now that the work of Hovanitz has shown that habit differences produced by a gene can be accurately studied and established in the Lepidoptera, we may hope for conclusive information on the habits of the mimetic forms of a species; and there is already a strong indication, but not complete evidence, that these may differ from one another.

Carpenter (1946) has dealt with the non-genetic aspects of Goldschmidt's article and indicated numerous inaccuracies and fallacies in them. His criticism should be studied by all those who are interested in this matter. Two of Goldschmidt's statements to which Carpenter draws special attention are so much related to the genetic situation which he is developing that they must be mentioned here.

First, Goldschmidt says that "mimetism [that is to say 'mimicry'] is about as rare as extreme seasonal dimorphism, and that it is confined to a few nearly related members of a few systematic groups." Car-

penter compares the occurrence of the two phenomena and shows that this extraordinary conclusion is unfounded.

Second, Goldschmidt, in order to sustain his argument for parallel mutation, says (p. 157) that "the widely different models of different mimetic females of a species are related among themselves; it does not occur that one female form mimics a Danaine and another a poison-eater *Papilio.*" This is a misstatement of fact, but one which the circumstance that the poisonous butterflies are restricted to only a few groups tends to obscure. Consider *Papilio dardanus*: there are no "poison-eater" *Papilios* available in Continental Africa, but while the majority of the mimetic females copy Danaidae, one of them, *planemoides* Trim., mimics a member of the Acraeidae; a complete contradiction of Goldschmidt's statement.

Goldschmidt is at much pains to demonstrate an underlying similarity of pattern in mimics and models and "the presence of a developmental system which permits only phenotypical changes of a definite type" by which different genes, acting in different ways, give rise to approximately similar color-patterns and wing shapes. It is by no means clear to me how it is thought that comparable developmental systems are responsible for the production of chemically different pigments (and their deposition in corresponding places) whose only similarity is their color. As already mentioned, the reds of the poisonous *Polydorus* swallowtails and the *Papilio* species which so often mimic them are chemically distinct (Ford, 1944). Similarly, reds, yellows, and whites are uric acid compounds in the Dismorphiinae, but they are not built up from uric acid in their Ithomiine models (Ford, 1941, 1947).

Consider also the apparent similarity between the *anthedon* and *wahlbergi* forms of *Hypolimnas dubius* and their *Amauris niavius* models. These are black-and-white insects, but the white has a blue tint very characteristic in natural conditions. In *A. niavius* this is attained by an opalescence of the white scales. In its mimics, however, these are dead white, and the blue tint is produced by a scattering of bright blue scales among them. Goldschmidt's discussion of a correspondence between basic patterns of distinct butterfly groups does not explain such a situation as this.

It is an essential of Goldschmidt's theory that mimetic resemblances have arisen ready made by mutation, and therefore he says (p. 213): "Thus I confidently expect that future research will reveal that the differences between geographic races of mimics (and models) will be of a multiple allelic character involving the locus controlling the mimetic pattern." We shall have to await the necessary work for concrete evidence on this point, but some indication of what that will show is cer-

tainly available already. The west- and east-coast forms of *Hypolimnas dubius* would provide excellent material for such studies. But if the differences between *dubius* and *mima*, between *anthedon* and *wahlbergi*, are really due to multiple allelomorphs, why are all degrees of intermediacy between them found in the intervening country where the models are mixed or intermediate also? It is indeed straining Goldschmidt's suggestion to assert that these are all due to multiple allelomorphs. It is also straining his suggestion that intermediates between mimetic forms are due to "incomplete sex control on a genetic basis," for the two sexes are equally affected. On the other hand, such variation is exactly in accord with the view of gradual adjustment by selection operating on genetic variability supplied by general recombination within the gene-complex. We have not yet the appropriate data in mimetic species, but the gradual shift of the pattern of var. *corylaria* of *Angeronia prunaria*, within the ambit of the switch-gene concerned, has been produced by selection in this way. And the genetic differences, but apparent similarity, of the races of *Triphaena comes* in the Hebrides and Orkney are due to many genes operating on the effect of the *curtisii* switch-gene (p. 77).

Even if all other considerations were set aside, it is a great weakness of Goldschmidt's theory that, as he fully realizes (p. 215), it is inapplicable to the evolution of cryptic patterns copying inanimate objects (e.g., of a *Kallima* butterfly to a dead leaf), and that he has to suppose that this is attained by quite different means from the mimetic resemblances of one insect to another. Also Carpenter (1946) rightly asks how parallel mutation is responsible for the production of mimicry to another butterfly on the upper surface but a cryptic copy of a leaf, not shown by the model, on the lower (e.g., *Protogonius*). The view that these similarities are gradually attained by selection operating upon genetic variability supplied by recombination within the gene-complex is equally applicable to cryptic resemblances to leaves, to monomorphic and to polymorphic mimicry. It requires no separation of these cognate phenomena into distinct types, produced by quite different means, and accords with the known facts relating to them all.

4. *Evolutionary Studies on Balanced Polymorphism*

A balanced polymorphism provides special advantages for the study of evolution by experimental means. For the population is, as it were, "sensitized" to both the allelomorphs at the locus, or loci, responsible for maintaining it, since even the rarer of them must be far commoner than those which are eliminated by selection and maintained only by recurrent mutation. Consequently, the adjustment of the gene-complex

to any particular controlling gene may often respond quite quickly to changing conditions and to the environments of different habitats (Ford, 1953a).

Bearing this possibility in mind, Fisher and Ford (1947) began the study of a polymorphic colony of the moth *Panaxia dominula* L. (Arctiidae) in 1939 in an attempt to throw light upon certain evolutionary problems. From 1947 onwards the work has been continued by Sheppard (1951). In the locality in question, Cothill, Berkshire, the species occupies an area of about 20 acres of isolated marshland. It is a day-flying insect, with an average size of about 48 mm. across the expanded wings. Its chief larval food is a marsh plant, *Symphytum officinale*, and it has but one generation in the year.

The polymorphism is controlled by a single pair of allelomorphs, the heterozygotes being distinguishable. The normal form (homozygous *dominula*) has dark metallic-green fore wings with white spots, some tinged with yellow, and scarlet hind wings with black spots. In the rare homozygote, var. *bimacula* Cockayne, there is a great extension of the black pigment, which obscures most of the red on the hind wings and all the larger pale spots on the fore wings except the two basal ones. The heterozygotes, consisting of var. *medionigra* Cockayne, are much more like normal *dominula* than *bimacula*, but the central pale spot on the fore wings is reduced or absent and there is an additional black spot in the middle of the hind wings. It is the most, and normal *dominula* the least, variable of the three forms. The "mutant" gene is ordinarily so rare that only one or two specimens of *medionigra* have ever been found outside the Cothill colony, so that elsewhere it evidently has the status of a rare variety maintained by mutation and constantly eliminated by selection.

Since all three genotypes can be distinguished by inspection, it has been possible to determine the gene frequency at Cothill year by year, sampling the population and marking the individuals so that the same specimen was not counted twice. During the period 1939 to 1950, the *medionigra* gene has occupied at the maximum 11.1%, and at the minimum 2.9% of available loci.

From 1941 onwards the number of individuals in the population has also been established by the method of marking, release, and recapture. For that purpose, dots of cellulose paint, varying in color and position, were used. These dry in a few seconds, when they seal the scales on to the wing membrane and are then permanent and waterproof.

The results so far obtained as shown in Table 3. Since both population-size and the percentage-frequency of the two allelomorphs are

TABLE 3

Gene Frequencies and Population Size in a Colony of *Panaxia dominula*

Year	*dominula*	*medionigra*	*bimacula*	*Total sample*	*Gene frequency (%)*	*Estimated population size*
up to 1928	164	4	0	168	1.2	—
1939	184	37	2	223	9.2	—
1940	92	24	1	117	11.1	—
1941	400	59	2	461	6.8	2000–2500
1942	183	22	0	205	5.4	1200–2000
1943	239	30	0	269	5.6	1000
1944	452	43	1	496	4.5	5000–6000
1945	326	44	2	372	6.5	4000
1946	905	78	3	986	4.3	6000–8000
1947	1244	94	3	1341	3.7	5000–7000
1948	898	67	1	966	3.6	2600–3800
1949	479	29	0	508	2.9	1400–2000
1950	1106	88	0	1194	3.7	3500–4700
	6508	615	15	7138		

known each season from 1941 onwards, it was possible to calculate whether or not the observed fluctuations in gene-ratio are small enough to be ascribed to random variations, as required by Sewall Wright's theory of genetic drift (Wright, 1931, 1932, 1935, 1940). By the time that we produced our 1947 paper (Fisher and Ford, *loc. cit.*) it was possible to demonstrate that these fluctuations were too large to be accounted for in that way even in a population as low as a thousand, and that they must be controlled by selection in the wild population.

This conclusion was criticized by Wright (1948) on several grounds, all of which have since proved invalid (Sheppard, 1951), while at least one of them was evidently so at the time the criticism was made. For Wright questioned the deductions because no population-size was calculated for 1939 or 1940. Yet in order to imperil them, the population in 1940 would have had to be as small as the number of moths actually caught. But even if collecting had been carried out every day that the insects were present, which it was not, the population must have been much larger than the sample, since the data for subsequent years showed that we never, at the best, caught more than one-quarter of the population in a season.

Wright (*l.c.*) also objected to the original findings because no indication was given of the size of the fluctuations in the selective value

of the *medionigra* gene, the period of observation not then being long enough for that purpose. Indeed, from the recorded fluctuations he estimated that the selective value of the heterozygotes (*medionigra*) might in half a century vary between semilethality and an advantage of 50% over homozygous *dominula*. In arriving at this conclusion he assumed that their selective value "has no trend but varies according to non-secular fluctuations in conditions from year to year."

Time has proved him wrong. The data now give no indication that the selective advantages of the three genotypes have varied over the period 1939 to 1950. On the contrary, Sheppard (1951) has shown that they had been affected by a steady trend, such that the heterozygotes have been consistently at about a 10% disadvantage compared with normal *dominula* subsequent to 1940.

Wright (*l.c.*) made one other suggestion to escape the conclusions to which these results lead: that the effective and apparent populations of *P. dominula* at Cothill may be very different. He says that if each female lays the majority of her eggs in one place, the offspring will be subject to similar environments, and whole broods may tend to survive or to be killed. Sheppard (*loc. cit.*) has investigated this subject and has been able to demonstrate that such a criticism is inapplicable as there is considerable scattering within each brood.

Thus, by the use of polymorphic material, it was possible, for the first time, to compare the effects of random survival and selection in a wild population. The results showed that chance fluctuations in generatios, such as may occur in very small populations, could not be responsible for the changes observed, a conclusion which has been confirmed by the subsequent work of Sheppard.

There is good evidence (Fisher and Ford, 1947) that during the earlier years of this century, up to 1928, the *medionigra* gene was at a low frequency (1.2% of available loci or less). Between 1929 and 1939 it must have spread at a rate consistent with a selective advantage of about 20%, and it looks as if this process may still have been taking place during the first year of our work (1939-1940). This might be due to a reversal of selection, or conceivably to "drift," if the population were reduced to very small numbers. However, the latter alternative is excluded, since it is clear from the records of collectors that the moth never became rare during the period in question. We have here a valuable instance of a relatively long-term change in the balance of selective agencies maintaining a polymorphism.

As indicated by Fisher and Ford (1947, see p. 171), these results are of wide significance for evolutionary theory. For they are in accordance with work on observed gene-frequencies in other organisms,

and the conclusion that natural populations are in general affected by selection varying from time to time in direction and intensity, and of sufficient magnitude to cause fluctuations in gene-ratios, is fatal to the theory of genetic drift as an agent of any importance in evolution.

Since 1928, the Cothill colony of *P. dominula* has had a greatly increased incidence of the *medionigra* gene. It seemed possible, therefore, that the population might become adjusted so as to modify its expression, in degree or in kind, by selection acting on the gene-complex.

The possibility of this has been demonstrated by experimental breeding in which the more and the less extreme heterozygotes, respectively, were selected. By this means, the appearance of *medionigra* in laboratory stocks has been altered in either direction. The difference between the two means was already significant after four generations. A comparable, though of course much less marked, effect has now been detected in the wild population, for the heterozygotes had, after eight generations, become detectably more extreme in form, a condition still maintained; nor was this due merely to a chance fluctuation in degree of expression, for a slight general tendency in that direction had been observable for some generations previously. Since for the last ten generations the *medionigra* gene, though so frequent compared with ordinary mutants, has been at a disadvantage, we may suppose that this change represents some adjustment of the gene-complex to it rather than an over-all intensificaton of its effects.

Sheppard (1951) has now found a second isolated colony of *P. dominula* at Sheepstead Hurst, in the Cothill neighbourhood, rather more than a mile from the original one. The *medionigra* gene does not occur there. However, he has discovered a new form "var. A." which exists in both populations, but in different frequencies, for it is much the rarer at Cothill. This is also maintained as a polymorphism. It is already known to be genetically determined, but its expression may be subject to environmental modification; however, the exact nature of its control is still under investigation. Sheppard is now extending the studies on gene-frequency which have been carried out on *medionigra* at Cothill to include also the new variety in both colonies. He has established that they are effectively isolated from one another and that they are different, not only in genetic composition, but in their general environment. A comparison of these two populations and two polymorphic genes should greatly extend the scope of this evolutionary work, and it will be a matter of much interest to follow the fate of the *medionigra* gene when injected into the population from which it is at present absent.

The study of balanced polymorphism in the Lepidoptera has made

it possible to obtain some information on the genetics of geographic races. Unfortunately, this has up to now been done in one instance only, but an extension of this type of investigation may, in the future, throw much light on the evolution of taxonomic differences. Among other aspects of this question, it is desirable to analyze what is meant by the statement, frequent in the literature of systematics, "that the same form of species occurs in two isolated localities." So far "the same" in such a context has meant no more than visible similarity, whether qualitative or quantitative, and we have no information whether or not the apparent identity extends to the mechanism producing it.

Advantage has been taken of the polymorphism of the moth *Triphaena comes* Hb. (Agrotidae) in Britain to study the genetics of such a situation (Ford, 1953b). This is a common and widely distributed species. In England and southern Scotland it is, with rare exceptions, monomorphic. The fore wings are of a light ocherous-brown color, and the hind wings yellow with a black submarginal band. However, it is dimorphic in some parts of nothern Scotland and in many of the Scotch islands, for there a dark variety, *curtisii* Newmn., occupies a considerable proportion of the population. Unfortunately, its frequency in different areas has not yet been determined, but in some parts it is certainly as common as, or commoner than, normal *comes*. *T. comes comes* is subject only to minor variation in color, but *T. c. curtisii* is very variable: the fore wings range from a mahogany shade to intense black, while the hind wings are clouded with black scales, very slightly in some specimens, but occasionally so as almost to obscure the yellow.

Rather surprisingly, a study of these two forms of *Triphaena comes* was one of the earliest pieces of genetic work to be carried out on any animal after the rediscovery of Mendelism, for Bacot (1905) showed, correctly, that *curtisii* is unifactorial and nearly, but not quite, dominant. I find that the heterozygotes are usually distinguishable without difficulty from the recessives (the normal *comes*), but heterozygous and homozygous *curtisii* overlap one another. Thus, in segregating families, the darkest *curtisii* are nearly always homozygotes, and the lightest heterozygotes, but those of intermediate shade cannot, with confidence, be assigned to their correct genotype.

T. comes is dimorphic in the Outer Hebrides, which lie to the west of Scotland, and in Orkney off the eastern end of the north coast of that country. These two groups of islands are very isolated from one another.

Females of the *curtisii* form were obtained both from Orkney and from Barra (Outer Hebrides). No difference could be seen between the specimens derived from these two sources; their genetics also were sim-

ilar, with *curtisii* semi-dominant, as already described, the recessives being clearly distinguishable from the other two genotypes.

By appropriate crossings, the *curtisii* gene derived from Barra or from Orkney could be placed in a genetic setting in which the gene-complexes of Barra and of Orkney are equally represented. It was then found that its semi-dominance was lost and that a continuous series between normal *comes* and dark *curtisii* was obtained, in which the sharp distinction between homozygous *comes* and the heterozygotes disappeared. It was also shown that *curtisii* is due to the same gene (or to allelomorphs of it) in both islands. That is to say, the *curtisii* form is identical in appearance and in genetic behaviour in Barra and in Orkney, but it achieves that identity by different means; for the genes which adjust its dominance are different in these two places. Thus it is in this instance possible to analyze what is meant by the statement that "the same" form of a species occurs in distinct and isolated parts of its range.

Particularly in view of the fact that *curtisii* is found also on the Scotch mainland, which intervenes between Barra and Orkney, it is not likely to be controlled by different allelomorphs at the same locus. The data were not competent to decide this point, but it is established that the species has been adjusted to the *curtisii* gene by selection operating on the gene-complex in the manner which Goldschmidt excludes as the basis for the evolution of mimetic polymorphism.

III. Transient Polymorphism

The best example of transient polymorphism in the Lepidoptera is provided by industrial melanism. During the last hundred years, black or dark varieties of many species of moths belonging to several families have spread in industrial areas in Britain and Continental Europe and no doubt elsewhere, displacing the normal forms, which in such places have reached the status of rare varieties. While the change is in progress, these populations become polymorphic, yet the advance of the gene giving rise to melanism is not arrested by a balance of selective agencies but continues so as to displace its allelomorph unchecked.

Widely different, from the point of view of classification and in other respects, as are some of the moths affected, industrial melanics have certain general characteristics in common. They occur only in those species which normally rest in exposed situations and protect themselves by means of cryptic coloration, resembling bark, lichen, or other objects. The melanic forms usually differ from the normal by a single gene. In a few instances they are controlled by two or three genes (*e.g.*

Lymantria monacha L.), while they are so far known to be multifactorial only in *Oporinia dilutata* Bork. This is singular, and suggests a situation of a special type, for selection operates more rapidly on multifactorial than on unifactorial conditions, while there is here no need to maintain a simple switch-mechanism, as in balanced polymorphism. The dominance relationships involved are also noteworthy, for the only black forms which have been utilized in this type of evolution are dominants or semi-dominants. Recessive industrial melanics seem to be unknown, though rare recessive black varieties have been reported in many species, including some of those in which dominant melanics have proved a success.

The more striking instances of industrial melanism are summarized in Table 4.*

It must be understood, however, that the information on the countries involved is very imperfect. This table may be expanded in a few respects: the most important of the three genes responsible for industrial melanism in *Lymantria monacha* (Goldschmidt, 1921) is a sex-linked dominant *C;* the autosomal gene *B,* semi-dominant in effect, is responsible for a less marked increase in pigment; and a third autosomal dominant, *A,* intensifies the pattern and the effects of *B* and *C*. In *Aglaia tau* L., two dark forms, *meliana* Gross. and *ferenigra* Meig., have become very much commoner in manufacturing districts (Hasebroek, 1934). Each is unifactorial and semi-dominant, and the genes producing them are allelomorphs of one another; when present together, they give rise to a third dark variety, *weismanni* Standf., which combines their characteristics. That is to say, mm = *tau; Mm* and MM = less, and more, extreme *meliana;* M^Fm and M^FM^F = less, and more, extreme *ferenigra;* while MM^F = *weismanni* (Standfuss, 1910). A number of genes each with small and additive effects is responsible for the darkening of *O. dilutata.* Melanism in the remaining species is unifactorial and either semi- or fully dominant, as indicated in the table.

It is tragic that the wonderful opportunities for studying the course of an evolutionary change in nature provided by industrial melanism have been almost wholly neglected. In nearly every instance the occurrence has been reported only when the process is nearly complete, and we know little of its speed except that it has taken place very rapidly. It is certain that the majority of these melanics have advanced from the status of rare varieties to occupy over 90% of the population in twenty to thirty generations, and sometimes less. Thus, Gerschler

* Some of these are now well known, and it is unnecessary to add the references to the genetic work carried out on all of them, but this information can be obtained from Ford (1937).

TABLE 4

Industrial Melanism in the Lepidoptera

Family	*Species*	*Country or district*	*Genetics of melanic forms*
Tortricidae	*Acalla comariana* Zell.	Lancashire	Incomplete dominant
Thyatiridae	*Tethea or* Fb.	Germany, Belgium, N. England	Dominant
Geometridae	*Oporinia dilutata* Bork.	N. England	Multifactorial
Geometridae	*Gonodontis bidentata* Cl.	N. England	Dominant
Geometridae	*Phigalia pedaria* F.	N. England, Germany	Dominant
Geometridae	*Biston betularia* L.	England	Dominant
Geometridae	*Hemerophila abruptaria* Thnbg.	London area	Dominant
Geometridae	*Cleora rhomboidaria* Schiff.	Germany	Dominant
Geometridae	*C. repandata* L.	England, Germany	Dominant
Geometridae	*Boarmia punctinalis* Scop.	Germany	Dominant
Geometridae	*Ectropis crepuscularia* Hb.	Germany	Dominant
Geometridae	*E. extersaria* Hb.	Germany	Dominant
Saturniidae	*Aglaia tau* L.	Germany	2 multiple allelomorphs, both semi-dominant
Agrotidae	*Polia nebulosa* Hufn.	N. England	Semi-dominant
Agrotidae	*Meganephia oxyacanthae* L.	England	Dominant
Lymantriidae	*Lymantria monacha* L.	Germany	3 dominants (1 sex-linked, 2 autosomal)

(1915), who has made one of the few quantitative studies of melanics in nature, reports that the black variety of *Tethea or Fb.* was "very rare" in the neighbourhood of Hamburg in 1904 and that in 1911-1912 he collected large numbers of larvae there and found that 90 to 95% of them (according to the district) produced it. There is but one generation of the moth in a year. It is possible that industrial melanism will not occur so often or so rapidly in the future as it has done in the past, for the means of reducing pollution of the countryside in manufacturing areas are improving, while the laws for carrying them into effect are more strictly enforced.

Industrial melanism has affected larvae as well as imagoes. Harrison (1932) gives a good account of the spread of such larval melanism in northern England, from which the species involved, its first appearance, and status at the date of writing, may be obtained. Unfortunately its genetics are usually quite unknown. However, the black larvae are

simple dominants in *Meganephria oxyacanthae* L., while in *Selenia bilunaria* Esp., in which they are rare, they are recessives. The moths reared from them are normal, though black varieties of the imagines of both these insects occur. Occasionally black larvae of many other species have been bred and seem always to have produced normal insects. This indeed is to be anticipated, for the ecological status of larvae and imagines in the Lepidoptera is so profoundly different that an impossible situation would arise were not their characters adjusted to be under distinct genetic control.

The explanation of industrial melanism has not proved a simple matter. Harrison attempted to show that the condition is due to mutation produced by salts of lead and manganese which are present in the soot found on leaves in manufacturing districts. As this view has been disproved and is indeed contrary to what is known both of the artificial induction of mutation (requiring a mutation rate far in excess of what could be produced) and of the impact of mutation upon evolution, it is not proposed to discuss it here (a brief survey of the subject, and the relevant references, can be obtained from Ford, 1945).

It has been thought that industrial melanism is due to simple selection for dark forms in the blackened countryside of manufacturing areas. But this does not suffice to explain one important aspect of the situation: that the industrial melanics appear to be hardier than the normal forms which, nevertheless, they have in the past failed to displace. On the other hand, Goldschmidt suggests that the melanic mutants confer upon the larvae resistance to the ill effects of feeding upon leaves encrusted with metallic salts. This again fails at the outset as an explanation, since it does not even cover the facts; for, as will be pointed out, it is not only in industrial areas that the melanic forms have increased.

It has long seemed to me that the correct interpretation of industrial melanism must take into account the effects of the genes concerned both on cryptic coloring and on the physiology of the organism. Accordingly, in 1937 I put forward the view that all those genes which improved the viability of the organism must have spread through the population unless they carry with them some counterbalancing disadvantage, such as excess melanin production, which destroys the cryptic pattern upon which the safety of the individual depends. However, black coloring will be less of a handicap, perhaps even an advantage, in industrial districts. For there the black forms match their surrounding the better, while predators are probably fewer.

It had long been clear that reports of the superior hardiness of these industrial melanics must be well founded, for they have been made independently by a number of different workers who noticed the phe-

nomenon, though they were not looking for it. However, in every instance, save one rather doubtful one (Ford, 1945), the excess of melanics over normals in the segregating families is not significant, even when these are combined as fully as seems legitimate. It appeared essential, therefore, to study this subject more accurately, and accordingly I undertook an investigation of the comparative viability of melanics and normals in *Cleora repandata* L. (Ford, 1940b). When I reared the forms in favorable conditions, I obtained a slight but non-significant excess of the melanics which, however, was fully established in other broods which were subject to a high mortality. This was deliberately produced by means of partial starvation. By increasing the effect of selection in this way, a difference in viability became detectable which might otherwise have required very large numbers to reveal.

It should not be supposed that the metabolism which leads to excess melanin production is necessarily favorable to the physiology of the organism. It is well known that some melanics are much less hardy than the normal forms (e.g., *Selenia bilunaria* Esp., *Ectropis bistortata* Goeze). These have never become industrial melanics, and they have been sufficiently handicapped in the past to be pressed into total recessiveness.

The explanation of industrial melanism advocated here involves a competition between the selective importance of cryptic color-pattern on the one hand and of physiology on the other. Since the only melanics which have spread are dominants or semi-dominants, it is probable that in normal circumstances the balance of these two opposing tendencies is but slightly weighted against them, so they may be able to establish themselves wherever the ecological adjustment of the organism has been upset in such a way as to favor increased viability relatively more. This is likely to happen not only in industrial areas but here and there in rural districts also, owing to the changes brought about by civilization. Indeed, in England most of the woodlands now differ greatly from their primeval condition and provide an environment of a type which the Lepidoptera have not previously experienced. Thus the occasional spread of melanic specimens in the unpolluted countryside is quite consistent with this view but not with the alternative explanations of industrial melanism already mentioned.

It is very noteworthy that in a few instances exactly this type of evolutionary change has been observed. It is worthwhile to refer briefly to the more striking of these.

Biston betularia L. and *Meganephria oxyacanthae* L. have become industrial melanics in the English "Midlands," but their dark forms

carbonaria Jordan and *capucina* Mill have now spread widely in the normal countryside of the southern counties.

Biston betularia is white speckled with black dots, but *carbonaria* is entirely black, except for a white dot at the base of the fore wing. There is also another and less extreme melanic form, *insularia* Th. Mieg., of this species, in which the black speckling is greatly extended, largely, but not wholly, obscuring the white ground color. It looks indeed as if it were a heterozygote between *carbonaria* and normal *betularia*, but it is controlled by a separate gene (Lemche, 1931). This is generally described as fully dominant, but I believe that the heterozygotes are often slightly less extreme than the homozygotes. It cannot be detected when present in addition to the wholly black *carbonaria*. There is not yet sufficient evidence to decide whether the *insularia* and *carbonaria* genes are multiple allelomorphs, but the indications obtained by Lemche suggest that they are at different loci. *Insularia* is rarer than *carbonaria*, in the sense that it nowhere occupies so high a proportion of the population; but, even so, it is in some places much too common to be maintained by recurrent mutation and it must be a polymorphic form. It is most frequent in or near industrial areas, a fact which proved rather difficult to establish considering that nearly all the specimens in such places are usually *carbonaria*, which wholly obscures *insularia*. The inter-relationship of forms requires further study.

Boarmia punctinalis Scop. var. *humperti* Hum., *Ectropis extersaria* Hb. var. *cornelseni* Hoff., and *E. crepuscularia* Hb. var. *delamerensis* White are now industrial melanics in Germany but not in England, where they exist only as great rareties, save in a few rural localities: *Ectropis crepuscularia* in Delamere Forest, Cheshire, the other two in woods in Kent. There the black forms have almost displaced those of normal coloring.

On the other hand, *Ectropis consonaria* Hb., *Cleora ribeata* Cl., and *C. rhomboidaria* Schiff. have become black in certain woods in Kent and Surrey but are not known as industrial melanics. However, it does not seem that the first two of them are to be found in any manufacturing districts, though *C. rhomboidaria* certainly is. Its black variety *rebeli* Auriger may well become an industrial melanic in the future. The melanics are dominants in all three species.

It is not clear what are the predators which normally eliminate the black forms in rural areas, except that they must be birds. The rapid act of picking a moth off a tree is difficult to observe and has, in fact, seldom been seen, but it may be that members of the Paridae sometimes feed in this way. It is true that the more habitually bark-searching species seem to find their prey by tapping or searching with the

beak, but it is likely that they may turn aside to eat a conspicuous object near them as they move up and down the tree trunk.

Some years ago I liberated populations of the moth *Gonodontis bidentata* Cl. in rural conditions and in a wood polluted by smoke. Each consisted of known proportions of black and normal specimens, and the fate of the two forms was followed in the succeeding seasons. Owing to the destruction of one of the woods where the work was being carried out, it is as yet too early to report in detail on the results, though a significant elimination of melanics has already been detected in the uncontaminated area (Ford, 1953a). It is now proposed to re-establish a similar colony in a manufacturing district and to begin the observations upon it again. We hope also to develop at Oxford other experimental methods for the study of industrial melanism in the Lepidoptera, for this represents the most striking evolutionary change ever actually witnessed in any organism, animal or plant.

IV. Summary

1. Polymorphism is the occurrence together in the same invironment of two or more discontinuous forms of a species in such proportions as the rarest of them cannot be maintained merely by recurrent mutation.

2. Two distinct types must be recognized: (a) *balanced polymorphism,* which is due to a balance of selective agencies which favor diversity; (b) *transient polymorphism,* which exists only while a gene spreads and displaces its allelomorph.

3. A balanced polymorphism must be controlled by some switch mechanism, and this usually consists of a pair, or a very few pairs, of segregating genes whose expression is adjusted by selection operating on the gene-complex.

4. Owing to the rarity and slow spread of genes whose survival value is effectively neutral compared with their allelomorphs, these are not normally concerned in producing polymorphism.

5. In polymorphism, the homozygous dominants tend to become disproportionately rare, owing to the accumulation of recessive lethals, or semi-lethals, in the section of chromatin sheltered by the "dominant" gene in heterozygotes.

6. An account is given of polymorphism in the Lepidoptera: (a) in species with a dimorphism affecting both sexes; (b) in those with multiple forms; (c) where sex-controlled inheritance limits the polymorphism to males or females.

7. Instances are described in which polymorphism affects the habits of the contrasted forms, so throwing some light on their maintenance.

8. A general survey of the control of mimetic polymorphism is given. It is shown that Batesian mimics will usually tend to be polymorphic (while the Müllerian types will not) and the different phases will be adjusted to such frequencies that they each possess equal advantages, whether all, or only some, of them are mimetic.

9. The evolution of mimetic polymorphism is discussed:

(a) It is suggested that the origin of mimicry by means of "parallel mutation," whether in the sense used by Punnett or by Goldschmidt, should, on a number of grounds, be excluded as the agent responsible for the production of mimetic resemblances.

(b) It is concluded that the gradual evolution of mimicry by selection operating on the gene-complex is responsible for mimetic polymorphism, and that this is as effective within the ambit of the switch-genes as with monomorphic species (e.g., Sesiidae) or as in the production of cryptic resemblances (e.g., to leaves).

10. Two pieces of work are described in which balanced polymorphism has facilitated the application of experimental methods to the study of certain evolutionary problems.

(a) A quantitative study of a population of the moth *Panaxia dominula* has helped to demonstrate that "genetic drift" is not an agent of importance in evolution. Furthermore, in the course of this same investigation, it has been possible to detect an evolutionary change in natural conditions and to forestall it in the laboratory.

(b) It has in one instance been possible to analyze what, in terms of genetics, is meant by the statement, common in taxonomy, that "the same" form of a species occurs in two isolated localities.

11. The most striking example of transient polymorphism in the Lepidoptera, and indeed in any organism, animal or plant, is provided by industrial melanism.

12. Instances of this phenomenon are reviewed and its evolution discussed. It is held that the available evidence favors the conclusion that industrial melanism is due to a balance of selective advantages and disadvantages between the cryptic coloring of the normal and black forms on the one hand and of their physiology on the other, as suggested by Ford (1937).

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shire, England. This should become a model for the arrangement of this kind of entomological exhibit, for the genetics of the British species are illustrated by abundant material, the relevant references to the literature being included with each set of specimens.

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Inheritance in Bacteriophage

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CONTENTS

I. Biology of Bacterial Viruses

Bacteriophages are viruses that multiply exclusively inside bacterial cells. They occur wherever bacteria occur. Bacteriophages that infect intestinal microorganisms were the first to be investigated (d'Herelle, 1917); they are found in animal feces, polluted water, and sewage.

In natural environments, bacteriophages form relatively stable associations with bacteria, called lysogenic, that carry the virus intracellularly, but are resistant to its lytic action. Extracellular virus originates by the lysis of exceptional cells in lysogenic cultures (Lwoff and Gutmann, 1950). The extracellular virus may infect sensitive bacteria. This kind of infection results in the destruction of the bacteria and a tremendous increase in the number of extracellular viral particles. In general, bacteriophages cannot be detected except by introducing materials containing them into pure cultures of sensitive bacteria of appropriate kind; the presence of virus is recognized by the more or less complete lysis of the culture.

Little is known about the stable virus-bacterium associations, except that lysogenic cultures can be produced by bringing suitable bacteria

and extracellular virus together (Burnet and Lush, 1936). Information about the behavior of bacteriophages comes from the study of the transient infections obtained by exposing sensitive bacteria to resting virus. This study has shown that bacterial viruses exist in unlimited numbers of kinds, the kinds being distinguished by morphologic and antigenic character, and especially by the range of bacterial strains, usually of one or a few related species, they can infect. The diversity is so great that two viruses isolated independently by propagation on the same bacterial species can nearly always be distinguished (Burnet and McKie, 1933). The taxonomic problems presented by the bacteriophages have been summarized recently by Adams (1952).

Several bacterial viruses have been identified as characteristic tadpole-shaped structures consisting of a spherical or elongated head, 20 to 100 mμ in diameter for different viruses, and a slender tail that may be the organ of attachment to bacteria (Luria, Delbrück, and Anderson, 1943; Anderson, 1951). Some of the larger viruses possess an osmotic membrane (Anderson, 1949) consisting largely of protein, which forms a sack whose only known content is desoxypentose nucleic acid (Herriott, 1951). The membrane is responsible for the attachment to bacteria (Herriott, 1951) but has no function in the intracellular growth of the virus (Hershey and Chase, 1952). In favorable instances, bacteriophage particles are almost perfectly infectious (Luria, Williams, and Backus, 1950).

The bearing of these facts on viral inheritance may be stated as follows:

1. The properties of bacterial viruses point to a long history of biologic variation and specialization.

2. The observable properties are sufficiently numerous and well-defined to encourage studies in hereditary variation.

3. A clone of bacteriophage, as propagated under the usual experimental conditions, is an initially homogeneous population of viral particles originating from a single particle.

4. Bacteriophage particles can be enumerated with precision by simple infectivity measurements.

5. Bacteriophage particles possess a structural and functional simplicity that is favorable to inquiry into the chemistry of inheritance.

II. Methods of Study

A series of dissimilar bacteriophages called T1 (Type 1), T2, · · · T7 (Delbrück, 1946a), all lysing a single strain of *Escherichia coli* known as B, have been used in all recent genetic experiments. Among

these, only T2 and T4 have been intensively studied.* These are related viruses, large in size, which possess morphologically and functionally differentiated structure (Luria, Delbrück, and Anderson, 1943; Anderson, 1949; Hershey and Chase, 1952).

Genetic experiments with bacteriophage call for one or more stocks of phage and suitable bacterial hosts. A phage stock consists simply of the products of lysis of a sensitive bacterial culture infected with phage from a recently started clone. It may contain anywhere from 10^9 to 10^{12} phage particles per milliliter. The bacterial hosts required consist of a sensitive strain, used for propagation and titration of virus, and one or more indicator strains that are selectively resistant to certain viruses, used for their identification. Except for trivial selective effects, the genetic composition of a viral stock is independent of the bacterial strain on which it is propagated.

Stock bacteriophages are presumably haploid in genetic constitution, and hereditary variants of them are called mutants. Nothing is known about the mechanism of viral mutation, nor is it known whether different mutations have a common mechanism. For the present, therefore, a mutant bacteriophage is any particle producing a subclone unlike the parent clone in which it arose.

Viral mutants are enumerated, as are bacteriophages in general, by counting the number of clearings or plaques that develop when a measured volume of viral stock is introduced into nutrient agar plates seeded with sensitive bacteria. The plaques represent clones of virus made visible by their lytic action on the bacteria. Mutants of altered host specificity (Luria, 1945), called *h* mutants, can be counted selectively by "plating" on a bacterial host resistant to the wild-type virus. This method is essential for the isolation of *h* mutants but cannot be relied on for their enumeration in mixed populations because of nongenetic effects of interactions between unlike viral particles (Novick and Szilard, 1951; Hershey *et al.*, 1951). For the enumeration of *h* mutants in mixtures containing wild type, a mixture of bacterial strains, one sensitive both to *h* and to wild type, one sensitive only to *h*, must be used. The "mixed indicator" (Delbrück, 1945) identifies the virus particles in terms of the *h* phenotype of their offspring: *h* particles produce clear plaques; wild-type particles produce turbid plaques. Plating on mixed indicator sacrifices the advantage of the selective host, but is necessary for reliable counts (Hershey and Rotman, 1949).

Plaque-type mutants are enumerated by inspection of plaques formed on plates seeded with sensitive bacteria (Hershey, 1946a). They

* The work on recombination in T4 (Doermann and Hill, 1952) appeared too late to be included in this review.

are also recognizable on mixed indicator. Plaque-type mutants and host-range mutants can, therefore, be counted in mixtures with wild type by plating on mixed indicator.

Current methods of studying the multiplication of bacteriophage originate from the one-step-growth experiment of Ellis and Delbrück (1939) and its subsequent refinements by Delbrück and others. This experiment permits one, among other things, to infect a known number of bacteria with a known number of phage particles and to examine the extracellular viral progeny released by the lysis of the infected cells. This progeny is prevented from further interaction with bacteria by diluting the culture before lysis begins. A progeny of this kind is called a one-step yield to distinguish it from a stock or lysate resulting from more than one cycle of infection and lysis.

Four variations of the one-step-growth experiment must be distinguished.

1. Single infection, resulting when the input ratio of virus to bacteria is so small (*ca.* 0.1) that nearly all the infected bacteria receive only one viral particle.

2. Multiple infection, resulting when the input ratio of virus to bacteria is so large (greater than three) that nearly all the bacteria become infected.

3. Mixed infection, obtained by infecting bacteria with two or more kinds of virus. If the mixed infection is also multiple, nearly every bacterium is infected with one or more particles of each kind.

4. Single burst, obtained by allowing a single infected bacterium (usually isolated in the statistical sense, by preparing small-sample cultures) to lyse by itself. The single burst is, in effect, a one-step clone of statistically known parentage, in contrast to the clone represented by a single plaque, which contains the progeny of repeated cycles of infection and lysis.

The average yield of virus per bacterium may vary from a few to several hundred particles, depending on the virus and on conditions of growth, but it is largely independent of which variant of the one-step-growth experiment is used. The yield is liberated from the infected cells by lysis after a characteristic latent period (21 minutes for T2), defined as the time that elapses between infection and the liberation of the first viral progeny. The latent periods of individual cells vary between this minimum and 30 minutes or more.

Several methods have been introduced for obtaining viral yields by artificial lysis of infected cells (Doermann, 1948; Anderson and Doermann, 1952; Levinthal and Fisher, 1952). These techniques are important in that they make available for examination the infective

virus particles present in the cells at any stage of intracellular multiplication. Yields of virus obtained by artificial lysis can be called premature yields.

For a detailed discussion of methods of working with bacteriophages, the review by Adams (1950) should be consulted.

III. Intracellular Growth of Bacteriophage

The growth of T2 starts with the attachment of one or more resting phage particles to a sensitive bacterium. This is followed immediately by the release from its protein coat of the phage nucleic acid (and possibly other materials) into the cell (Hershey and Chase, 1952). The injected materials form, in close collaboration with the cell (Cohen, 1949), non-infective structures (vegetative phages) that can multiply. The multiplication itself is geometric (Luria, 1951) and is already well advanced 10 minutes after infection. This must be so, because at this time, when infective particles are just beginning to reappear in the cell, recombination between genetic markers has already occurred (Doermann and Dissosway, 1949).

From the tenth minute until the cell lyses, multiplication of vegetative phage, genetic interaction, and the conversion of vegetative into infective phage, are going on simultaneously, as shown by the analysis of premature yields by genetic (Doermann, 1952) and biochemical (Stent and Maaløe, 1952) methods. The conversion of vegetative into infective particles (maturation) necessarily consists of the reformation of the protein membrane characteristic of the resting phage particle and the inclusion in it of genetic material from the vegetative phage. At some step in the maturation process, the capacity of the phage to multiply and to exchange genetic characters must be lost.

When the cell lyses, its content of mature phage is released together, presumably, with any immature particles that may remain. Only the mature particles are at present amenable to either genetic or biochemical examination.

IV. Analysis of Mutations

The first systematic studies of mutation were the descriptions of host-range (*h*) mutants by Luria (1945) and of certain plaque-type (*r*) mutants by Hershey (1946a). Host-range mutations consist of the acquisition or loss of the ability of the virus to attach to cells of a particular bacterial strain. They occur in all types of bacteriophage, with the result that stocks always contain *h* mutants, whose number depends on the indicator strain used to detect them, as well as on the accidental

character of the mutations. The *r* mutations occur only in T2, T4, T6, and their relatives. A small-plaque mutant (*m*) of T2 has also been isolated (Hershey and Rotman, 1949), and plaque-type mutations occur in other viral lines, but these have not been studied.

Mutants analogous to *h*, *r*, and *m* had been seen earlier (Sertic, 1929a, 1929b; Craigie, 1946), but serious genetic study begins with the paper of Luria (1945).

The *h* and *r* mutations in T2 exhibit the following characteristics in common.

1. They occur during the multiplication of the virus, producing mutant clones that vary greatly in size (Luria, 1945, 1951).

2. The proportion of mutants in a bacteria-free stock of virus remains constant, except for obvious instances of selective survival (Luria, 1945; Hershey, 1946a).

3. The mutants of either class are represented by more than one hereditary phenotype (Hershey, 1946b), which arise by independent, well-defined steps.

4. The *h* and *r* mutations occur independently of each other (Hershey, 1946a).

5. Both mutations are reversible. Serial propagation of mutant clones invariably leads to the step-wise reappearance of the wild phenotype (Hershey, 1946b). In many instances tested, the reverted stocks cannot be distinguished from wild type by crossing (Hershey and Rotman, 1948, and unpublished). Needless to say, serial propagation is likely also to select mutants that are phenotypically distinct from wild type (Hershey and Davidson, 1951).

6. Two mutants of identical phenotype, one having arisen in one step, the other in two steps, can usually be distinguished by the patterns of reversion. The one-step mutant can revert to the wild phenotype in a single step; the two-step mutant reverts in two steps (Hershey, 1946b; Hershey and Rotman, 1948; Hershey and Davidson, 1951).

The properties listed establish the existence of independent sites of mutational change in the viral particle. These sites may be called "mutative loci" to distinguish them from genetic factors identified by recombination tests, which may be called "combinative loci."

An attempt has been made to measure rates of viral mutation (Hershey, 1946a). This aim now appears fruitless, for the following reasons.

1. The rate of the *r* mutation, which is great enough to measure by non-selective methods of counting, is really the sum of the rates of mutation at an indefinitely large number of loci (Hershey and Rotman, 1948).

2. The rate of one of the phenotypically recognizable *h* mutations

of T2, which must approach the rate for a single gene change (Hershey and Davidson, 1951), is so low that selective methods of counting are necessary. The use of the selective host for this purpose is unreliable owing to the abnormal phenotype of the *h* mutant resulting from mixed infections with wild type (Hershey *et al.*, 1951).

Certain features of the mutational process observed in T2 seem to distinguish viral mutations from mutations in other organisms. These are: the failure to detect mutations in resting populations of bacteriophage, and the failure to induce mutations by ultraviolet irradiation of the naked virus (Latarjet, personal communication). This may mean simply that the resting virus is not comparable metabolically to resting organisms of other biologic classes. Latarjet (1949) has reported the induction of *h* mutations in intracellular virus by irradiating infected bacteria. This report, as yet unconfirmed, suggests a unique opportunity to analyze the relation between metabolic states and susceptibility to mutagenic action.

V. Genetic Recombination

The discovery of a second mutation in T2 suggested at once the test of mutational independence (Hershey, 1946a). One might suppose that it would also have suggested a test of genetic recombination. In fact, it did not; recombination was discovered accidentally. The historical reasons for this are of more than passing interest.

Technically, the demonstration of genetic recombination was made possible by the first experiments on multiple and mixed infection (Delbrück and Luria, 1942). These experiments led to the generalization, later proved correct (Delbrück, 1945), that two unrelated bacteriophages were incapable of multiplying together in the same bacterial cell. They also suggested the idea, later proved incorrect (Hershey, 1946a), that only one virus particle could initiate an infection in one cell. When viral mutants became available, it was this idea that had to be tested.

Luria (1945) made the first test of mixed infection with related viruses (T2 and its *h* mutant). For technical reasons of no interest here, he obtained the expected but incorrect result. Hershey (1946a) made the same test with T2 and its *r* mutant. For technical reasons of no interest here, his relatively crude experiments yielded the unexpected, correct result.

Delbrück and Bailey (1946) designed an experiment well suited to the study of mixed infection with pairs of related viruses. They infected bacteria with T2r^+ and T4*r*, which could be distinguished both

by type of plaque and by host range. The experiment proved also fairly well suited to the demonstration of genetic recombination: the mixedly infected bacteria yielded the two recombinants, T2*r* and T4*r*$^+$, in addition to the two parental types.

The paper of Delbrück and Bailey is entitled "Induced Mutations in Bacterial Viruses," though of course they, and others (Hershey, 1946b; Muller, 1947), foresaw the possibility of genetic recombination. In fact, their experiments could be interpreted in several ways. One could admit that the two infecting viruses both entered the cell and multiplied, in which case genetic recombination might be the simplest interpretation of the result (Hershey, 1946b). Or one could imagine that one of the infecting viruses was excluded from the cell but somehow managed to produce directed mutations in its more successful competitor (Delbrück, 1946b). Finally, even if these were genuine instances of mixed infection, the genetic results could be explained in terms of some unique lability of the *r* genetic factor, which was known to be rather unstable also in unmixed infections (Delbrück and Bailey, 1946).

As matters now stand, it would be premature to assert that genetic recombination and directed mutation, in viruses or elsewhere, are mutually exclusive ideas. It is clear, however, that genetic recombinations in bacteriophage conform in detail to all that the phrase implies. The items of this conformity, as observed in mixed multiple infections with genetically defined stocks of T2, are summarized below.

1. Recombination need not involve the *r* genetic factor. It occurs also in crosses between certain phenotypically distinct *h* mutants of T2 (Hershey, 1946b; Hershey and Davidson, 1951), between *h* and *m* in T2 (Hershey and Rotman, 1949), between mutants of T1 (Hershey, unpublished), and in interspecific crosses with T5 (Adams, 1951).

2. Genetic recombination undoubtedly occurs in cells in which both parental viruses multiply. The yield from nearly every cell infected with any two mutants of T2 so far tested contains both parental viruses, and the proportion of offspring particles containing a given marker shows a unimodal distribution among the individual yields, the average being close to the proportion in the infecting viral mixture (Hershey and Rotman, 1949).

3. Non-parental types of virus arise in mixed infections only when the infecting pair of viruses differ by two or more mutational steps.

4. Tests on a small scale show that recombinants cannot be distinguished from the parental viruses of identical phenotype by backcrossing or outcrossing (Hershey and Rotman, 1949).

5. The recombination frequency with respect to a given pair of

markers is independent of other genetic differences between the viruses entering into the cross, and is the same in coupling and repulsion (Hershey and Rotman, 1949).

6. Recombinants of the expected complementary types are always found, and the numbers of the complementary types are equal in yields averaged over many bacteria (Hershey and Rotman, 1949).

7. Recombination frequencies are characteristic for pairs of combinative loci, not for individual loci (Hershey and Rotman, 1948).

8. Rare recombinants of a given phenotype do not appear in clones in yields from single bacteria. Rather, their numbers form a nearly random distribution, as if the individual particles arose more or less independently of each other (Hershey and Rotman, 1949). This shows that recombinants do not multiply in the cell or, at any rate, appear late in the course of multiplication. Spontaneous mutations, on the other hand, occur at all times during the multiplication of the virus and produce clones of mutant virus (Luria, 1951).

VI. The Linkage System

The analysis of the structure of bacteriophage T2 by genetic methods was made possible by the finding that most of the plaque-type (r) mutations in this virus occur at different combinative loci (Hershey and Rotman, 1948). Crosses were made between all possible pairs of ten of these mutants, and selected crosses were made with others, with a host-range mutant (h), and with a small-plaque mutant (m). The linkage system revealed by these crosses is illustrated in the form of a map of recombination frequencies in Fig. 1.

This figure is not a linkage map in the usual sense, because the data are not obtained from individual breeding tests, but from mixed multiple infections in which the unit of observation is the viral population produced during one cycle of intracellular growth. The data have to be interpreted in terms of the genetics of small populations. The nature of the problem can be seen from the following qualitative considerations.

1. Not all the progeny from a mixed viral infection descend from particles that have had an opportunity to interact with their opposite number (Hershey and Rotman, 1949). This is evident, since factors shown by three-point tests (Hershey and Rotman, 1948; Visconti and Delbrück, 1952) to be unlinked yield only 40% of recombinants in two-factor crosses.

2. Some of the progeny descend from lines in which more than one interaction between unlike particles has occurred. This is true because

triparental recombinants are found after infection with three kinds of virus (Hershey and Rotman, 1948; Hershey and Chase, 1951) and also because a sufficiently large excess of one parental type in dual infections causes the yield of the minority parental type to be smaller than the yield of either recombinant (Doermann, 1952).

It follows that the data of Fig. 1 could be converted into a linkage map of T2 by appropriately increasing the large distances (e.g., 50 instead of 40 units for the unlinked factors) and decreasing the smaller ones. Figure 1 nevertheless shows the principal features of the genetic structure of T2 that can be deduced from present data. There are at least three independently assorting regions of genetic material. Most of the *r* mutants so far examined carry alterations in a group of closely linked loci. In addition, it is known that most, but not all, of the independently occurring *h* mutations produce alleles and duplications at the locus shown in Fig. 1 (Hershey and Davidson, 1951).

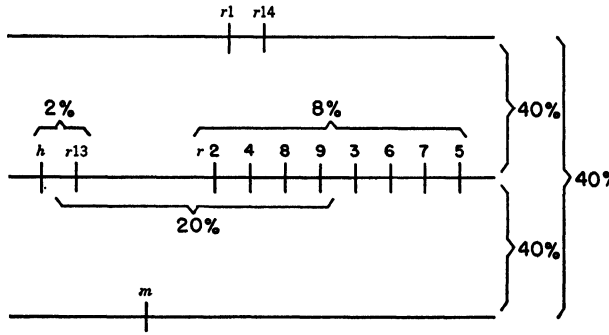

FIG. 1. Yields of recombinants in two-factor crosses between mutants of T2.

Further questions about structure are inseparable from the problem of mechanism of recombination. This problem cannot be discussed very intelligently at this time, and we shall limit ourselves here to the question: can the present genetic data be reconciled with the idea of matings between phage particles?

Hershey and Chase (1951) tested this idea from one point of view, namely, for the consistency of linkage measures as obtained from biparental and triparental recombination data. They found that the measures were consistent, and called for a minimum of three successive rounds of random mating between phage particles during the span of intracellular growth. Their theory was inadequate to deal with other features of the recombination data.

Visconti and Delbrück (1952) worked out a more complete mating theory that proved competent to explain some results of three-factor crosses. These results were obtained in the following way. Bacteria were infected with mutant viral pairs of the type *hr*2 and *r*1, and the r^+ virus in the yield was classified with respect to the *h* or h^+ character. The proportion of hr^+ within the class r^+ has the possible range 0 to 50% and measures the recombination frequency between *h* and *r*2 among particles that have all recombined with respect to *r*1 and *r*2. This method of measurement eliminates from the tally those particles that have had no opportunity to recombine at all.

In the cross *hr*2 × *r*1, the yield of hr^+ among r^+ was found to be 25 to 30%. In the cross *hr*2 × *r*7, however, the yield of hr^+ among r^+ was increased to 38%. Both of these are measures of linkage between the same two factors, *h* and *r*2. The only difference lies in the frequency of recombination between the reference markers; large for *r*1 and *r*2, small for *r*2 and *r*7. Seen in conventional breeding tests, this result would be called negative interference, since the second cross would differ from the first in measuring recombinations that are double crossovers. Visconti and Delbrück (1952) show that this result is an inevitable consequence in population genetics of any type of successive mating. In the second cross, the particles selected for the linkage test have already undergone one rare recombination (*r*2 × *r*7), which means that they have been selected for multiple matings that would favor other rare recombinations as well. In the cross *hr*2 × *r*1, on the other hand, this selective influence would be almost negligible. The results obtained show that there is no strong interference between two recombinations in the same linkage group.

The mating theory of Visconti and Delbrück (1952) is a special case of random mating—random with respect to partner and random with respect to time—in non-equilibrium populations. It accounts satisfactorily for the main results of two-factor, three-factor, and triparental crosses with T2, and for the effects of unequal numbers of parents. It provides an estimate of the average number of successive rounds of mating (five), and permits the calculation of recombination frequencies per mating. Some results that are not yet explained are summarized in the discussion of heterozygosis that follows.

VII. Heterozygosis

It is a curious fact that the discovery of heterozygous phage particles (Hershey and Chase, 1951) made no immediate contribution to the thinking about genetic recombination. The reasons for this are twofold.

1. The heterozygotes are resting phage particles, which means that maturation and perhaps other unknown processes intervene between the primary mating products and the recognizable heterozygotes. For this reason, we shall speak of the latter as residual heterozygotes.

2. The residual heterozygotes themselves are novel in structure, as revealed by segregation patterns. These and other properties are described in the following paragraphs.

Residual heterozygotes are detected in the following way. If the viral yield from any mixed infection with r and r^+ virus is plated on sensitive bacteria to obtain isolated plaques, about 2% of the plaques are mottled and prove to contain mixtures of stable r and r^+ virus. It can be shown, less conveniently, that a similar proportion of offspring particles resulting from crosses involving h and h^+ virus produce mixed clones of h and h^+ virus. The mixed clones arise from unit particles, and not from clumps of particles, as shown by the normal sensitivity of heterozygotes to various inactivating agents.

The virus in the mixed clones does not show any genetic abnormalities detectable by backcrossing to the parental stocks and appears to arise by segregation before multiplication of the heterozygote. This behavior distinguishes the residual heterozygotes from lines of T2 (of rare, unknown origin) containing a genetic factor for instability (Hershey, Coon, and Chase, unpublished). These yield unstable as well as stable progeny, and the stable progeny may give rise to unstable ones again in crosses with normal stocks.

The residual heterozygotes are, therefore, particles containing allelic genes that segregate completely from each other during a single cycle of intrabacterial multiplication.

The frequency of residual heterozygosis is the same for five different r loci and for an h locus in T2. This shows that the formation of heterozygotes does not depend on local abnormalities of structure resulting from mutation, or at any rate not on abnormalities peculiar to individual mutations.

Further information about the structure of the residual heterozygotes is obtained by analyzing crosses of the type $hr \times$ wild type. The heterozygotes that are produced in such crosses belong to the segregation classes h, hr; wild type, r; wild type, h; r, hr; and wild type, hr. Thus all possible classes except particles segregating into h and r (the two recombinants) are found. For technical reasons, the existence of this class is not excluded by the data, but it is shown to be rare.

The heterozygotes found consist of four singly heterozygous classes (heterozygous for h or r, but not both), and one doubly heterozygous class (segregating to yield the two parents). Since the four singly

heterozygous classes are equal in size, the composition of the heterozygous population can be described simply in the manner shown in Table 1. These data represent a slight extrapolation from the actual experimental data (Hershey and Chase, 1951).

The unexpected feature of these data is the small proportion of double heterozygotes, even for the closely linked factors *h* and *r*13. This shows that the residual heterozygotes are not direct products of random matings among the intracellular virus particles, which would yield a very much larger proportion of double heterozygotes. Moreover, the terminal intracellular virus population in the crosses *hr*1 × wild type and *hr*7 × wild type differ considerably, yet these crosses produce identical numbers of double heterozygotes. An effect of linkage on the structure of residual heterozygotes is seen, however, for the closely linked factors *h* and *r*13, and a similar effect is obtained with another closely linked pair not shown in the table.

Two hypotheses have to be considered regarding the structure of the residual heterozygotes.

TABLE 1

Per Cent Yields of Recombinants and Heterozygotes from Crosses of the Kind *hr* × Wild Type

Cross	*hr*1 × wild	*hr*7 × wild	*hr*13 × wild
Recombinants	40	20	2
Double heterozygotes	0.12	0.12	1.48
Single heterozygotes	3.76	3.76	1.04

The recombinants consist of equal numbers of *h* and *r*.

The double heterozygotes belong principally to the class segregating into *hr* and wild type; a few may segregate into *h* and *r*.

The single heterozygotes comprise four classes of equal size, segregating respectively into the pairs, *h*, *hr*; *h*, wild; *r*, *hr*; and *r*, wild.

The data are idealized to make the total frequency of heterozygosis with respect to a given marker exactly 2%.

1. They may be diploid zygotes that failed to segregate before maturation. In this case, to explain the preponderance of single heterozygotes, it must be supposed that recombination strongly predisposes to failure of segregation.

2. They may not be diploid in structure at all, but haploid particles carrying local doublings of genetic material. In this case they could arise either by abnormal segregation of zygotes or by some other process of genetic transfer.

The heterozygotes thus present in more explicit terms the same

questions raised by the phenomenon of genetic recombination. One of these can be generalized as follows. The data of Table 1 show that heterozygosis for one marker is nearly independent of heterozygosis for a second, excepting closely linked markers. If this is a general rule, the progeny of a mixed infection with two particles differing by many markers would nearly all be heterozygous for one marker or another. By extension, nearly all phage particles must carry unmarked doublings of genetic material. The alternatives 1 and 2 above presumably apply, therefore, to all viral progeny and lead to the question: are phage particles diploid or not? An answer to this question would in large measure answer other questions about genetic recombination and heterozygosis.

In principle, this question could be answered independently of recombination tests: by analysis of mutational behavior, for instance. Unfortunately, the failure to observe mutations in naked virus precludes any simple approach of this kind.

It remains to consider whether the heterozygotes provide any clue to the recombination process. On entering a new bacterial cell, the residual heterozygotes segregate for the most part to yield one recombinant type and one parental type of virus. We may ask whether any of the recombinants produced in crosses result from segregations of analogous kind.

One suggestion that they do is the following. The numbers of the two complementary recombinant types are practically uncorrelated in the viral yields from single bacterial cells (Hershey and Rotman, 1949). This is conveniently explained if only one recombinant type is produced per segregation. It can be explained in other ways, however (Visconti and Delbrück, 1952).

A second suggestion that structures comparable to the residual heterozygotes contribute to the recombinants produced in crosses, is somewhat more direct. At present, the withdrawal of a viral particle from the cellular pool of multiplying virus has to be explained in terms of the maturation process. When residual heterozygotes multiply, they segregate. Accordingly, we imagine that genetic structures that mature into residual heterozygotes must either be formed at the time of maturation or must possess the alternative capacity to segregate before maturation. But if recombinants are produced from zygotes at all, they must be produced from zygotes that are formed and segregate independently of maturation: otherwise triparental recombination would be impossible. It follows that residual heterozygotes are produced from structures that can segregate before maturation or else they are not formed from

zygotes at all. And if residual heterozygotes are not produced from zygotes, there is no reason to suppose that recombinants are either.

Certain peculiarities of the recombination data reappear in the segregation pattern of heterozygotes. Three-point tests (Hershey and Rotman, 1948, 1949) show that the loci *h*, *r*4, and *r*7 are arranged in the order listed. The cross $h \times r4$ yields 20% of recombinants, and the cross $r4 \times r7$ yields 8%. These distances are not additive, because the cross $h \times r7$ also yields 20% of recombinants. The discrepancy cannot be explained solely by assuming that the distance between *h* and *r*4 is large, because crosses between unlinked factors yield twice as many recombinants as the cross $h \times r4$.

A strictly analogous discrepancy is seen in the independent tests of linkage provided by the segregation pattern of heterozygotes (Table 1). These show that the factors *h* and *r*7 are just as "unlinked" as the independently assorting factors *h* and *r*1, in spite of the twofold difference in recombination frequencies.

The explanation of the earlier data suggested by Hershey and Rotman (1949) is also applicable here. The hypothesis can be stated as follows. (Only recombinations within linkage groups involve pairing. In the absence of sex, pairing must be random with respect to partner, and not more than one-fourth of the pairs can yield recombinants even when the markers are distant. Recombinations between linkage groups represent assortments of independently multiplying structures brought together at the time of maturation. Half of these assortments can yield recombinations of unlinked markers.

This hypothesis is revived here because an independent test of the possibility that matings do not occur between genetically intact phage particles is now within reach (Visconti and Delbrück, 1952). Suitable materials for this test—two pairs of linked markers belonging to two different linkage groups—should be looked for.

VIII. The Viral Gene

Only one example of alternative mutations occurring at the same locus in T2 is known (Hershey and Davidson, 1951). They produce different alleles of the *h* factor. In this instance the alternative mutations occur at a single mutative and combinative locus, which occupies a map position that is independent of the mutational state. Conversely, independent mutations always occur at different combinative loci. For as long as genetic tests continue to yield consistent identifications of mutative loci, combinative loci, and map positions there can be no ambiguity in referring to the loci as genes. When inconsistencies arise, the

more explicit terms will have to be used. In the meantime they are useful when it is desired to make clear what method of identification of the locus has been used.

IX. General Inferences

The details of viral inheritance suggest that bacterial viruses originated in much the same way as other organisms: that is to say, out of a dim past. They are unique only because of their structural and physiologic simplicity, which corresponds to an extremity of parasitic adaptation.

Bacteriophages do not appear to possess anything analogous to sex. Yet they have developed efficient mechanisms of genetic recombination and genetic isolation that substitute admirably for sex. These depend on the facts, not yet understood in mechanistic terms, that related viruses can multiply together in the same bacterial cell, whereas unrelated ones cannot (Delbrück and Bailey, 1946). This generalization must be stated with the reservation that the rules of mixed viral growth in lysogenic bacteria have not yet been worked out and may prove to be different.

Bacteriophage particles are haploid, or at least are incapable of passing through a cycle of intrabacterial multiplication in the heterozygous condition. This limitation also seems to have been circumvented by taking advantage of the fact that genetically different viruses can infect the same cell. Thus if a heat-sensitive *h* mutant and the relatively stable wild type of T2 infect the same cell, the offspring particles carrying the *h* marker possess some of the stable characteristics of the wild phenotype (Hershey *et al.*, 1951). This effect is not hereditary and must be due simply to the close metabolic collaboration between cell and virus and between virus and virus. The situation is not dissimilar to that in heterozygous cells of other organisms, and may be thought of as the functional equivalent of diploidy.

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Biochemical Genetics of *Bombyx mori* (Silkworm)

HIDEO KIKKAWA *

CONTENTS

I. Introduction

In view of the practical importance of *Bombyx mori*, its life history has been investigated from various viewpoints. More than one thousand papers concerning the genetics and chemistry of this insect have been published. But researches combining genetics with chemistry are rather scarce in number. With the progress of biochemical genetics in recent years, however, much attention has been paid to this new field.

II. Gene-Controlled Chemical Reactions

1. *Relations between Genes and Enzymes*

The gene-enzyme hypothesis is not new. Goldschmidt (1916), Troland (1917), Wright (1941), and many other investigators have given it serious consideration. But, among classical works in this field, there may be no study so brilliant as that of Matsumura (1934) concerning the amylase of *Bombyx mori*. He investigated amylase functions in digestive and body fluids of many races of silkworms and found that the

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enzymes concerned are controlled by two different genes. If we use the symbol *Ae* to represent a positive amylase in digestive fluid versus *ae* (negative amylase), and the symbol *Be* as a positive amylase in body fluid versus *be* (negative amylase), we may expect the following four types according to gene combinations.

Type	Phenotype	Amylase in digestive fluid	Amylase in body fluid
I	*Ae, Be*	+	+
II	*Ae, be*	+	—
III	*ae, Be*	—	+
IV	*ae, be*	—	—

These four types were actually found in many races of silkworms, and they also have been obtained artificially by means of matings. It is of interest that the two genes *Ae* and *Be* are located in close proximity in the same chromosome, *viz.*, *Ae* 0.0, *Be* 1.1 in the VIII chromosome. As pointed out by Komai (1950), these two genes are semi-allelic.

Strictly speaking, however, amylase activities differ according to the developmental period of the silkworm, even if an individual possessing the gene *Ae* or *Be* is taken into consideration. For example, amylase activity in digestive fluid is low in the early stage of the fifth-instar larva; it increases gradually to a peak in the middle stage, and decreases again in the mature stage just before spinning. A similar phenomenon is observed in the amylase activity of body fluid. Moreover, it has been ascertained that the gene *Ae* controls amylase not only in digestive fluid, but also in the salivary gland, the crop, and other tissues or organs.

Recent studies show further that amylase activities differ according to races. For example, employing various races with different amylase activities in the digestive fluid, Matsumura (1948, 1951) obtained the following results: (1) mating of *ae* races *inter se* always gives only *ae* offspring; (2) mating of an *Ae* race with an *ae* race gives offspring which are intermediate in amylase activity; (3) a similar phenomenon is recognized for the mating of any two *Ae* races having different enzyme activities. Thus, it seems likely that the amylase activity in digestive fluid is controlled by iso-alleles or multiple alleles of the gene *Ae*.

A similar relation is observed in the amylase activity of body fluid (Kikkawa, 1950a). In connection with this matter, an interesting phenomenon has been found by the writer (*loc. cit.*). When a *Be* ovary is implanted into a *be* larva, the body fluid of the host in the late pupal stage shows a positive activity for amylase. This result seems to indicate that the implanted ovary releases amylase itself or some activators into the body fluid of the host.

It may be asked whether or not the silkworm having *Ae* or *Be* gene is stronger in vitality than that carrying an *ae* or *be* gene. It is surprising that no difference in vitality is found among individuals with any combination of these genes (Muroga, 1947; Matsumura, 1948). Presumably, the silkworm utilizes only mono- or disaccharides present in mulberry leaves, instead of polysaccharides. In fact, Bertholf (1927) observed a similar phenomenon in the bee.

However, in regard to amylase in the silkworm, many questions need to be reconsidered in the light of recent advances in this field. At least two kinds of amylase, *i.e.*, α- and β-amylase, are found in various organisms. Phosphorylase also is involved in the decomposition of starch or glycogen.

Seki and Kuroda in our laboratory have been studying the problem from these new standpoints. A few liters of digestive fluid were collected from the fifth-instar larvae of *Ae* type, and the amylase was purified by treating with acetone, ammonium sulfate, and potato starch powder. Similar procedures were carried out on the digestive fluid of the *ae* type. At present, the difference between the two types seems to concern the quantity of protein responsible for the amylase function and not the quality of the protein (Seki, 1951 and unpublished). Moreover, this experiment indicates that the digestive amylase of the silkworm belongs among the α-amylases. Kuroda has found that the ebb and flow of glycogen in fat bodies of silkworm larvae and pupae has no bearing on the amylase activity in the body fluid, notwithstanding the fact that fat bodies are in direct contact with the body fluid (Kuroda, 1951). Presumably, the metabolism of glycogen in fat bodies is controlled by other enzymes like phosphorylase, rather than by amylase.

Catalase is another example of an enzyme controlled by a special gene. There are many reports concerning catalase in the silkworm, but they have not been investigated from the viewpoint of genetics. Kikkawa and Ishizaki (1947) used dark and pale eggs segregated in one batch (black *vs.* white or black *vs.* red), and compared their catalase activities with each other. Generally speaking, the catalase activity in dark eggs is higher than that in pale ones. As is well known, catalase is composed of a special protein containing hematoporphyrins. But how the genes concerning egg colors control catalase activities is still obscure. It is of interest that the artificially pigmented eggs which are obtained by injecting 3-hydroxykynurenine into white-1 mutant pupa, as will be shown later, have a higher catalase activity than the uninjected white-1 eggs. This fact suggests strongly that the pigmentation of the egg has a close connection with catalase activity.

Watanabe (1933) discovered a strain in European races in which the body fluid had a barely measurable tyrosinase activity. According to Bito (personal communications) who is continuing the work of the late Watanabe, two dominant genes concerned with tyrosinase in the silkworm have been recognized. Representing the genes with symbols *T-1* and *T-2* respectively, the F_1 individuals which are derived from the mating of *T-1, T-2* × *t-1, t-2,* show an intermediate tyrosinase activity of the parent types. In the F_2 generation, segregation occurs, and one-sixteenth of the offspring lacks tyrosinase, while the remainder has tyrosinase in various degrees.

Thus, if various enzymes of the silkworm are studied genetically, we may reach the conclusion that each enzyme is controlled by a special gene. In fact, recent advances in biochemical genetics of *Neurospora* and other microorganisms seem to justify this view.

2. *Relations between Genes and Selective Permeabilities*

As pointed out by Jucci (1949), evidence has been accumulated indicating that genes control cell permeabilities in silkworms. For instance, let us consider the case of cocoon colors. Cocoons of silkworms are, in general, snowy white in color, but yellow, green, or scarlet cocoons are frequently found in various shades. Many genes affect these cocoon colors. Oku (1930) isolated in crystalline form, for the first time, the yellow pigments from cocoons of European and Chinese races and identified them as xanthophylls. Lutein ($C_{40}H_{56}O_2$) was the main substance; it amounted to 20 to 54 mg./100 g. cocoon. In addition small amounts of violaxanthin ($C_{40}H_{56}O_4$) and of carotene ($C_{40}H_{56}$) were found. Since these carotenoids were present in mulberry leaves, Oku (1931-34) assumed that the pigments found in cocoons might have been derived from the mulberry leaves. The validity of this assumption has been demonstrated by Hatamura and Harizuka (1944), who compared the amounts of carotenoids present in the excrement of larvae of white cocoons and of yellow ones. They found that the amount of carotenoids involved in the excrement of white-cocoon larvae was greater. This suggests that carotenoids present in mulberry leaves are absorbed through the digestive glands of the larvae of the yellow-cocoon types.

According to genetic investigations, three genes, at least, are involved in the formation of a yellow cocoon. They are represented by the symbol *A* (chromosome, unknown), *Y* (25.6 in the II chromosome) and *Yc* (0.0 in the XII chromosome; sometimes it is represented by the symbol *C*). Combinations of these three genes show the following characteristics:

No.	Phenotype	Color of body fluid	Color of cocoon
1	*A, Y, Yc*	Yellow	Yellow
2	*A, Y, yc*	Yellow	White
3	*A, y, Yc*	White	White
4	*A, y, yc*	White	White
5	*a, Y, Yc*	White	White
6	*a, Y, yc*	White	White
7	*a, y, Yc*	White	White
8	*a, y, yc*	White	White

(*A*, fundamental cocoon color; *Y*, yellow blood (body fluid); *Yc*, yellow cocoon)

From these results, it is concluded that, in order to form yellow blood, *A* and *Y* genes must be present simultaneously (Nos. 1-2), and that the formation of a yellow cocoon necessitates further the presence of *Yc* gene (No. 1). This relation has been proved more thoroughly by the following experiments.

Employing a strain which produces many mosaics (Goldschmidt and Katsuki, 1931, Katsuki, 1935), Harizuka (1940, 1948) obtained several mosaics involving silk glands; one had *Yc* gene, and the other, *yc* gene. Although these silk glands were located in the silkworms with yellow blood, the gland of *yc* constitution was unable to absorb carotenoids from the blood. Recently, more crucial experiments were performed by Fujimoto (1949a,b). He implanted a colorless silk gland, which was picked out from a young larva and which had a certain genetic constitution, into the body cavity of the other larva of a different genetic constitution, and examined the pigmentation of the implanted gland in the matured stage. His experimental results are summarized in Table 1.

These results indicate that the implanted silk gland is unable to absorb carotenoids from the body fluid unless it contains *Y* and *Yc* genes simultaneously (Nos. 1-9), and further that the implanted silk gland having both *Y* and *Yc* genes can take up pigment slightly, even if it is present in the colorless body fluid of *y, Yc* or of *y, yc* constitution (Nos. 10-11). This curious phenomenon has been explained consistently by the fact that the *A, y, Yc* or *A, y, yc* individual which was used in the above experiments, carried a small quantity of carotenoids in its body fluid. When an individual having the *a* gene, rather than the *A*, was used as a host, the implanted silk gland could never become pigmented even if it had *A, Y, Yc* constitution.

In connection with this problem, the case of the dominant white cocoon is of interest. This trait is represented by the symbol *I* (Inhibitor, 0.0 in the IX chromosome). In the individual having this gene, neither the body fluid nor the silk gland become pigmented even though the

TABLE 1

Relation between Blood Color and Cocoon Color (Fujimoto, 1949a)

No.	*Host*			*Implant*			*Result*
	Phenotype	Color of body fluid	Color of cocoon	Phenotype	Color of body fluid	Color of cocoon	
1	*Y, Yc*	Yellow	Yellow	*y, yc*	White	White	White
2	*Y, yc*	Yellow	White	*y, yc*	White	White	White
3	*y, Yc*	White	White	*y, yc*	White	White	White
4	*Y, Yc*	Yellow	Yellow	*y, Yc*	White	White	White
5	*Y, yc*	Yellow	White	*y, Yc*	White	White	White
6	*y, yc*	White	White	*y, Yc*	White	White	White
7	*Y, Yc*	Yellow	Yellow	*Y, yc*	Yellow	White	White
8	*y, Yc*	White	White	*Y, yc*	Yellow	White	White
9	*y, yc*	White	White	*Y, yc*	Yellow	White	White
10	*y, Yc*	White	White	*Y, Yc*	Yellow	Yellow	Yellow in various degrees
11	*y, yc*	White	White	*Y, Yc*	Yellow	Yellow	Yellow in various degrees
12	*Y, yc*	Yellow	White	*Y, Yc*	Yellow	Yellow	Yellow

The genetic symbol *A* is omitted in this table since all materials used in this experiment contain the *A* gene.

individual has *A*, *Y* and *Yc* genes simultaneously. This is analogous to the case of the white leghorn in domestic fowl. Hatamura and Harizuka (1944) have shown that the quantity of carotenoids in the excrements of *I* larvae is less than that in the larvae with yellow blood. Accordingly, they have assumed that the digestive organ of the *I* larva may have had enzymes which decompose carotenoids into leuco-substances.

Jucci (1949) states that the dominant white individual accumulates a greater quantity of carotenoids in its intestinal mucosa than the recessive white or the yellow individual. According to his opinion, the digestive organ of dominant white larva should have the ability of absorbing carotenoids present in mulberry leaves. Perhaps those carotenoids are not released from the intestinal mucosa into the body fluid or they are decomposed in the body fluid by some enzymes.

Strictly speaking, however, the problem of permeability in the silk-gland is more complicated and histological analyses are necessary for its solution. Yamanouchi (1922) and the writer (unpublished) have found the following facts (see Fig. 1).

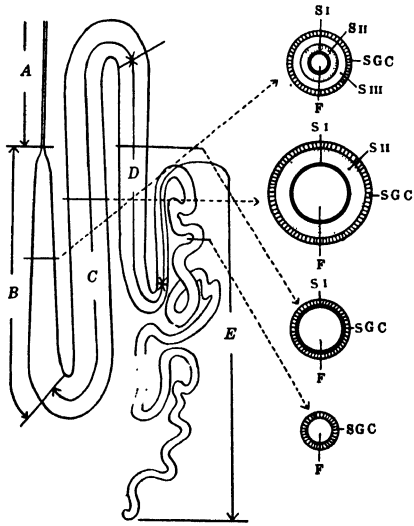


FIG. 1. Schematic figure of one silk gland. *Right*, cross sections. *A*, anterior silk gland; *B*, anterior division of middle silk gland; *C*, middle division of the same; *D*, posterior division of the same; *E*, posterior silk gland; SI, sericin I; SII, sericin II; SIII, sericin III; *F*, fibroin; *SGC*, silk gland cell.

The A part (anterior silk gland) represented in this figure does not secrete either fibroin or sericin; the B part (anterior division of middle silk gland) secretes a sericin called "sericin III" which is the most mucous type; the C part of middle silk gland secretes a sericin called "sericin II" which is the most abundant; the D part (posterior division of middle silk gland) secretes a sericin called "sericin I" which adheres very closely to the fibroin; and fibroin alone is secreted from the E part (posterior silk gland).

The pigments belonging to the carotenoids are present only in sericin I and II and are absent in sericin III and fibroin. Careful observations, however, reveal that the distribution of pigments in the silk gland differ according to the cocoon colors. In Fig. 2 are shown two types which are observed most frequently. Type A is found commonly in Chinese yellow cocoon races and type B in European yellow cocoon races.

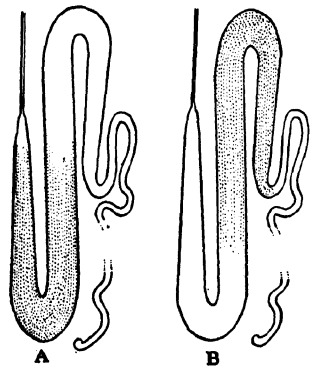


FIG. 2. Two types of silk glands related to yellow cocoon just before spinning. Type *A* produces a cocoon in which the outer layer is golden yellow in color and the inner layer is pale yellow. Type *B* produces a cocoon in which the outer layer is yellow in color and the inner layer is golden yellow. Dotted part represents yellow pigments.

Thus, the permeability of carotenoids depends on the genetic constitution of silk-gland cells. The *Yc* or *C* gene described before determines the distribution of carotenoids as shown in type A, and the *Ci* gene (an allele of *Yc*), is responsible for type B. Besides these genes, *F* gene (Flesh, 13.6 in the VI chromosome) deposit a kind of carotene in siricin I, and *Rc* gene (Rusty, 31.8 in the II chromosome) accumulates an unknown rusty carotenoid in sericin I. These genetic and chemical analyses are very complicated, and no definite conclusion has been obtained from them.

Another type of pigment is found in the cocoon; it is water soluble and yellowish green in color. This pigment is involved in cocoons of such races as *Seihaku, Daizo, Hekiren, etc.*, and is called "green cocoon." But careful analysis shows that it is distributed widely in many other races of silkworms. Among them, the paler ones are colled "Sasamayu," which means a cocoon having a color of little bamboo.

Iwaoka (1927), Jucci and Manunta (1932), Barbera (1933), Oku (1934), and others have stated that the pigment is a kind of flavone. Oku obtained a brownish-yellow substance from green cocoons in almost pure state. His chemical analyses showed that it was a glucoside of a flavone-like substance. The pigment was named "bombycin," and the aglycone obtained by hydrolysis was called "bombycetin." The most striking characteristic of this substance was that it contained the nitrogen in the molecule. Such a flavone was never previously found in nature. Moreover, since bombycin has not been detected in mulberry leaves, Oku (1934) concluded that the pigment is synthesized by the silkworm itself.

Meanwhile, Adachi (1936) and Hashimoto (1941) investigated the genetics of green cocoons and reached the conclusion that at least two dominant genes, *Ga* (Green cocoon-a) and *Gb* (Green cocoon-b), are necessary for a perfect manifestation of green cocoon. Hashimoto found that *Gb* is located at 7.0 in the VII chromosome.

TABLE 2

Relation between Blood Color and Cocoon Color (Fujimoto, 1949c)

No.	*Phenotype of host*	*Phenotype of implant*	*Result*
1	*ga, Gb*	*Ga, Gb*	Green
2	*ga, Gb*	*Ga, gb*	Green
3	*Ga, Gb*	*Ga, gb*	Green
4	*Ga, Gb*	*ga, Gb*	White
5	*Ga, gb*	*ga, Gb*	White
6	*Ga, gb*	*Ga, Gb*	White

Recently, Fujimoto (1949c) obtained the results summarized in Table 2 by means of his implantation method. They show that: (1) when a silk gland having the gene *Ga* is implanted into the body cavity of a *Gb* larva, the implanted silk gland becomes greenish (Nos. 1-3), but the *ga* silk gland does not take up pigment (No. 4); (2) when a *Ga* or *ga* silk gland is implanted into a *gb* larva, no pigmentation takes

place in the implanted silk gland (Nos. 5-6). On the basis of these findings, Fujimoto assumes that the *Gb* gene has the ability to produce a substance responsible for a green cocoon in the body fluid, and that the *Ga* gene is concerned with the permeability of the silk gland cell.

Kikkawa and Nakanishi (1950) have studied this problem by means of paper chromatography. The pigments extracted from various green cocoons by water or alcohol were developed by a butyl alcohol-acetic acid mixture solvent. Since the substances related to green cocoons give strong yellowish or reddish-yellow fluorescence, spots distributed on the paper chromatogram are detected without difficulty. Although the experiments are still in progress, it may be stated that at least three kinds of pigments are found. The characteristics of those pigments are as follows:

Tentative name of pigment	Rf value	Color of fluorescence
Bombycin-1	0.34	Yellow
Bombycin-2	0.19	Yellow
Bombycin-3	0.05	Reddish yellow

Thus, the bombycin described by Oku (1934) may have been a mixture of three different substances which are closely related to one another. In our opinion, these substances seem to belong among the pterins.

In the late stage of the fifth-instar larva having *Gb* gene, bombycin-1 begins to appear in the body fluid, but such a phenomenon is never seen in *gb* larva. When *Ga* gene is present in the silk gland, the latter is capable of absorbing bombycin-1 from the body fluid. Very light green cocoons contain only bombycin-1, but in more greenish cocoons a part of bombycin-1 is converted into bombycin-2 in the silk gland. In the most greenish cocoon, the majority of bombycin-1 is probably converted into bombycin-3. But it is also possible to assume that bombycin-3 is derived from bombycin-2. Presumably, a few genes have to do with these chemical transformations. Contrary to carotenoids, the bombycins are distributed in all silk substances, *i.e.*, sericin I, II, III, and also in the fibroin.

No matter how many examples may accumulate, the nature of permeability of the plasmatic membrane remains a mystery. There is little doubt that carbohydrates, lipides, proteins, and some inorganic substances like Na, K, Ca, Mg, *etc.*, take part in this phenomenon, but how their action is controlled by genes is a difficult problem to solve.

III. Problems Relating to Tryptophan Metabolism

1. *Formation of the Brown Pigment*

In the silkworm, this problem was investigated, for the first time, by the writer (1937) in connection with the mechanism of eye and egg color formation. Early results were published in *Genetics* in 1941. The work in this field is increasing rapidly, and our knowledge concerning it is becoming more and more extensive (Ephrussi, 1942; Becker, 1942; Kikkawa, 1943 a, b; Hanser, 1948; Caspari, 1949, *etc.*).

As pointed out by many workers, the brown pigments (skotommins) in insects seem to be formed by the following chemical reactions: tryptophan → kynurenine → 3-hydroxykynurenine (+ chromogen) → brown pigments. Presumably these chemical reactions are carried out by enzymes under the control of certain special genes. For example, the white-1 mutant in *Bombyx mori* (0.0 in the X chromosome) lacks the enzyme which converts kynurenine into 3-hydroxykynurenine. Therefore a large amount of kynurenine accumulates in the pupa or in the egg of this mutant (Kikkawa, 1941a,b, 1943a). According to my experimental results, the amount of kynurenine present in a white-1 pupa reaches about 0.3 to 0.5 mg./g. of fresh weight. This substance seems to be produced largely in fat bodies and ovaries during the pupal stage. In the male, most of the kynurenine is excreted in the urine, while in the female, a large quantity of kynurenine is preserved unchanged in eggs.

In 1939, Suzuki described another type of egg color mutant called "white-2." The gene controlling this trait is located very near the locus of white-1, *i.e.*, at 3.5 in the X chromosome. In a pupa or an egg of this mutant 3-hydroxykynurenine is accumulated instead of kynurenine.

The discovery of 3-hydroxykynurenine constitutes a dramatic story, but the details will be not discussed here. It corresponds to the cn^+ substance of Drosophila, the *A* hormone of Ephestia and the + chromogen of Bombyx. Crystalline 3-hydroxykynurenine was obtained for the first time by Butenandt, Weidel, and Schlossberger (1949) from pupae of *Calliphora erythrocephala,* and independently by Hirata, Nakanishi, and Kikkawa (1949, 1950a) from eggs of *Bombyx mori.* It was soon synthesized independently by several organic chemists (Butenandt *et al.,* 1949, 1950; Hirata and Nakanishi, 1950; Musajo *et al.,* 1950; Kotake, Sakan, and Senoh, 1951). The synthetic material was identical with the natural product in all chemical properties except the decomposition point (about 190-223° C. for the synthetic material and *ca.* 180° C. for

the natural one) and the specific rotatory power (approximately $[\alpha]^{17}_{4561}$: $-30°$ for the natural material).

3-Hydroxykynurenine, as well as kynurenine, seems to be formed in fat bodies and ovaries during the pupal stage of normal or white-2 types. In the male, most of the 3-hydroxykynurenine is converted into a brownish substance called "urochrome" in the excretory organ and is excreted in the urine, while in the female, a greater part of this substance is preserved in eggs, to be utilized as essential materials for pigments formed in the serosa after fertilization.

There is a noticeable change of 3-hydroxykynurenine in the egg of the normal type. It decreases rapidly with the formation of brown pigment in the serosa (Table 3). Such a rapid decrease of 3-hydroxykynurenine indicates clearly that the substance does not act as a hormone in the pigment formation but is utilized as a real substrate or precursor of the pigment.

TABLE 3

Changes in 3-Hydroxykynurenine Concentration in the Normal Egg (Kikkawa, unpublished)

Days after oviposition	*Egg color*	*Content of 3-hydroxykynurenine (mg./g.) in fresh eggs*
1	Yellowish white	1.449
2	Pale reddish brown	0.645
3	Brown	0.370
4	Purplish brown	0.247
5	Purplish brown	0.236

The brown pigments are present in the granular or needlelike chromoplasts existing in cytoplasm. These chromoplasts are very small in size and colorless in the early stage of development, but with the deposition of pigment they enlarge gradually and reach a certain volume. In the mutants with whitish eyes or egg colors, these granules are very small and remain colorless for a long time. In Ephestia, Hanser (1948) and Caspari and Richards (1948) have demonstrated that the granules contain ribonucleoproteins. In experiments using Malpighian-tube cells of Drosophila and Bombyx, the writer (1943b) and Tsujita (1948) have shown that the precursor granules have an ability of self-reproduction and closely resemble chondriosomes. The writer has represented the

relation between genes and the formation of brown pigment in a schematic model shown in Fig. 3.

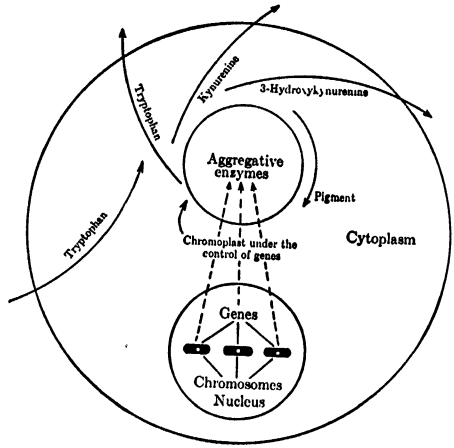


FIG. 3. Schematic figure illustrating the pigment formation in insects (from Kikkawa, 1943b; slightly modified).

In the writer's opinion, the chromoplast contains all enzymes responsible for tryptophan metabolism. However, in a leucoplast, which is seen in whitish color mutants, the granule may lack one or more of these enzymes.

At present, none of the enzymes concerned with tryptophan metabolism in insects has been demonstrated *in vitro,* though Itagaki and Nakayama (1941), Caspari and Richards (1948), and Knox and Mehler (1950) have isolated them from animal livers. Kato and Hamamura (1950) reported that they succeeded in isolating the enzymes from silkworm eggs, but in the writer's opinion their results should be re-examined.

The brown pigment is insoluble in water or alcohol, but it can be extracted by acid methyl alcohol or formic acid from chromoplasts (Ephrussi and Herold, 1944). Hirata (1950) attempted to isolate the pigments from silkworm eggs by means of chromatographic techniques and obtained at least three types, which were assumed to belong to a new category of natural pigment. But attempts at crystallization are still unsuccessful.

Recently, on the basis of their experiments using locusts, Drosophila, and Ephestia, Goodwin and Sprisukh (1950) advanced the opinion that the pigment may contain a pyrrole nucleus, and they have called it

"insectorubin." But, the writer is hesitant to subscribe unconditionally to their conclusion.

Many questions arise as to intermediate substances which are presumably formed between tryptophan and the brown pigment. In particular, the situation of α-hydroxytryptophan is being discussed. This substance was isolated by Wieland and Witkop (1940) from a toadstool, *Amanita phalloides,* and Butenandt, Weidel, and Becker (1940), by using the vermilion mutant of Drosophila, have concluded that it should be an intermediate between tryptophan and kynurenine. This view, which was also advanced by Y. Kotake and Masayama (1938), was accepted by many investigators without any question. Recently, M. Kotake, Sakan, and Miwa (1950) have succeeded in synthesizing α-hydroxytryptophan, and this compound had no effect on Drosophila and Bombyx (unpublished experiments by the writer). A similar negative result has been obtained by Sakan and Hayaishi (1950) who used the method of adaptive enzymes in Pseudomonas, and also by Mitchell (personal communications) in Neurospora.

Formylkynurenine, which was obtained by Amano, Torii, and Iritani (1950), and independently by Sakan and Senoh (1951), has been found by the writer (unpublished) to be effective on the vermilion mutant of Drosophila. This result suggests that formylkynurenine is an intermediate between tryptophan and kynurenine. In fact, the validity of this assumption has been proved by Amano and his coworkers (1950), and by Knox and Mehler (1950). It is assumed on chemical grounds that there is another intermediate between tryptophan and formylkynurenine, but its chemical nature is yet unknown.

Recently, Sakan and his coworkers have suggested to the writer that 3,4- or 3,6-dihydroxykynurenine may be an intermediate between 3-hydroxykynurenine and the brown pigment, and they have synthesized the former substance. Tests of this substance with vermilion or cinnabar mutants of Drosophila, however, were negative (Kikkawa, unpublished). Whether 3,6-dihydroxykynurenine will be positive is not known.

Thus, at present, the formation of brown pigment in insects may be represented as shown in Fig. 4.

In closing this section, the writer wishes to discuss briefly the problem of the physiological significance of the brown pigment. As pointed out by many investigators in Ephestia and in Bombyx, the brown pigment exerts a great influence on the viability of these insects. Mutant individuals producing pale eggs are, on the whole, less viable than the normal ones. But in instances where white-1 eggs have been pigmented artificially by implanting an ovary of normal type or by injecting 3-hydroxykynurenine, the pigmented eggs show a high percentage of

hatching and a more rapid development of the embryo than the white-1 eggs (Kikkawa, 1948).

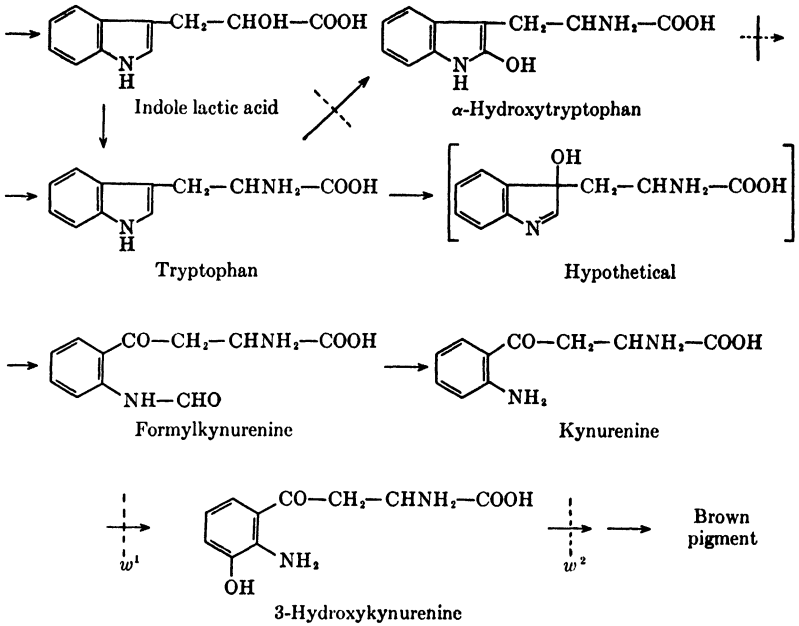


FIG. 4. Tryptophan metabolism in the synthesis of brown pigment in *Bombyx mori*. w^1, white-1; w^2, white-2. α-Hydroxytryptophan cannot be utilized by the insect.

It may be mentioned here that the brown pigment plays an important role in the phototaxis of the insect. A silkworm is not suitable material for this study, but the close relationship between brown pigment and phototaxis is easily observed in Drosophila. In general, the more brown pigment that is present in the eyes, the stronger the phototaxis becomes.

2. *Problems Concerning Syntheses of Tryptophan and Nicotinic Acid*

Many investigators are studying tryptophan metabolism by using mammals and microorganisms. According to Beadle and his coworkers (*cf.* Haskins and Mitchell, 1949), tryptophan metabolism in Neurospora proceeds as follows: anthranilic acid → indole + serine → tryptophan → kynurenine → 3-hydroxykynurenine → 3-hydroxyanthranilic acid → nicotinic acid (niacin). The writer (1950b) has performed an experiment to find out whether or not Bombyx is able to utilize anthranilic acid

or indole as a precursor of tryptophan. Anthranilic acid or indole was injected into young pupae of the white-1 or of the normal type, and the change of the injected substance was traced. If anthranilic acid or indole was utilized by Bombyx as a tryptophan precursor, the amount of kynurenine in the white-1 type or that of 3-hydroxykynurenine in the normal type would have increased as compared with the control. But the outcome of the experiment was negative. It has been found that anthranilic acid injected into either the white-1 or the normal pupa is converted into some other substances. Recently, one of these has been identified as anthranilylglycine (Hirata, Iwasaki and Kikkawa, 1951). These derivatives of anthranilic acid and the indole itself are finally excreted in the urine in the adult stage. These facts seem to indicate that the genes converting anthranilic acid or indole into tryptophan have become inactive or have been lost in Bombyx.

Thus, it is assumed that the major portion of tryptophan in Bombyx is derived from mulberry leaves, but a doubt still remains as to whether Bombyx is able to produce tryptophan from other substances. In order to test this possibility, various substances were injected into young pupae and their chemical changes were followed. Indole derivatives like skatole, indole acetic acid, indole propionic acid, indole lactic acid, and various amino acids like glycine, alanine, and tyrosine were tested. So far, only indole lactic acid has been utilized by Bombyx as a tryptophan precursor. A transformation of indole lactic acid into tryptophan has been demonstrated in mammals by Ichihara and Iwakura (1931), but whether or not it is a normal precursor of tryptophan in insects has not yet been established.

The most interesting problem concerning tryptophan metabolism is the relation between tryptophan and nicotinic acid. Since tryptophan, kynurenine, and 3-hydroxykynurenine are abundantly present in Bombyx, there is a good possibility that nicotinic acid is produced by Bombyx itself.

The writer (1951 and unpublished), employing paper chromatographic methods, has investigated tryptophan metabolism in various egg-color mutants of Bombyx. Alcohol extracts of eggs were used for this purpose. Butyl alcohol-acetic acid mixture was used as a solvent, and the characteristic fluorescence of each derivative of tryptophan was detected under ultraviolet light with a filter. Although the experiment is still in progress, the results so far obtained are summarized in Table 4.

When using extracts from white-1 mutant eggs, as the fluorescence of kynurenine fades before hatching, three kinds of spots with purple fluorescence begin to appear on the chromatogram. One of them (Rf, 0.95) was identified as anthranilic acid, and the other substance,

TABLE 4

Changes in Tryptophan Derivatives in Eggs of Various Types (Kikkawa, 1951 and unpublished)

Substance	*Color of fluorescence*	*Rf value*	*White-1 type*		*White-2 type*		*Normal type*	
			Eggs before incubation	Eggs just before hatching	Eggs before incubation	Eggs just before hatching	Eggs before incubation	Eggs just before hatching
Anthranilic acid	Purple	0.95	—	+	—	—	—	—
3-Hydroxyanthranilic acid	Bluish-purple	0.86	—	—	—	+	—	—
Anthranilylglycine	Purple	0.77	—	+	—	—	—	—
3-Hydroxyanthranilylglycine	Bluish-purple	0.67	—	—	—	+	—	—
X	Purple	0.65	—	++	—	—	—	—
Y	Bluish-purple	0.57	—	—	—	++	—	—
Kynurenine	Blue	0.50	++	±	—	—	—	—
3-Hydroxykynurenine	Green	0.44	—	—	++	±	±	—

with an Rf value of 0.77, as anthranilylglycine. But the nature of the third substance, with an Rf value of 0.65, *i.e.*, X, is still unknown.

In the white-2 mutant type, at least three spots with bluish-purple fluorescence appear on the chromatogram at the time of hatching. One of those spots (Rf value, 0.86) is in the same position as of 3-hydroxyanthranilic acid, and the chemical nature of the other substance with an Rf value of 0.67 is 3-hydroxyanthranilylglycine. The remaining substance with an Rf value of 0.57, *i.e.*, Y, is probably an oxidative form of X in the white-1 eggs.

In the normal eggs, these spots, except a pale one of 3-hydroxykynurenine, are scarcely recognized on the chromatogram. In Table 4, fluorescent spots which are assumed to be due to riboflavin or pterins are disregarded intentionally.

These derivatives of tryptophan are detected in the urine in the late pupal stage. From the facts mentioned above, we are able to offer the following assumption. Supposing that nicotinic acid is derived from tryptophan, the quantity of it in the white-1 type should be less than that in the white-2 or the normal type, inasmuch as the white-1 mutant lacks the ability to produce 3-hydroxykynurenine or 3-hydroxyanthranilic acid. The amounts of nicotinic acid in the white-1, white-2, and normal pupae have been measured by the bioassay method (Kikkawa and Kuwana, 1952 and unpublished) (Table 5). As seen in this table,

TABLE 5

Nicotinic Acid in Pupae of Various Types (Kikkawa and Kuwana, unpublished)

Type	*Contents of nicotinic acid (μg/g. dried pupa)*					
	Early stage		Middle stage		Late stage	
	♀	♂	♀	♂	♀	♂
Normal	157	145	158	175	174	178
White-1	137	155	156	171	152	156
White-2	155	165	163	175	167	191

Since the weight of fresh pupae decreases gradually with development, the content of nicotinic acid per individual does not necessarily correspond with the values shown in this table.

no marked differences in the amount of nicotinic acid have been found among these three types. This result suggests that the mechanism involved in the formation of nicotinic acid in the silkworm does not correspond with that in mammals or microorganisms.

In order to answer the question more adequately, various substances

which are assumed to be precursors of nicotinic acid, *e.g.*, tryptophan, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid, were injected either into the white-1 or the normal type pupa. If these substances are utilized as precursors of nicotinic acid by the silkworm, the amount of nicotinic acid should increase as compared with the control, except when tryptophane was injected into the white-1 type pupa. These experiments gave negative results (Kikkawa and Kuwana, 1952).

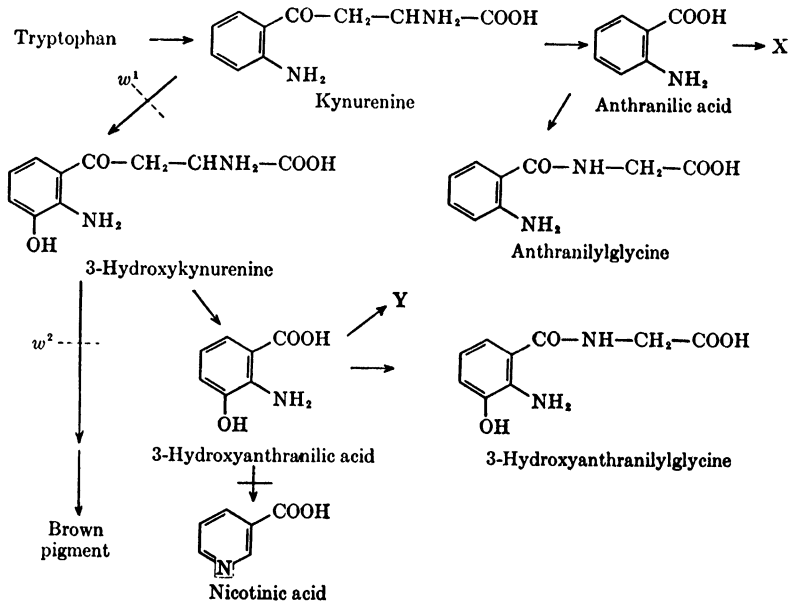


FIG 5. Pathways of tryptophan metabolism found in pupa and egg of *Bombyx mori*. w^1, white-1; w^2, white-2. Conversion from 3-hydroxyanthranilic acid into nicotinic acid may be interrupted in the insect.

This finding is supported further by the fact that the amount of nicotinic acid is nearly constant during the pupal stage in any mutant or normal type. Presumably, most of the nicotinic acid in the silkworm is derived from mulberry leaves, as is the case with tryptophan. It appears probable that the genes responsible for the conversion of 3-hydroxyanthranilic acid into nicotinic acid have become inactive or have been lost in Bombyx. These results agree with those obtained by Schultz and Rudkin (1948) in Drosophila, by Fraenkel and Stern (1951) in Tenebrio and Tribolium, and by Ito (1950, 1951, 1952) in Bombyx.

But, Kato and his coworkers (1950-52) hold the opinion that nicotinic acid in Bombyx may be synthesized in the pupal stage.

Thus, as far as available data show, pathways of tryptophan metabolism found in Bombyx may be illustrated as shown in Fig. 5.

3. *Problems Concerning Pterins, Riboflavin, and Uric acid*

It is well known that in insects water-soluble pigments are present together with skotommins. The pigments of this group are generally called "red pigments" in Drosophila and are being investigated by many workers (Maas, 1948; Heymann *et al.*, 1950; Chan *et al.*, 1951, *etc.*). In Bombyx, as well as in Ephestia, such a red pigment has not been recognized, but several kinds of water-soluble pigments with beautiful fluorescence are commonly found.

A typical case is a mutant called "lemon" which has a yellow skin; its gene is located at 0.0 in the III chromosome. On the basis of their chemical studies, Hirata, Nakanishi, and Kikkawa (1950b) have called this new xanthopterin-like substance "xanthopterin-B," its suffix "B" coming from Bombyx. It should be pointed out that xanthopterin-B is present with xanthopterin in the wings of such yellow butterflies as Eurema and Colias. A part of xanthopterin-B in the epidermis of *lem* mutant larvae is excreted in the matured larval stage, but most of it accumulates in the body fluid during the pupal period and is finally excreted in the urine in the adult stage. Xanthopterin-B is scarcely found in the egg of *lem* mutant.

The study of xanthopterin-B present in the *lem* mutant suggested the existence of other pterins in the epidermis of normal larva. Chemical investigations of this problem have been carried out by the writer in collaboration with Hirata and Nakanishi (1950b). At present, the existence of two kinds of pterins, *i.e.*, leucopterin-B and leucopterin, has been ascertained. They are found not only in the normal type, but also in the eggs of all races of silkworms so far studied. Although the chemical nature of xanthopterin-B and of leucopterin-B is yet unknown, the former may be a derivative of xanthopterin, and the latter seems to be identical with fluorescyanine (probably isoxanthopterin) which was obtained from fish scales (Polonovski *et al.*, 1948, 1950; Busnel *et al.*, 1950; Hirata and Nawa, 1951).

The most common substance present in the epidermis of the silkworm is uric acid. Jucci (1932) states that it is present in the epidermis as a salt. Hirata, Nakanishi and Kikkawa (1950b) have obtained uric acid from the epidermis of normal larvae in a crystalline form and have assumed that it is present as a calcium salt. Aruga and his coworkers (1951) state, however, that uric acid in the epidermis is present

as a sodium urate. Thus, the solution of this problem is left for the future.

The distribution and amount of uric acid in the epidermis and in other organs of the silkworm are controlled by the genes called "oily" or "translucent." Many different mutants showing a similar trait, *viz.*, a translucent skin instead of an opaque one of a normal type, are found in Bombyx, as in the case of vermilion eye groups in Drosophila. Jucci (1932) pointed out that in oily mutants, the amount of uric acid is less than in the normal type.

A similar result has been obtained by Shimizu (1943) using three kinds of oily mutants, *viz.*, *oc* (Chinese translucent, 40.0 in the IV chromosome), *ok* (Kinshiryu translucent, 4.7 in the V chromosome), and *og* (Giallo Ascoli translucent, 7.4 in the IX chromosome). He has selected normal and oily larvae derived from one batch and estimated the amount of uric acid involved in the epidermis. The results are shown in Table 6.

TABLE 6

Uric acid (mg./g. dried epidermis) in Normal and Oily Types (Shimizu, 1943).

oc series		*ok series*		*og series*	
$+^{oc}$	*oc*	$+^{ok}$	*ok*	$+^{og}$	*og*
57.6	10.3	106.8	4.9	103.7	3.1

Generally speaking, the more translucent the skin becomes, the less uric acid larvae contain. Presumably oily mutants have a characteristic in common—to be able to produce or accumulate less uric acid in various organs or tissues than the normal type (Inagami *et al.*, 1952, Yoshitake *et al.*, 1952).

Hatamura (1943) also has investigated the distribution of uric acid in an oily mutant named *od* (distinct translucent, 49.6 in the I chromosome) and has found that the amount of uric acid in the *od* larvae and eggs is less than in the normal type. The most interesting phenomenon found by Hatamura was that in both types the amount of uric acid present in the egg decreased just before hatching. This result implies that uric acid is converted into some other substances. But he was not able to determine what they were.

Allantoin is a well-known derivative of uric acid. But Manunta (1949) detected the presence of allantoic acid and glyoxylic acid instead of allantoin in normal and oily type larvae of silkworms. If this finding is right, uric acid in a silkworm may be decomposed by the steps shown in Fig. 6.

According to Koyanagi (1938, 1941), the amount of riboflavin present in the Malpighian tube is about 60% of the total content in the mature larva. Employing various mutant races mentioned above, the writer (1946, 1948) demonstrated that the total amount of riboflavin present in the pupa is roughly proportional to the degree of pigmentation of the Malpighian tube in the larva. A similar relation was found by Koyanagi and Hatamura (1944), who used a special mutant, "oily-mottled" (an allele of *od*).

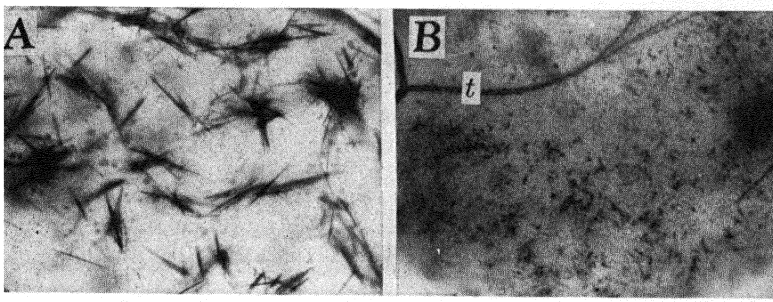


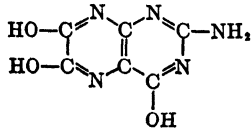
FIG. 7. Photomicrographs (*ca.* x300) of chromoplasts found in a cell of the Malpighian tube of *Bombyx mori*. *A*, Normal type (crystal-like); *B*, *od* type (small, granular or needle-like); *t*, branch of the trachea.

Now the question arises as to the origin of riboflavin in the silkworm. In 1941, Koyanagi suggested that riboflavin in the silkworm might be derived from mulberry leaves. This suggestion, however, has been opposed by Maruo and Koike (1941), who observed a greater increase of riboflavin in the early pupal stage than in the mature larvae. A similar phenomenon has been observed by the writer (1946). Thus, unlike the case of nicotinic acid, it is very likely that riboflavin can be synthesized by the silkworm itself, even though a part of it might be derived from the food. As pointed out by the writer (1946), the ability to preserve riboflavin in the silkworm is controlled by certain genes. The majority of oily genes, such as *od*, *oα*, and *ol*, decrease riboflavin content, while the white-1 gene increases it. These results imply that there is a close relation between the formation of uric acid and that of riboflavin. Hatamura (1949) suggested that riboflavin may be converted into uric acid and *vice versa*.

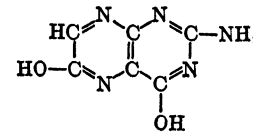
Several investigators have proposed that the transformation of ribo-

mins. Thus it is assumed that the chromoplasts involved in tryptophan metabolism are concerned also in purine or pyrimidine metabolism. It seems probable that competition takes place between the two metabolic pathways.

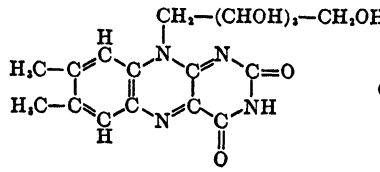
flavin into pterins may occur in various insects (Bodine *et al.*, 1948; Burgess, 1949, etc.). A similar relation has also been observed in Bombyx (Kikkawa, unpublished). The genetic and physiological phenomena mentioned above suggest strongly that there are close connections between uric acid, riboflavin, and pterins. In chemical properties also, they are similar to one another, especially in their chemical structures (cf. Fig. 8). Presumably these substances are derived from a common precursor.



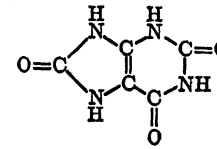
Leucopterin



Xanthopterin



Riboflavin



Uric acid

FIG. 8. Chemical structure of pterins, riboflavin, and uric acid.

It should be further noted that some oily genes like *od*, *oα*, and *ol* considerably influence the formation of brown pigments in eyes, eggs, and other organs; in these mutants the amount of brown pigment is lower than in the normal type. Pleiotropic phenomena are common in these oily mutants. Thus, we are obliged to conclude that the metabolism of purines or pyrimidines is closely bound with that of tryptophan.

4. *Problems Concerning Melanin Pigments*

A blackish pigment present in the cuticle of the silkworm, which is insoluble in water or alcohol, is generally called *melanin*. The mutants Striped, Moricaud, Black, Zebra, Multilunar, *etc.*, show this pigment. Aruga (1943, 1944) and Ito (1949) have found that the epidermis underlying the cuticle with melanin in Striped and Zebra mutants has little uric acid, namely, it shows oily-like features. Using a mutant called "S-mottled," which is mosaic for Striped and normal skin, Aruga (1943) obtained good evidence in favor of this assumption. Thus, it may be assumed that the genes responsible for melanin pigment have some

bearing on the formation of uric acid, as well as brown pigments. But the mechanism involved in those interactions is very complicated, and no definite conclusion has been obtained.

Harizuka (1942, 1947) has investigated the genetics and chemistry of a recessive mutant black pupa (its gene, *bp*, is located at 17.1 in the XI chromosome). Although this mutant shows only in the pupal stage, it is possible to separate *bp* larvae from normal ones with the help of the linkage between *bp* and *K* genes (Knobbed, 0.0 in the same chromosome). The gene *bp* is sensitive to temperature. Its temperature sensitive period is very short, ranging for several hours from the end of spinning till pupation. Below 15 to 20°C., the *bp* trait shows up, but above 25°C., it does not.

Harizuka (1942) once proposed that the *bp* pupa has a strong tyrosinase activity in the body fluid as compared with the normal one. In a later report (1947), he has advanced a different hypothesis, namely, that the amount of water-soluble proteins present in the cuticle may play an important part in the hardening and coloring processes of puparium, inasmuch as the tyrosinase activity present in the *bp* cuticle is the same as that in the normal one.

The problem of melanin formation in the cuticle of Bombyx is being studied by several investigators in Japan (Aruga and Kawase, 1952, *etc.*).

IV. Maternal Inheritance in the Egg Color

The phenomenon of maternal inheritance in the egg color of the silkworm was once an enigma. But, with the advance of knowledge concerning pigment formation in insects, the problem has been solved little by little.

In 1943, adopting the writer's hypothesis (Kikkawa, 1941a,b, 1943a), Suzuki attempted to explain his large body of data dealing with various cases of maternal inheritance in the egg color of Bombyx. Later, this problem was discussed more thoroughly by the writer (Kikkawa, 1948). At present, there are two fundamental types of maternal inheritance of the egg color.

(1). Type I (white-1 type). As shown in Fig. 9, in the cross of $w^1/w^1 \times +/+$, the egg color of the F_1 generation does not show the whitish color of the P generation, that is, its color is intermediate. In this sense, it may not be regarded as typical maternal inheritance.

As stated before, the white-1 mutant lacks the enzyme that converts kynurenine into 3-hydroxykynurenine, though it has enzymes converting 3-hydroxykynurenine into pigments. Accordingly, in the cross $w^1/w^1 \times +/+$, a great amount of kynurenine is accumulated in the egg, while

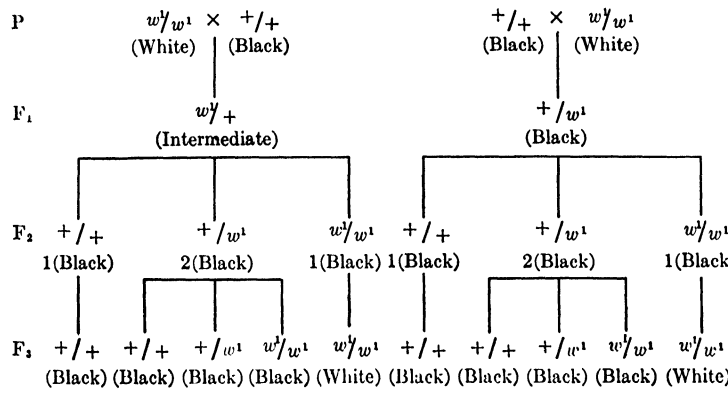


FIG. 9. Schematic figure illustrating maternal inheritance of white-1 type. w^1, white-1; +, normal. The symbols +/+, +/w^1, and w^1/w^1 designate the genotype in the serosa.

in the reciprocal cross it is replaced by 3-hydroxykynurenine. In either cross, the genotype of the F_1 serosa has the constitution +/w^1, which is able to convert tryptophan into pigments. In the cross of w^1/w^1 × +/+, kynurenine present in the egg has to be converted, in the first place, into 3-hydroxykynurenine, then into pigments, while in the reciprocal cross, the chemical step of conversion from kynurenine into 3-hydroxykynurenine may be omitted. Thus, the advantageous chemical pathway in the cross +/+ × w^1/w^1 favors production of dark pigments, since the enzymes involved are assumed to be limited in function.

In the F_2 generation, three types of eggs may be expected, *viz.*, +/+, 1; +/w^1, 2; and w^1/w^1, 1. All these show black color, although the last type has the w^1/w^1 constitution. This is due to the presence of 3-hydroxykynurenine which has been brought in the F_2 egg from the mother with +/w^1 constitution.

(2). Type II (brown-1 type). The manner of inheritance of this type is shown in Fig. 10. This type resembles type I and shows a typical maternal inheritance. But it has been found that the mechanism of maternal inheritance in this type is quite different from that in type I. Although the ovary and other organs, like eyes and fat bodies, of the brown-1 type have the ability to produce 3-hydroxykynurenine from kynurenine, 3-hydroxykynurenine cannot pass into the ooplasm, that is, brown-1 shows the phenomenon of selective permeability. Thus the egg contains little 3-hydroxykynurenine in the ooplasm (about 10 to 20%

in the reciprocal case), although the chromoplast contains the enzymes concerned with pigment formation. The chromosome in which the gene responsible for this behavior is located has not been established.

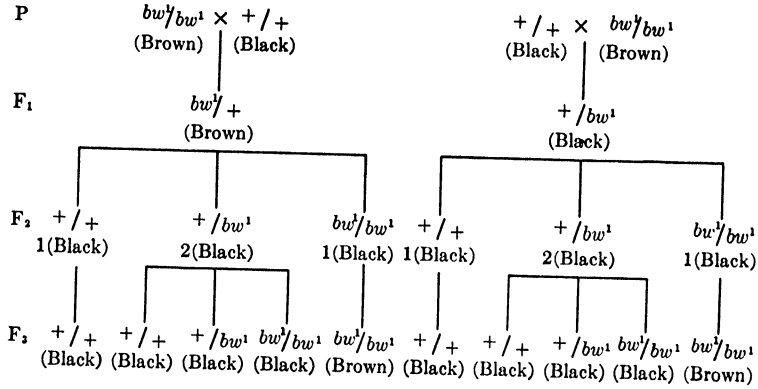


FIG. 10. Schematic figure illustrating maternal inheritance of brown-1 type. bw^1, brown-1; +, normal.

(3). Complex type. Beside the two types described above, there are several complex types of maternal inheritance of egg color. For example, the brown-2 mutant (bw^2, 8.0 in the VI chromosome) investigated by Uda (1923, 1924) and Tanaka (1924) belongs to one of them.

In this type, segregation occurs in the F_2 generation, *viz.*, black and brown eggs appear in the ratio of 3:1 in the same batch layed by a single female, but in the cross $bw^2/bw^2 \times +/+$, brown eggs show a peculiar color which is different in different generations, *viz.*, light-brown in P, purplish-brown in F_1, and dark-brown in F_2.

In order to account for this complex behavior, the writer (1941c, 1948) proposed that the brown-2 mutant has little 3-hydroxykynurenine in the ooplasm (as in the brown-1 type), and further that the enzyme present in the chromoplast is too weak to convert 3-hydroxykynurenine into pigments. These relations are shown schematically in Fig. 11. But the problem whether such physiological differences in enzyme function and permeability are controlled by only one gene or by pseudoalleles has not been solved as yet.

V. MISCELLANEOUS CASES

Many important problems in the biochemical genetics of Bombyx are still unsolved. In 1939, Morohoshi reported that he could obtain a trimolting larva by injecting the body fluid of a trimolting larva into

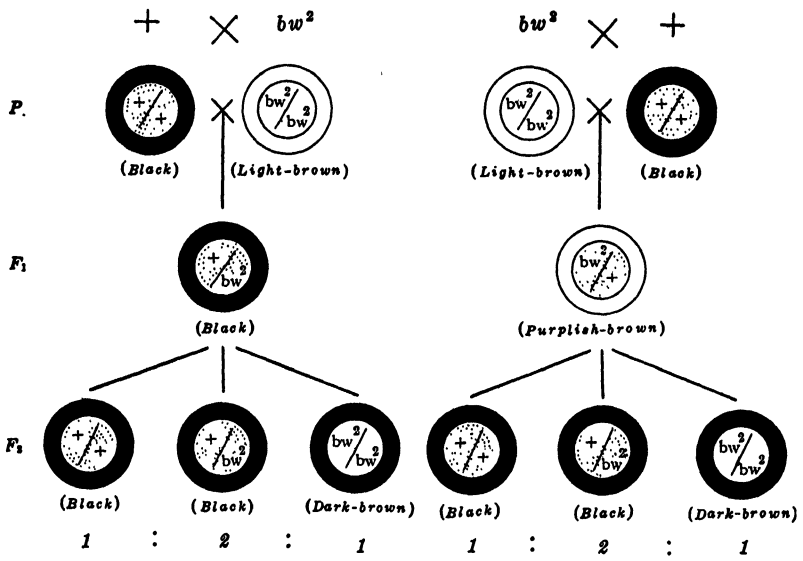


FIG. 11. Schematic figure illustrating maternal inheritance of brown-2 type. Larger circles indicate the constituents of ooplasma determined by the genotype of the mother moth. Black circles represent large quantities of 3-hydroxykynurenine; white ones, small quantities. Smaller circles indicate the constituents of genotypes in the serosa which are determined by the combination of parental gametes. The symbols $+/+$ and $+/bw^2$ (dotted circle) represent the enzymes capable of converting 3-hydroxykynurenine into pigments. The symbol bw^2/bw^2 (white circle) represents the enzymes with weaker abilities.

a tetramolting one. His result seems to indicate that molting is controlled by one or more diffusible substances present in the body fluid. Bouhniol (1937), Kin (1939), Fukuda (1944), Ichikawa (1950), and others have proved that the prothoracic gland and corpora allata are concerned with molting and pupation. Probably multiple alleles of the molting gene which is present in the VI chromosome control the morphological or physiological characteristics of those organs. But no detailed study has been carried out as yet.

A similar situation exists in the problem of voltinism. Employing transplantation methods, Umeya (1926) and Fukuda (1940) have clearly proved that a diffusible substance or substances present in the body fluid is concerned with voltinism. The writer (1943a) pointed out that there is a close connection between voltinism and tryptophan metabolism. Recently, Hasegawa (1952) and Fukuda (1952) have found independ-

ently that the suboesophageal ganglion and brain play an important role in determination of voltinism.

There are many reports in which chemical differences are recognized between the two sexes. In Bombyx, as pointed out by Hashimoto (1933), and by Tazima (1941), the W-chromosome has a decisive function in determining the female sex. Accordingly, it is likely that sexual differences in chemical components may be controlled by the gene or genes involved in the W-chromosome. But this case, as well as that of polyploidy, is too complicated to discuss in terms of the chemical nature and function of genes.

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(J), Japanese; (J, ER), Japanese with English résumé; (J. GR), Japanese with German résumé.

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The Genetics of *Aspergillus nidulans*

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CONTENTS

I. Introduction

In 1945 a search was made for a microorganism suitable for a genetic approach to certain problems of spatial organization of the cell. The outlines of these problems have been given elsewhere (Pontecorvo, 1950, 1952b, 1952c). Tentative work with a number of species of molds led to the conclusion that *Aspergillus nidulans* (Eidam) Winter, a homothallic ascomycete, was on the whole the most suitable. Genetically this species was unexplored: a not surprising fact, since no homothallic fungus had been investigated before, homothallism usually being assumed to be too serious an obstacle in the way of experimental breeding. Henrard (1934), who made an attempt at genetic analysis in *A. nidulans*, concluded: "Malheureusement nous avons affaire à une espèce homothalle, ce qui augmente considerablement la difficulté, á la supposer surmontable. Si nous confrontons deux souches homothalles . . . il y a peu de chance pour que, parmi les fructifications obtenues, il y a des hybrides; et s'il y en a, comment les distinguer?"

The present work shows that these difficulties are unreal and that homothallism may even constitute an advantage in certain technical respects. It is now clear that the innumerable homothallic species of fungi are not necessarily less suitable for genetic investigation than the heterothallic ones. The principles underlying our techniques (Pontecorvo, 1949a, and present paper) together with those underlying Lederberg's (1947) technique for bacteria, and the recent ones for asexual filamentous fungi (Roper, 1952; Pontecorvo and Roper, 1952) open the way for the genetic investigation of an enormous range of microorganisms with a wide variety of life cycles. In the applied fields of industrial fermentations, "microbial breeding" is now as obvious a possibiilty as plant breeding in horticulture. The choice fell on *Aspergillus nidulans* because:

1. It lends itself to standard genetic analysis, since it has a normal sexual cycle. In the greatest part of the cycle the nuclei are haploid; the fusion of two haploid nuclei—presumably when the diploid nucleus of the young ascus is formed—alternates with meiosis which restores immediately the haploid condition in the ascospores.

2. It lends itself to the techniques of balanced heterokaryosis (Dodge, 1942; Beadle and Coonradt, 1945; Kniep, 1920; Pontecorvo, 1947) because its hyphae are multinucleate, and hyphal anastomosis followed by migration of nuclei between hyphae occurs readily.

3. Since it forms uninucleate vegetative spores (conidia) of dark-green color, and spore color mutations supply excellent "markers," plating of conidia is equivalent to sampling individual nuclei, a very

convenient fact for the study of a variety of problems: e.g., segregation of nuclei from heterokaryons (Gossop, Yuill, and Yuill, 1940; Pontecorvo, 1947); detection of cytoplasmic inheritance; isolation of artificially induced diploids (Roper, 1952); isolation of mutants; measurement of mutation rates; selection of somatic recombinants (Pontecorvo and Roper, 1952).

4. Its minimal nutritional requirements being as simple as they can be in a fungus—one source of organic carbon, nitrate as source of nitrogen, and inorganic salts—there is ample scope for the study of the genetics of biosyntheses.

5. Since it forms compact colonies on agar, its ascospores or its conidia lend themselves to plating techniques as those used for yeasts and bacteria: 48 hours after plating and incubation at 37°C. the colonies are classifiable as to morphology, color of conidia, etc.

6. Its fruiting bodies—"perithecia" or, more accurately, "cleistothecia"—do not eject the ascospores when ripe: the mature ascospores can therefore be preserved for months or years within the cleistothecia and used when required. This is particularly useful when it is necessary to reinvestigate an old cross.

7. Its asci (eight ascospores) are easy to micromanipulate and lend themselves to tetrad analysis. However, the spores are not arranged in linear order within the ascus; this makes the location of centromeres more laborious (Lindegren, 1949; Whitehouse, 1950) than in forms (e.g., *Neurospora*) where a linear order occurs. The new technique of centromere location by means of somatic crossing-over in heterozygous diploids (Pontecorvo and Roper, 1952; Pontecorvo, 1952a), however, might replace that of ascus analysis for this purpose.

8. Random samples of mature ascospores from many perithecia are easily taken, and enormous numbers of ascospores can be tested. This means that with selective techniques one can detect and estimate recombination rates as low as 10^{-5} (Roper, 1950a, and unpublished).

9. Its conidia are produced in bundles of parallel chains, with all the conidia of each chain deriving their nucleus from a single nucleus in the sterigma. This is most convenient for the study of a number of problems requiring exact knowledge of cell lineages and nuclear lineages.

The present work has been substantially the result of team activity. The following members of the staff and present or former research students of the Department of Genetics, University of Glasgow, have contributed to it in various measure: Dr. J. A. Roper, Miss L. M. Hemmons, Mr. K. D. Macdonald, Mr. E. C. Forbes, Mr. A. W. J. Bufton, and Miss O. B. Adam. Some of the sections of the present paper appear under authorship other than that of the senior author or under joint author-

ship. This is an attempt to apportion these contributions where they have been exclusive or predominant.

II. Life Cycle

1. *Vegetative Cycle*

As in most other filamentous fungi in which sexual reproduction occurs, *Aspergillus nidulans* (Eidam) Winter, an ascomycete (Order Plectascineae, Family Aspergillaceae) shows a vegetative cycle side by side with a sexual cycle (Fig. 1). The species was first described in

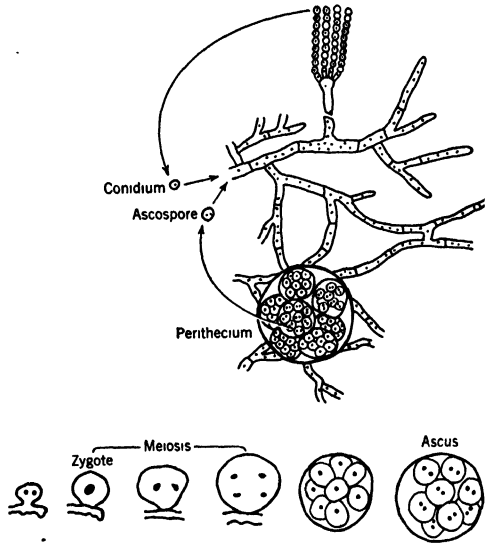


Fig. 1. Life cycle of *A. nidulans*.

detail by Eidam (1883); further information is to be found in papers by Dangeard (1907), Schwarz (1928), and Henrard (1934); and an up-to-date review is in Thom and Raper's *Manual of the Aspergilli* (1945). Schwarz stated, without details, that *A. nidulans* is homothallic; and Henrard, by showing that single ascospores give origin to self-fertile strains, went most of the way toward proving it.

On germination, a uninucleate (Dangeard, 1907; Yuill 1950) haploid vegetative spore (conidium) produces colorless septate hyphae with multinucleate "cells." Some of these (foot cells) differentiate to form

a multinucleate stalk (conidiophore), 100 μ in length and 6 μ in diameter, growing out of the medium and ending in a globose multinucleate vesicle, 10 μ in diameter. The foot cell, the stalk, and the vesicle have a brownish thick cuticle. From the surface of the vesicle, a number of uninucleate elongated buds, 5 μ in length, (primary sterigmata) develop synchronously and, again synchronously, each one of these gives origin to a second series of one, two, or, rarely, more uninucleate secondary sterigmata. How the nuclei of the two or more sister secondary sterigmata are related to the nucleus of the primary sterigma is not known for certain (see Yuill, 1950).

The nucleus in each secondary sterigma divides repeatedly, and at each division one daughter nucleus remains in the proximal part of the sterigma; the distal part with the other daughter nucleus is then constricted out and differentiates into a conidium. This process is repeated many tens of times. The conidia are thus formed in long unbranched chains, with the last formed near the sterigma and the older ones, further away, gradually attaining full size and full green color. All the conidia of a chain derive their nucleus from the nucleus in the sterigma, a deduction familiar to mycologists, which can now be fully confirmed on genetic grounds (section V-1).

FIG. 2. Conidial head of *A. nidulans*.

In *A. nidulans* the chains produced by one vesicle remain parallel to one another, forming a columnar head of up to 40 μ in diameter and containing up to 100 chains (Fig. 2). It is possible to follow a single

chain of conidia throughout its length under a stereoscopic microscope. In cultures left undisturbed, the length of the chains may become enormous, say 400 μ; this implies that the nucleus in each sterigma divided about 100 times. The conidia are 3 to 3.5 μ in diameter (see table 30). They remain capable of germination for at least one year at room temperature, probably for several. In the wild type their color varies from dark green to dull gray-green according to age, media, and other external conditions. In a liquid medium after 5 hours at 37°C. most conidia have germinated.

One important feature of the vegetative cycle is hyphal anastomosis. It occurs readily between branches of one hypha, between different hyphae of one monosporous mycelium, or between hyphae of two mycelia of the same or of different strains, when their hyphae meet on the surface of the medium. As a consequence of anastomosis between hyphae of genetically different strains, two or more different kinds of nuclei may come to be included within the same cell, which can thus give origin to heterokaryotic mycelium.

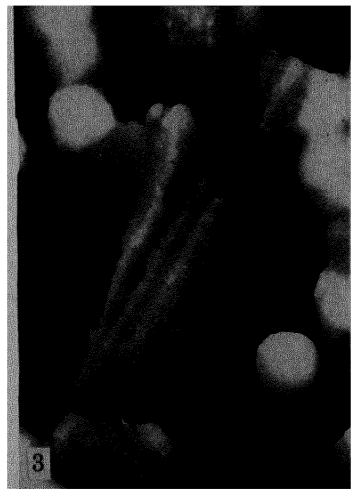


FIG. 3. Conidial head of heterokaryon between a white and a green strain; chains of conidia are either white or green.

When a heterokaryotic hypha forms a conidiophore, the vesicle may carry nuclei of more than one kind, but each secondary sterigma, being uninucleate, can carry only one kind of nucleus. All the conidia in one chain will therefore have the same kind of nucleus, but different chains in the same head may differ in this respect. Thus, in a hetero-

karyon, the mechanism of formation of conidia leads inevitably to segregation of the different kinds of nuclei into different conidia. As shown by Gossop, Yuill, and Yuill (1940), this segregation can be verified by inspection when the two kinds of nuclei determine differences in color of the conidia and (Pontecorvo, 1947) the difference is genetically cell-localized (Fig. 3).

2. *Sexual Cycle*

The sexual cycle is concentrated in specialized organs, the fruiting bodies (cleistothecia or perithecia). There are large blanks in the knowledge of its morphologic details, and even certain details of the nuclear cycle have to be deduced, so far, from the genetic results reported in the present work.

Mature perithecia are present about 8 to 10 days after incubation of cultures at 37°C. They are spherical bodies, mostly 100 to 200 μ in diameter, with an outer coat of yellowish loose hyphae carrying giant cells of unknown function—the "Hülle cells" (Eidam, 1883)—characteristic of the *Aspergilli* of the *nidulans* and a few other groups. Inside the coat of Hülle cells there is a shining, hard, dark red-brown wall 1 to 2 μ thick, originating from a single layer of cells, which constitutes the outer casing of the perithecium. This wall does not break at maturity unless crushed rather hard. The mature perithecium is full of asci, from 10 to 100,000 in perithecia of more than 100 μ in diameter, and each ascus of spheroidal shape, about 10 μ in diameter, contains within its colorless thin sheath eight brown-red ascospores, each binucleate (Adam, unpublished) discoidal, (diameter 3.5 × 4 μ) with two characteristic ridges. The mature ascus breaks very easily, liberating its eight ascospores, which germinate as quickly as the conidia.

I have traced back the processes leading to the mature ascus as far as the ascus primordium. The immature perithecium is filled with thick contorted hyphae of such irregular shapes that it is difficult to grasp their details. On these hyphae ("ascogenous hyphae"), the ascus primordia bud out like grapes in a bunch. The primordia are almost spherical, about 6 μ in diameter, with one conspicuous nucleus, which seems to result from fusion of two smaller nuclei (Fig. 4). The nucleus of the ascus primordium undergoes the two meiotic divisions, and the four products of meiosis divide again, giving in all eight haploid nuclei. The content of the ascus is then cut out into eight spores, each with one nucleus, and this nucleus divides again before the spore is fully mature (Adam, unpublished).

As to the nuclear cycle, from the study of meiosis (section II-3), from ascus analysis, and in general from the genetic evidence there is

little doubt that the nucleus of the ascus primordium is diploid ($2n = 8$), the two nuclei of the ascospore are haploid and sisters, the eight ascospores of an ascus represent the four products of meiosis in duplicate, and the nucleus of the conidium is haploid. As to the nuclei in the mycelium, a direct cytological examination is of no avail because of the smallness of the chromosomes. But we may take it that from the haploid nuclei of the ascospore the nuclei of the mycelium derive by mitosis, and from these the nuclei of the conidia.

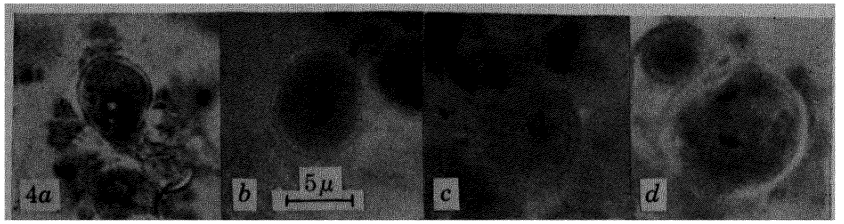


FIG. 4. Four stages in the ascus primordium: (*a*) two nuclei presumably about to fuse; (*b*) the nucleus of the zygote; (*c*) first meiotic metaphase—four structures, one out of focus; (*d*) first meiotic anaphase.

As to the nuclei in the ascogenous hyphae, the analysis of the asci of individual perithecia produced in a cross shows that the asci of any one perithecium tend to be either all selfed of one parental kind, or all selfed of the other parental kind, or *all crossed* (section V-4). This last type of perithecium could arise only with one of two mechanisms: (1) two nuclei, one of each kind, fuse at the beginning of the perithecium and give origin to a diploid heterozygous nucleus from which the nuclei of the 10 to 100,000 ascus primordia of that perithecium derive; or (2) two nuclei, one of each kind, enter into conjugate divisions at the beginning of the perithecium, or very early in its development, and fusion between two descendants, *one of each lineage,* takes place at some later stage, presumably in each ascus primordium.

The first alternative must be rejected if fusion of two nuclei in the ascus primordium does really occur (Fig. 4). On the other hand, conjugate divisions are believed to be widespread in the *Ascomycetes* (but see Martens, 1946, for a critical discussion). In the absence of crucial evidence, we shall take it, tentatively, that conjugate divisions in the ascogenous hyphae and karyogamy in the ascus primordium occur in *A. nidulans.*

3. *Meiosis*

Only a very superficial study of meiosis has been carried out, the main point which was urgent to ascertain being the number of chromosomes in *A. nidulans*. After having succeeded in isolating strains with diploid nuclei in their vegetative cells (section VII-1), it also became important to verify cytologically the occurrence of tetraploid meiosis.

The best material is obtained from young perithecia when the first asci with fully colored ascospores begin to appear. This is usually the case after 4 to 6 days of incubation in plate cultures on a complete medium. Older cultures can also be used by selecting immature perithecia when they just show a pink color in their walls.

We have made use extensively of fresh crushed preparations in aceto-lactic-orcein following McClintock (1945). The staining improves on keeping for about two weeks.

The stages which we have been able to recognize with some confidence are shown in Fig. 4*a*, *b*, *c*, and *d*. Stage *a*, which we interpret as that of two nuclei about to fuse in the ascus primordium, could be taken, of course, as a division. However, comparison with stage *d*, which is undoubtedly an anaphase seems to exclude this interpretation. As to stage *b*, we interpret it as that of the diploid zygote; it shows a nucleus larger and better staining than at any other stage, and the ascus primordium is somewhat larger than in the preceding stage. Stage *c* is undoubtedly the first metaphase of meiosis, seen in almost polar view. In the many cases in which a count has been possible, we have seen four bivalents in haplo-diploid strains. Two of these are of medium size, one is much larger, and one very small, almost dotlike. We conclude, provisionally, that *A. nidulans* has four chromosome pairs (in the zygote), and of these one is very large, one very small, and two of medium length. Though *A. nidulans* is certainly not easy material for cytological work, it is probably not more difficult than *Neurospora* (McClintock, 1945).

Tetraploid meiosis has been observed in diplo-tetraploid strains, i.e., strains (section VII-1) which presumably have diploid nuclei in their vegetative cells and tetraploid nuclei in most ascus primordia. In these strains, there are plenty of asci in meiosis, but the majority degenerate afterwards. Not more than a few hundred mature asci are present at best in one perithecium. These asci (section VII-1) are mainly 16-spored, and the ascospores have a germinability of less than 1 to 50. The most striking, though expected, feature of metaphase of meiosis in diplo-tetraploids is that instead of the four bivalents invariably seen in haplo-diploids, bodies varying in number from four to seven have been

observed. No case of eight bodies has been seen, but observation is difficult enough not to give too much weight to this. The bodies are often of complex structure, suggesting multivalent associations.

The observations on diplo-tetraploid strains permit only the conclusion that tetraploid meiosis certainly occurs in a high proportion of their asci.

III. Methods of Culture

1. *Strains*

Three original strains of *A. nidulans* have been the starting point of our work. One, kindly supplied by Mr. J. L. Yuill, we call the "wild type" and we designate it "+." It is the green-spored strain to which Yuill (1939, 1950) gives the symbol A69 and Thom and Raper (1945, p. 159), the symbol NRRL 194. A second strain, also received from Mr. J. L. Yuill, is the white-spored mutant "alba" (Yuill, 1939) which arose spontaneously in 1937 from A69. It is given the symbol A70 by Yuill (1950), and NRRL 195 by Thom and Raper. This strain differs by a single known allele (w_a) from wild type (section VI-1). A third strain is a mutant, unable to utilize sulphate as a source of sulphur, obtained by Dr. Hockenhull (1948) from A69 following nitrogen mustard treatment and given by him the symbol S_5. This strain again differs by a single known allele (s_d) from wild type (section VI-1).

All other strains produced in our work are spontaneous or induced mutants from these three or recombinants obtained from ascospores in crosses or by mitotic crossing-over from heterozygous diploids. Strains not requiring additional growth factors are kept on slopes of "minimal medium" (see below) at room temperature, subcultured by conidial transfer every 6 to 12 months. They are kept pure and fertile by occasional isolation, with the micromanipulator, of a single ascospore or of a single conidium or by single-colony isolation from plated conidia or ascospores. Most mutant strains requiring additional growth factors are kept on slopes of "complete medium" (see below). Despite the risk of accumulation of further hereditary nutritional differences in the strains kept in this way, the extra cost and labor of keeping them on minimal medium, supplemented only with the specific growth factor(s) required by each mutant would not be justified. Certain nutritional mutants, however, are inhibited by substances present in the complete medium and these must be kept in this way.

2. *Media*

Minimal medium: sodium nitrate, 6 g.; potassium chloride, 0.52 g.; magnesium sulphate ($7H_2O$), 0.52 g.; potassium di-hydrogen phosphate 1.52 g.; iron and zinc,

traces; dextrose, 10 g.; pH adjusted to 6.5 with sodium hydroxide (before sterilization); distilled water, 1000 ml. All ingredients of analytical reagent standard.

Sporulation minimal medium: used for production of abundant perithecia; same as minimal medium but sodium nitrate reduced to 1 g. and dextrose increased to 20 g.

Complete medium: It has been repeatedly modified since the beginning of the present work; in its present formula it consists of solution in 1000 ml. tap water as for minimal medium supplemented with: Difco Bacto Peptone, 2.0 g.; yeast extract "Yeastrel", 1.0 g.; casein hydrolyzate, 5 ml.; acid and alkali hydrolyzates of yeast nucleic acid, 3 ml.; acid and alkali hydrolyzates of thymus nucleic acid, 2 ml.; peptic and tryptic casein digest, 5 ml.; hydrolyzed plasma, 3 ml.; hydrolyzed corpuscles, 3 ml.; B vitamin solution, 1 ml.; pH adjusted to 6 ± 0.2.

The various solutions are prepared as follows:

Casein hydrolyzate: (according to the method of McIlwain and Hughes, 1944; (*Biochem. J.* 38, 187). One liter of solution made from 200 g. B.D.H. light white soluble casein. Kept in the dark over chloroform for up to three months.

Nucleic acid hydrolyzate (yeast and thymus): 2 g. nucleic acid in 15 ml. *N* NaOH; 2 g. nucleic acid in 15 ml. *N* HCl. The two mixtures heated at 100° C. for 20 minutes, then mixed, brought to pH 6 and filtered hot. Volume adjusted to 40 ml. and solution kept in dark over chloroform: to be shaken before taking samples.

Casein digests: 30 g. B.D.H. light white soluble casein mixed with 250 ml. water and divided into equal portions. Portion 1, brought to pH 8 with NaOH, and 2 g. trypsin added. Incubated at 40°C. over chloroform for 40 hours, then centrifuged and the supernatant boiled, adjusted to pH 6. The sediment mixed with water to a paste, adjusted to pH 1 with HCl, and 1 g. pepsin powder added; incubated at 40° C. for 40 hours, adjusted to pH 6, and boiled. Portion 2 treated in the same way but the order of digestion inverted (first peptic, then tryptic). All four solutions mixed, brought up to 240 ml. and pH 6, stored in dark over chloroform.

Hydrolyzed plasma and corpuscles: Plasma from oxalated horse blood (25 ml.) mixed with 25 ml. 2 *N* HCl, autoclaved at 120° C for 10 minutes, adjusted to pH 7, filtered, brought up to 50 ml. volume, stored in dark over chloroform. Corpuscles from same, treated same way, but pH adjusted to 10, and volume brought up to 75 ml.

Vitamin solution: riboflavin, 10 mg.; nicotinamide, 10 mg.; *p*-aminobenzoic acid, 1 mg.; pyridoxin-HCl, 5 mg.; aneurin-HCl, 5 mg.; biotin, 0.02 mg.; Ca-pantothenate, 20 mg.; choline chloride, 20 mg.; irositol, 40 mg.; folic acid, 1 mg.; distilled water, 10 ml. Koch sterilized.

To all media, when required, 1.5% Davies powdered agar is added, and after melting the medium is filtered through asbestos pulp. Sterilization of all usual media is carried out at 10 lb. for 10 minutes.

3. *Incubation and Growth Rates*

A. nidulans grows within a very wide range of temperatures; the optimum (Eidam, 1883) is unusually high, i.e., near 40°C. In our laboratory, cultures are incubated at 36 to 37°C.

On agar minimal medium at 36 to 37°C. colonies of the wild type started from point inoculum of conidia grow in radius at a constant rate of about 5.9 mm. per 24 hours after an initial lag. On complete medium, this constant rate is about 6.5 mm. per 24 hours (Table 1). When well-

TABLE 1

Radius of Colonies of *A. nidulans* on agar at 37°C.

Duplicate petri dishes, with 20 ml. medium, point-inoculated with conidia. Distance in millimeters of growing edge at successive times measured to the nearest millimeter from point of inoculation.

	Hours after inoculation									
	48	96	120	144	168	192	216	240	264	288
Complete Medium										
Dish 1 (mm.):	12	24	30	36	43	49	56	63	70	75
Dish 2 (mm.):	12	25	32	38	45	52	58	65	72	78
Minimal Medium										
Dish 1 (mm.):	7	19	24	30	36	42	47	53	60	66
Dish 2 (mm.):	8	19	25	30	36	41	47	53	59	66

separated colonies are wanted (e.g., for isolating from individual colonies), plating of conidia or ascospores should be aimed at not more than 50 colonies per petri dish (Fig. 5). When only counts and/or

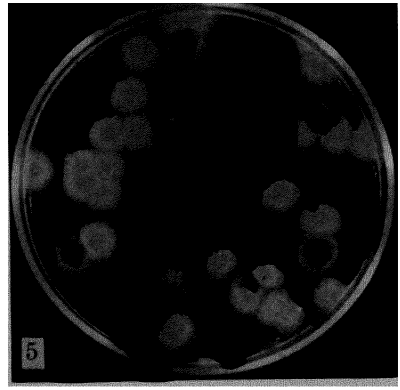


FIG. 5. Colonies from a cross segregating for yellow and green conidia; 48 hours after plating the ascospores.

classification as to conidial color are required, up to 200 colonies per dish are still manageable. Colonies of strains with normal speed of development of conidia can be classified as to colors about 48 hours after plating.

4. *Plating*

The conidia are non-wettable, and those in a chain are not easily separated. Conidial suspensions for counting and plating are made in

sterile distilled water or saline containing 1:10,000 calzolene oil as a wetting agent. The conidia are separated from one another by sucking them up and down vigorously for at least 100 times in a Pasteur pipette with a capillary spout. After counting with a hemocytometer dilutions are made in screw-top containers each with 9 ml. distilled water or saline without calzolene. For serial dilution, 1 ml. suspension is added to the 9 ml. of a container and so on in order to give serial steps by a factor of 1/10. Plating is done by spreading with a glass rod not more than 0.1 ml. suspension over the agar surface of each petri dish.

For the plating of ascospores, if a pooled sample from many perithecia is required, the perithecia are picked with a platinum wire into a test tube of saline-calzolene and sucked up and down repeatedly with a Pasteur pipette in order to remove conidia and Hülle cells. The clean perithecia are then transferred to a new test tube of any liquid medium without wetting agent and crushed against its wall. The ascospores—dark red—do not cluster if the perithecia are ripe. When the ascospores of a single perithecium are needed, the perithecium is carefully cleaned of mycelium, Hülle cells, and conidia by rolling it with a needle on the surface of agar medium, and it is then crushed into 0.1 to 0.2 ml. of liquid. In fully fertile strains the content of ascospores of one perithecium varies from about 50,000 to 900,000. Germination of ascospores is almost complete after 6 hours at 37°C.

IV. Isolation of Mutants

1. *General Methods*

The first step in our work was that of securing a good supply of mutants; about 600 are now available. Two of these—the white conidia mutant "alba" differing in one gene (w_a) from wild type, and a parathiotrophic mutant also differing in one gene (s_d) from wild type—were supplied by other laboratories. All the others are spontaneous or induced mutants obtained in our laboratory in one or more steps from the wild type or from "alba." Since the conidia of *A. nidulans* are uninucleate and haploid, the isolation of mutants consists in the isolation of colonies originated from single conidia.

All sorts of "visible" mutants, differing from the wild type in a way detectable by inspection (color of conidia growth habit, secretion of a pigment, secretion of an enzyme detectable by visible reaction, etc.), arise with the greatest ease, especially after irradiation. We have, however, kept and made use of only the following six including "alba" (see p. 185 and Table 17):

w_a ("alba"): white conidia; incompletely cell-localized effect; spontaneous from wild type (Yuill, 1936).

y: yellow conidia; cell-localized effect; X-ray induced from wild type (1947).

$w\ ad_1\ y$: white conidia; incompletely cell-localized; adenine-requiring; X-ray induced from y; simultaneous occurrence of the two mutant alleles w and ad_1 (1947).

$w_n\ paba_1\ bi_1$: white conidia; P.A.B.A.- and biotin-requiring; spontaneous from $paba_1\ bi_1$ (1951).

$bi_1\ pr_1$: reduced extracellular proteolytic activity (Fig. 6); biotin-requiring; ultraviolet induced from bi_1 (1951).

$paba_1\ y\ co$: compact colony; yellow conidia; P.A.B.A.-requiring; origin of co unknown; detected 1951 in strain $paba,\ y$.

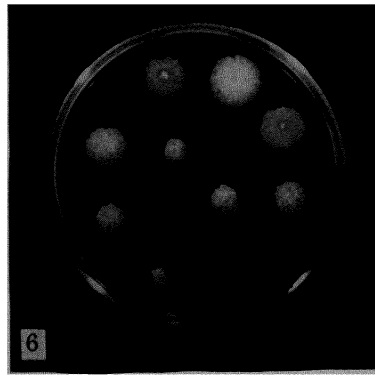


FIG. 6. Plate-tests on milk medium of segregants for the "visible" characters: colors of conidia and proteolysis; the latter revealed by presence or absence of clear ring around the colonies.

These "visible" mutants are most convenient as markers. With the possible exception of *co*, the analysis of which has not yet been completed, the visible mutant effect is due to one locus in every case. The same locus seems to be involved in all three mutants with white conidia because (*a*) from more than 2000 ascospores out of zygotes w_a/w_n and from more than 300 w/w_a, no colored recombinant was obtained; (*b*) diploids (section VII-1) heterozygous for each of the three alleles and the wild type allele have colored conidia, whereas heterozygotes w/w_a have white conidia.

"Nutritional" mutants constitute the bulk of those which have been isolated and made use of in our work. Needless to say, of the about 600 available, only a minor fraction, i.e., 27, have been analyzed genetically, and some only in a preliminary way. Nutritional mutants, or auxotrophs, differ from the wild type (prototroph) in being unable to grow

on minimal medium unless a growth factor(s) is added (Beadle and Tatum, 1941).

To obtain auxotrophs in quantity, there are practical difficulties well known in microbial genetics since the pioneer work of Beadle and Tatum. Among the colonies originating from single conidia, the auxotrophs constitute a small proportion of the total, not more than 3% even after the most effective mutagenic treatment. The auxotrophs can only be identified by testing on non-supplemented medium, on which the parent strain grows and they do not. After having been identified as an auxotroph, each strain has to be characterized; i.e., its growth factor(s) requirement must be identified. All this is laborious, and devices to reduce labor are necessary.

In our work it was found that a very considerable economy of labor could be achieved in the characterization of the auxotrophs simply by rationalizing the sequence of successive approximations required. The details are given in Section IV-3. As to the isolation of auxotrophs, one selective technique, based on the differential survival of auxotrophs under specific starvation, was prompted by the work of Fries (1940a,b) with *Ophiostoma;* it has made the isolation of auxotrophs as easy in *A. nidulans* as the penicillin technique (Davis, 1948; Lederberg and Zinder, 1948) has made it in bacteria. The "starvation" technique, as we call it, has provided about 500 of the 600 auxotrophs available; it is dealt with in Section IV-2. A few auxotrophs were obtained by an adaptation of Lederberg and Tatum's (1946) "delayed enrichment" technique. The bulk of the remainder were obtained by "total isolation," modifying slightly the adaptation of the original technique of Beadle and Tatum (1941) previously worked out for *Penicillium notatum* (Pontecorvo, 1946).

As mutagenic agents, X-rays (85 kv.) and ultraviolet (90% output in the 2537 A region from Hanovia XI low-pressure mercury lamp) were used. With X-rays, the conidia were treated dry by putting under the beam a piece of agar carrying sporulated mycelium 6 to 10 days old. The suspension of conidia was made after irradiation. A single dose of irradiation—50,000 r.—was used throughout. The viable counts from conidia so treated and plated on complete medium were about 1/200; the proportion of auxotrophs among survivors, pooling all results, was 2.35% (81/3438).

With ultraviolet, the conidia were suspended in 10 ml. saline, the suspension placed in a petri dish 45 cm. distant from the lamp, and the dish rocked gently during irradiation. The time of treatment was kept constant: 8 minutes. However, because of changes in mains voltage and other non-controlled conditions, the actual treatment varied vastly

between experiments, giving survival rates between 5% and 25%. We shall therefore express the dose in terms of viable counts relative to conidia plated (hemocytometer estimate). With ultraviolet the highest proportion of auxotrophs obtained among survivors was 1.25% in two experiments with viable counts of 5%.

Total isolation. With this technique the conidia are treated as mentioned and plated on complete medium at such a density as to obtain 20 to 50 colonies per plate. After incubation for 48 hours, isolations are made from each colony well separated from the others, and the tests for identifying the auxotrophs and characterizing their growth-factor requirements are carried out (Section IV-3).

At the beginning of this work, each colony was isolated onto slants of complete medium, and the further tests were carried out from these. Later it was found that Fries' (1948b) plate tests save much labor. The technique is now as follows:

1. From each colony a small amount of mycelium is spot-transferred onto dishes of minimal medium, 20 isolates per dish.

2. After 48 hours of incubation, the transfers that show much less growth than the majority (usually at the expense of the small amount of complete medium carried over with the inoculum) are "rescued" onto complete medium slants, and from these the further tests are carried out (Fig. 7).

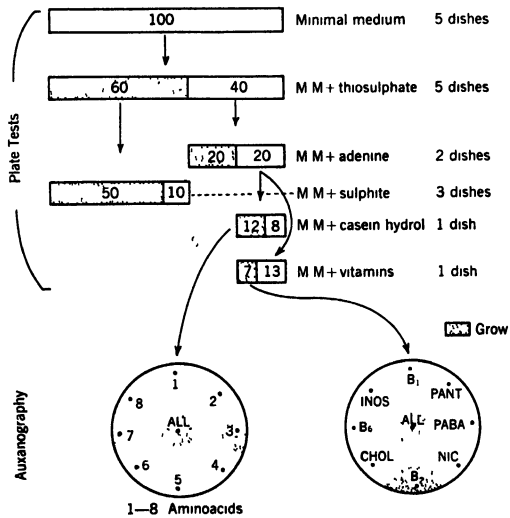


FIG. 7. Diagrammatic sequence in the routine for characterization of auxotrophs: plate-tests followed by auxanography.

TABLE 2

Mutants Obtained by "Total Isolation"

Series	*Strain treated*	*Isolates* (no.)	Total (no.)	Total (%)	*Auxotrophs* * S	N	A.A.	N.A.	Vit.	Untested
X-rays, 50,000 r.										
19-11-46	*y*	201	7	3.5	0	1	0	2	1	3
28-1-47	w_a	338	7	2.1	1	1	1	2	0	2
28-2-47	+	262	8	3.1	0	2	0	0	2	4
15-3-48	*y*	364	8	2.2	1	4	0	0	3	0
J.A.R.-1	*y thi*	1372	21	1.5	3	2	2	2	7	5
J.A.R.-2	bi_1	671	26	3.9	1	3	7	3	2	10
S-1, S-2	bi_1	230	4	1.7	0	0	1	0	3	0
	Total X-rays	3438	81	2.4	6	13	11	9	18	24
U.V. (in brackets survival rates)										
S-3, S-4 (5%)	bi_1	400	5	1.3	1	0	1	2	1	0
S-5 (25%)	bi_1	500	2	0.4	0	0	0	0	2	0
S-6 (12.5%)	bi_1	1070	5	0.5	0	4	0	0	1	0
	Total U.V.	1970	12	0.6	1	4	1	2	4	0

* S, unable to utilize sulphate; N, unable to utilize nitrate; A.A., requiring an amino acid, N.A., requiring a purine or pyrimidine; Vit., requiring a vitamin.

Results of series J.A.R-1 and J.A.R.-2 supplied by Dr. J. A. Roper. Results of series S1-S6 are the "0-hr. controls" of the experiments with starvation (Section IV-2).

Table 2 gives the results of all the series carried out so far by total isolation. The ultraviolet results are the "controls" of the experiments in which "starvation" was used as a selective means (section IV-2). The pooled results from "total isolation" give 93 auxotrophs out of 5408 isolates. Of the 93 auxotrophs, 69 were fully tested (section IV-3), and for 67 a growth factor was identified as capable *singly* to permit growth when added to minimal medium. Only two of the 69 strains required simultaneously more than one growth factor. As to the requirements: 7 strains were unable to utilize sulphate but could grow on more reduced inorganic sulphur compounds, e. g., thiosulphate; 17 were unable to utilize nitrate but could grow on nitrite or ammonium salts; 12 required an amino acid; 11 a purine or pyrimidine; and 22 a vitamin or a mixture of vitamins. The details of the spectrum of mutants will be discussed later (section VI-4).

Delayed enrichment. A small-scale attempt at increasing the yield of mutants was made by adapting to *A. nidulans* Lederberg and Tatum's

(1946) "delayed enrichment" technique for bacteria. This is based on the following reasoning: if a mixture of auxotrophic and prototrophic cells is plated on minimal medium, the latter give origin to colonies, but the former do not. After incubation, as soon as prototrophic colonies are barely visible (when they can be marked on the reverse of the petri dish) complete medium is added, and the auxotrophic colonies may then come up. The auxotrophs are therefore identified, because they grow only after addition of complete medium.

With Lederberg and Tatum's technique the plated cells are covered by a layer of agar medium to make it possible to add, after incubation, a further layer of complete medium. With *A. nidulans* it was found that the hyphae growing vertically reached the surface of the covering layer too soon. This difficulty was overcome by using not a wild-type strain but a strain requiring aneurin and by controlling its growth rate by means of a limiting amount of aneurin in the minimal medium. Irradiated conidia of the aneurin-requiring strain (*y thi*) were spread over a bottom layer of 5 ml. of agar minimal medium, covered with a second layer of 5 ml. of the same medium and incubated for 24 hours. The barely visible colonies were then spotted, and a further layer, this time of complete medium, was poured on top. After further incubation, all the colonies developed *after* the addition of complete medium were isolated and tested.

Unfortunately, as already found in the case of *Aerobacter aerogenes* (Devi, Pontecorvo, and Higginbottom, 1951), only a minor proportion of the delayed colonies turned out to be auxotrophs (i.e., requiring an *additional* growth factor besides aneurin, which all required). In fact, for irradiation with 50,000 r. the yield of auxotrophs among isolates was raised only from 2.4%, as in "total isolation," to about 5% (Table 3).

TABLE 3

Comparison of the Efficiency of Total Isolation, Delayed Enrichment, and Starvation for the Isolation of Auxothrophic Mutants

	X-rays (50,000 r.)			*U.V.* (5% survival)	
	Total isolation	Delayed enrichment	Starvation (≧ 96 hr.)	Total isolation	Starvation (≧ 96 hr.)
Isolates	3438	71	344	400	777
Mutants	81	4	94	5	308
Per cent	2.4	5.6	27	1.3	39

The efficiency of the starvation technique in the experiments tabulated above is considerably lower than the best now attainable, which approaches 60%.

The reason for this is that irradiation produces an enormous scatter in the germination times of the conidia; many delayed colonies are therefore not auxotrophs.

The technique was discontinued.

2. *"Starvation" Technique*

by K. D. Macdonald and G. Pontecorvo

This technique, prompted by Fries' (1948a; 1948b) work with *Ophiostoma,* has proved to be of extraordinary efficiency, yielding up to 60% auxotrophs.

In the first place, it was found that the conidia of a biotin-requiring *A. nidulans* mutant (*bi_1*) when plated embedded in (biotin-free) minimal medium died off quickly, after 100 hours less than 1% survived. In the second place, it was found that conidia of strains requiring, besides biotin, another growth factor (adenine or aneurin) died in minimal medium at a *slower* rate than those of the strain requiring only biotin. These results were in full agreement with Fries' findings.

That Fries' results were paralleled in *Aspergillus* was shown by appropriate "reconstruction" experiments, in which conidia (green or yellow) of two biotin-requiring strains (*bi_1* and *y bi_1*) were mixed in known proportions with conidia (white) of a biotin-adenine requirer (*w ad_1 bi_1*) or with conidia (yellow) of a biotin-aneurin requirer (*y bi_1 thi*). The mixed conidial suspensions were spread in a series of dishes over a bottom layer of minimal medium, covered by a second layer of minimal medium, and incubated at 37°C. No colonies, of course, developed after this first incubation, the medium lacking the required growth factors. After different lengths of incubation, different dishes were enriched by the addition of a top layer of complete medium. The number of colonies developed after further incubation indicated the number of conidia still viable at the moment of addition of the complete medium. By making use of the color differences (white, yellow, green) between the strains, the classification of the survivors of each strain could be done by inspection. Figure 8 shows the results of such reconstruction experiments: after about 100 hours "starvation" (i.e., time from inoculation in minimal medium to addition of complete medium) very few of the *bi_1* (biotin-requiring, green) or of the *y bi_1* (biotin-requiring, yellow) conidia were viable, whereas more than 20% of those of the other two strains were still capable of giving origin to colonies. Thus, selection of the latter was almost 100% effective.

The next step was the investigation of whether double-auxotrophic conidia, *arising as a consequence of induced mutation among irradiated*

conidia, would behave in the same way as the conidia from double auxotrophic strains—i.e., die more slowly under starvation than those of the parent strain. The conidia of the *bi* strain were irradiated, plated embedded in minimal medium, and incubated as in the "reconstruction" experiments. Complete medium was added as a top layer to different dishes at different times after inoculation, and a random sample of all

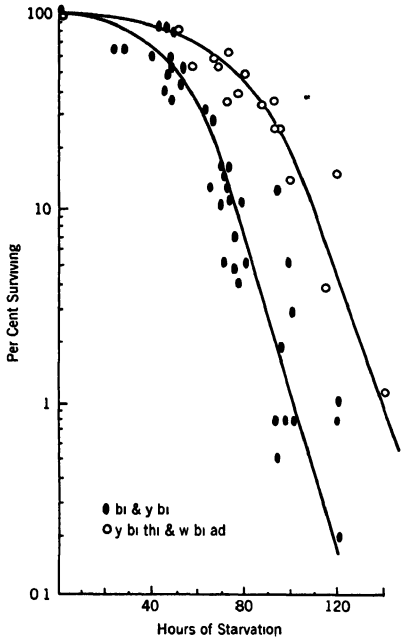


FIG. 8. Reconstruction experiments: under starvation the conidia of strains requiring biotin and aneurin or adenine (*y bi, thi* and *w ad, bi*) die off at a slower rate than conidia of strains requiring biotin only (*bi* and *y bi*).

the colonies developed thereafter was isolated and tested for growth-factor requirements *additional to that for bi,* which all had. The results of a number of experiments (Series S-1 to S-5), both with ultraviolet and X-rays, covering periods of starvation from 0 to 160 hours are graphically shown in Fig. 9 and given in detail in Table 4. In each of the curves of Fig. 9, two points are most relevant. One is the percentage of (double) auxotrophs among survivors at 0 time; this percentage represents the relative yield of mutants recoverable without selection, i.e., by "total isolation." The other is the maximum percentage of double

TABLE 4

Mutants Obtained by Starvation after Irradiation

Series S-1 and S-2: 50,000 r., X-rays. Series S-3 and S-4: U.V., 5% survival. Series S-5: U.V., 25% survival.

Hours of starvation	*Conidia plated* *	*Colonies*	*Isolates*	*Auxotrophs among isolates*		*Auxotrophs per 10^8 spores plated*
	(no.)	(no.)	(no.)	(no.)	(%)	
X-rays, 50,000 r.						
0	63,000	335	230	4	1.7	92
46	45,000	184	115	2	1.7	71
74	45,000	129	88	3	3.4	97
96	630,000	165	119	18	15.6	39
111	1,010,000	130	93	31	33.3	42
117	560,000	44	40	12	30	24
123	1,120,000	42	38	11	28.9	11
147	2,240,000	57	54	22	40.8	10
U.V. (5% survival)						
0	23,100	1010	400	5	1.25	545
114	2,640,000	889	300	56	18.7	63
137	1,980,000	191	137	89	65	62
143	2,640,000	362	200	91	45.5	66
161	2,640,000	254	140	72	51	49
U.V. (25% survival)						
0	3,000	734	500	2	0.4	975
96	56,700	348	160	2	1.25	77
112	112,800	383	175	12	6.9	234
123	56,700	147	123	10	8.1	212
136	112,800	104	86	24	27.9	260
159	225,000	185	160	35	21.9	180

* Hemocytometer estimate.

auxotrophs, obtained usually after periods of starvation of over 100 hours. Compared with the former this percentage gives an idea of the enrichment effected by starvation. In the best of the curves of Fig. 9 this enrichment is by a factor of about $\times$ 70.

It will be noted that the enrichment factor in the case of double auxotrophs produced by irradiation is considerably smaller than in the reconstruction experiments. This is probably the result of the scatter in germination times produced by irradiation, noted already in section IV-1 for the "delayed enrichment" technique.

The qualitative spectrum of mutant types selected by starvation is superficially similar to that of "total isolation" (Table 6). Quantitatively, however, there are some striking differences, such as the high proportions of mutants unable to utilize sulphate ("parathiotrophic") and of those requiring adenine, and the low proportion of mutants requiring a vitamin and of those unable to utilize nitrate ("paraazo-

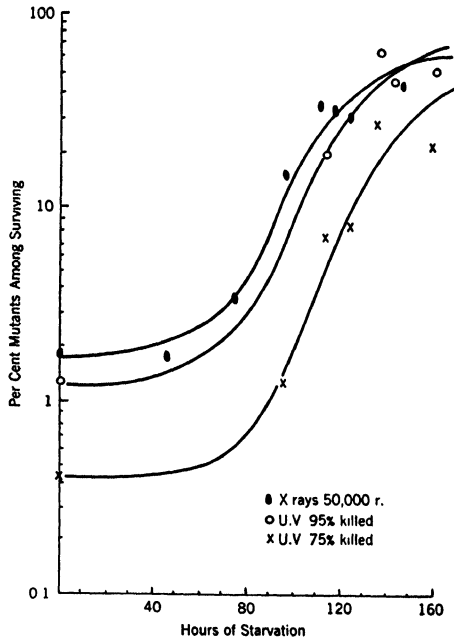


FIG. 9. Rise in the proportion of double auxotrophs (requiring biotin plus another growth factor) among survivors with increasing length of "starvation" of irradiated conidia.

trophic"). The proportion of parathiotrophic mutants increases steadily with an increase in time of starvation (Table 5); that of paraazotrophic and of vitamin-requiring mutants shows the opposite trend.

When we compare in detail the qualitative spectra of mutants (Table 6), important differences and similarities appear. For instance, among the arginine-requiring mutants, those responding to arginine only and those responding to arginine or ornithine were not found after starvation, whereas those responding to arginine, ornithine, or proline were abundant. The agreement between total isolation and starvation

TABLE 5

Effect of Time of Starvation on the Proportions of the Different Types of Auxotrophs Recovered

Hours of Starvation *	*Isolates* (no.)	Total (no.)	Not fully tested (no.)	*Auxotrophs* †					
				Fully tested					
				Total	S.	N.	A.A.	N.A.	Vit.
0	5408	93	24	69	7	17	12	11	22
				(100)	*(10)*	*(25)*	*(17)*	*(16)*	*(32)*
96–117	887	131	3	128	71	0	9	37	11
				(100)	*(55)*	*(0)*	*(7)*	*(29)*	*(9)*
118–139	384	134	0	134	81	2	6	42	3
				(100)	*(60)*	*(2)*	*(4)*	*(31)*	*(2)*
140–161	554	220	4	214	146	1	13	55	1
				(100)	*(68)*	*(.5)*	*(6)*	*(25)*	*(.5)*

* The 0-hour data are from all the results in Table 2. The other data are from series S-1 and S-2 (X-rays); S-3, S-4, and S-5 (U.V.), omitting the results at 46 and 74 hours in series S-1 because of the small numbers. Figures in brackets are %.

† S, unable to utilize sulphate; N, unable to utilize nitrate; A.A., requiring amino acids; N.A., requiring purines or pyrimidines; Vit., requiring vitamins.

in not yielding certain types of auxotrophs (e.g., tryptophan-, histidine-, guanosine- or inositol-requiring) is certainly remarkable.

After having perfected the starvation technique, we investigated two points by means of it. The first was whether the failure to obtain certain types of auxotrophs both in total isolation and starvation experiments might be due to the presence in the complete medium of substances specifically inhibitory for these mutants. This is the case for histidine-requiring mutants of *Neurospora* (Lein, Mitchell, and Houlahan, 1948) and guanosine-requiring ones of *Ophiostoma* (Fries, 1950). This possibility was probed by adding, after starvation, not complete medium, but minimal medium plus biotin, supplemented with some of the growth factors, mutants for which had not been previously obtained. The second point was whether, as suggested by Fries (1948a), the longer survival under starvation of the conidia of double-auxotrophic mutants was really due to their being more heterotrophic. This was probed in three ways: (1) by trying whether mutants of specific types would be eliminated in a starvation experiment when the starvation medium was supplemented with the corresponding growth factors; (2) by comparing the survival rates under starvation of conidia of strains with different

TABLE 6

Comparison of the Types of Auxotrophs Obtained from Total Isolation and Starvation (96 hr. or more)

Type of auxotroph	*Total isolation*	*Starvation (96 hr.)*	*Total*
Not fully tested	24	7	31
Parathiotrophic	7	298	305
Sulphite	5	284	289
Thiosulphate	2	14	16
Paraazotrophic	17	3	20
Nitrite	13	0	13
Ammonium	4	3	7
Amino Acids	12	28	40
Arginine	1	0	1
Arginine/ornithine	5	0	5
Arginine/ornithine/proline	1	14	15
Lysine	3	7	10
Proline	0	2	2
Ornithine	0	1	1
Methionine	1	0	1
Methionine/cystine	1	2	3
Phenylalanine	0	1	1
Isoleucine	0	1	1
Tryptophan	0	0	0
Nucleic Acid components	11	134	145
Adenosine	10	134	144
Cytidine/Uridine	1	0	1
Vitamins	22	15	37
Biotin *	4	0	4
P.A.B.A.	2	3	5
Pantothenate	1	0	1
Nicotinic/anthranilic acid	4	1	5
Nicotinic/tryptophan/anthranilic acid	3	1	4
Riboflavin	1	1	2
Pyridoxin	1	7	8
Choline	1	0	1
Aneurin *	3	2	5
Multiple	2	0	2
TOTALS	93	485	578
FROM ISOLATES	5408	1825	7233

* Quantitative data not homogeneous because in some series the strain used was either biotin- or aneurin-requiring.

single nutritional requirements; and (3) by comparing the survival rates of the conidia of strains with *double* requirements when starved of one, the other, or both required growth factors.

a. *Isolation of New Types of Auxotrophs.* The results of two experiments for the selective isolation of new types of mutants are shown in Table 7. The technique was the usual one except that, instead of

TABLE 7

Search for Previously Unobtained Tryptophan, Inositol, and Guanosine Auxotrophs by Adding, after Starvation, Minimal Medium Plus These Growth Factors and Biotin Instead of Complete Medium

Experiment	*Hours of starvation*	*Conidia plated*	*Colonies*	*Isolates*	*Auxotrophs* Total	*Auxotrophs* New types
S-6 (U.V., 12.5% survival)	124	450,000	133	112	12	1
	168	1,300,000	422	234	12	0
S-7 (U.V., 4% survival)	112	315,000	3,404	1,000	5	1
				1,346	29	2

The two new auxotrophs required tryptophan and guanosine, respectively. Of the other 27 auxotrophs, 26 were parathiotrophic and 1 adenine-requiring. These 27 evidently grew enough to be isolated even in the absence of the required growth factor.

complete medium, the top layer added after starvation was of minimal medium plus biotin (5 mg./l.), inositol (14 mg./l.), guanosine (60 mg./l.), and DL-tryptophan (10 ml. of 0.05 *M* solution per liter). The 1346 isolates yielded 29 auxotrophs; of these, 27 were of types already obtained (26 parathiotrophic, 1 adenine-requiring) and 2 were of the desired new types, i.e., 1 tryptophane-requiring and 1 guanosine-requiring. The experiment was therefore successful in providing two out of the three types, not previously obtained, which could have come up under the conditions used. Though the numbers are not sufficient for concluding that the previous failure was significant, the following evidence makes it very probable. Figure 10 shows that the two mutants barely grow on complete medium but that they grow well on minimal medium supplemented with the required growth factors.

b. *Elimination of Specific Types of Auxotrophs.* The longer survival of double auxotrophs (i.e., requiring biotin plus an additional growth factor) under starvation was investigated first as follows: if it were due to starvation for the additional growth factor, in the presence of this growth factor the difference should be annulled. Thus, for ex-

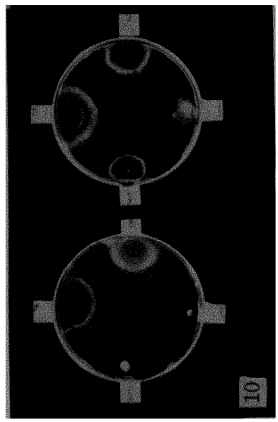


FIG. 10. Two mutants (TRYPT, requiring tryptophan; and GUAS, requiring guanosine), of types not obtained before the use of a selective technique, grow well on minimal medium plus tryptophan, adenine, and guanosine (*left*) but not on complete medium (*right*). Two other mutants (NIC, requiring nicotinic acid or tryptophan; AD, requiring adenine) grow well on both.

ample, parathiotrophic mutants might not be recovered preferentially from starvation experiments in which thiosulphate was added to the starvation medium. This expectation was fully borne out (Table 8). After irradiation with ultraviolet (survival 5%), the conidia of the biotin strain were embedded as usual in two sets of dishes: one of minimal medium, the other of minimal medium plus 1g./1. $Na_2S_2O_3 \cdot 5H_2O$. After incubation for 139 hours, complete medium was added, and of the colonies which developed, 400 from each set of dishes were isolated and characterized. Of the 35 auxotrophs recovered from the minimal medium dishes, 24 were parathiotrophic, and 11 were of other types; of the 17 recovered from minimal medium+thiosulphate dishes, 1 was parathiotrophic and 16 were of other types. Clearly, when not starved of thio-

TABLE 8

Elimination of Auxotrophs of an Unwanted Type by Supplementing the Starvation Medium with the Corresponding Growth Factor

			Types of mutants			
Starvation medium	*Total number of isolates*	*Total nutritional mutants*	Unable to utilize sulphate	Requiring amino acids	Requiring purines	Requiring vitamins
Minimal	400	35	24	1	8	2
Minimal supplemented with thiosulphate	400	17	1	3	8	5

Ultraviolet treatment (5% survival) followed by 139 hours of starvation on minimal medium or minimal medium + thiosulphate ($Na_2S_2O_3 \cdot 5H_2O$, 1g./l.).

sulphate the double auxotrophs, biotin-thiosulphate-requiring, do no longer survive preferentially; the other types of auxotrophs, however, are found in the expected proportions in both sets of plates.

A practical outcome of this finding is that the elimination of unwanted types of auxotrophs in order to save labor can be achieved simply by using as a starvation medium one which contains the corresponding growth factor(s).

c. *Survival Rates of Different Auxotrophs under Starvation.* A comparison was made of the rates of survival in minimal medium of four mono-auxotrophic strains:

bi$_1$, green conidia, biotin-requiring;
y bi$_1$, yellow conidia, biotin-requiring derived by crossing from the former;
w$_a$ *lys*, white conidia, lysine-requiring;
ad$_1$, green conidia, adenine-requiring.

The results, based on addition of complete medium at 0, 48, 72, 96, and 120 hours (approximately) and counting of survivors are shown

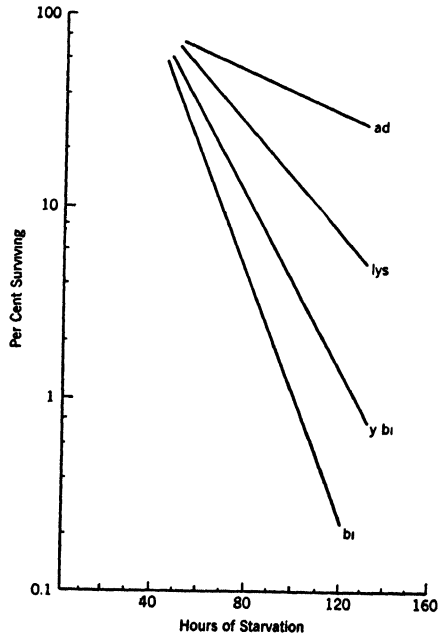


FIG. 11. Rate of dying off under starvation of conidia of strains with different single nutritional requirements: adenine (*ad*), lysine (*lys*), and biotin (*bi* and *y bi*).

graphically in Fig. 11. It is clear that there are enormous differences in the rates of dying-off between the different auxotrophs: e.g., after 120 hours in minimal medium, 30% of the conidia of the ad_1 strain are still viable, compared with 12% of those of the w_a *lys* strain, 1.2% of those of the *y* bi_1 strain, and 0.2% of those of the bi_1 strain.

The next step was that of determining the rate of dying off of a double auxotroph (*w* ad_1 bi_1: white conidia, adenine- and biotin-requiring) in minimal medium, in minimal medium plus biotin, and in minimal medium plus adenine, respectively. In the first, starvation is for both adenine and biotin, in the second for adenine alone, and in the third for biotin alone. Determination of survivors was done at the same intervals as in the previous tests. Furthermore, a proportion of conidia of both strains *y* bi_1 and ad_1 was added to the inoculum in minimal medium, a proportion of conidia of *y* bi_1 to the inoculum in minimal medium plus adenine, and a proportion of conidia of ad_1 to the inoculum in minimal medium plus biotin. The results are summarized graphically in Fig. 12.

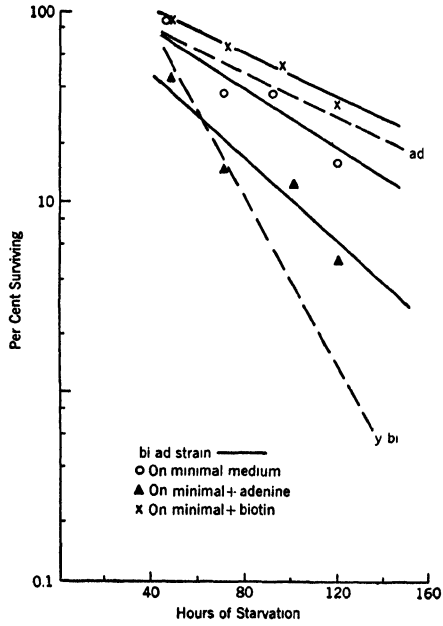


FIG. 12. Rate of dying off under starvation of a strain requiring biotin and adenine. x: starved of adenine alone; ▲: starved of biotin alone; o: starved of both. For comparison, broken lines show rates of dying off of two strains having either requirement only.

The rates of dying-off of *y bi*$_1$ and of *ad*$_1$ were the same in minimal medium or in minimal medium plus the irrelevant growth factor. The double auxotrophs, however, died off at a rate almost identical to that of *ad*$_1$ when starved of adenine only, at a rate approaching that of *y bi*$_1$ when starved of biotin only, and at a rate very nearly equal to that of *ad*$_1$ when starved of both. It could be concluded, therefore, that at least in respect to these two requirements, the rate of dying-off in minimal medium was largely determined by the requirement for which starvation led to the slowest rate of dying-off. Clearly, the degree of heterotrophy had no appreciable role in the results.

The next step was that of taking three double auxotrophs (requiring biotin plus another growth factor) from those obtained by total isolation and five from those obtained by starvation and comparing their survivals after 112 hours incubation at 41°C. in minimal medium, in minimal plus biotin, and in minimal plus the other growth factor, respectively. The growth factors involved, besides biotin, were thiosulphate, lysine, arginine, P.A.B.A., and pyridoxin.

TABLE 9

Survival after 112 hours in Starvation Media of the Conidia of Eight Auxotrophs Requiring Biotin and Another Growth Factor, Three of Which Were Obtained by Total Isolation and Five by Starvation

			*% Survival after 112 hours of starvation**		
Strain	*Origin*	*Requirements*	For biotin †	For the other growth factor ‡	For both §
S3–A2	Total isolation,	Biotin and SO_3^-	0.04	39.0	36.0
S5–F35	Starvation	Biotin and SO_3^-	0.2	51.0	44.0
bi$_1$ *lys*$_2$	Total isolation,	Biotin and lysine	0.6	2.8	1.2
S5–F2	Starvation	Biotin and lysine	1.7	0.9	1.5
bi$_1$ *orn*$_2$	Total isolation,	Biotin and arginine or ornithine	0.02	0.04	0.001
S4–C96	Starvation	Biotin and arginine or ornithine or proline	1.3	13.3	4.5
S2–D1	Starvation	Biotin and P.A.B.A.	0.02	0.08	0.003
S5–E1	Starvation	Biotin and pyridoxin	0.2	3.0	5.3

* Taking as 100 the viable counts without starvation.
† On minimal medium plus the other growth factor.
‡ On minimal medium plus biotin.
§ On minimal medium.

The results, summarized in Table 9, are rather disconcerting. They certainly confirm the tremendous differences in the rates of dying-off between different auxotrophs. They do not suggest any simple relationship between degree of heterotrophy and survival rate. It is remarkable that starvation for biotin alone increases, in all cases, the rate of dying-off and brings it close to that of the *bi*$_1$ strain.

Clearly the rate of dying-off under conditions of specific starvation could become a very powerful tool in the study of metabolic interrelationships. The investigation of which kinds of substances affect this rate could become as valuable as that carried out by the Hinshelwood school on what is, essentially, the opposite process: the lag phase in growing cultures.

As a tentative interpretation of our results we may suggest that, in our case, the efficiency of the starvation technique depended almost entirely on the happy choice, as the starting point, of the *bi*$_1$ strain which worked out to have an exceedingly high rate of dying-off under specific starvation. Any additional nutritional requirement induced by irradiation is therefore likely to induce a lower rate, hence the enrichment by starvation.

As a working hypothesis it may be suggested that growth-factor requirements, starvation for which has a generalized blocking effect on metabolism (e.g., adenine, thiosulphate), are likely to slow down the rate of dying-off relative to those which distort specifically processes with no *immediate* general effect.

3. *Characterization of Auxotrophs*

Any auxotroph obtained by means of one of the techniques described in the preceding two sections is identified as such because of its inability to grow on the simplest medium adequate for the parent strain and of its ability to grow on complete medium. The simplest medium adequate for the growth of the parent will be referred to, for short, as "minimal medium," though of course it is actually minimal medium in the case of auxotrophs derived from the wild type, w_a or *y*, but minimal medium + biotin in the case of auxotrophs derived from *bi*$_1$, and minimal + aneurin in the case of auxotrophs derived from *y thi*.

The identification of the additional growth factor, or factors, required by any auxotroph presents no theoretical difficulty, it is a matter of trying a number of individual supplements, or mixtures of supplements, with the minimal medium until the effective ones are found.

It is a fact that practically all newly arisen auxotrophs have a single growth-factor requirement: out of 612 so far subjected to systematic tests, we have failed to identify the additional requirement of only 33.

These may be cases of multiple requirements (due to multiple mutation, or to metabolic intricacies) or of requirements for substances not included in our routine tests. Thus, any procedure based on the test of individual growth factors would be successful in characterizing at least 95% of the auxotrophs. The practical problem is therefore one of efficiency.

In the first place, the procedure must restrict progressively the field of search. It would be absurd to try on each auxotroph one by one all available vitamins, amino acids, nucleosides, etc.; we must first identify the group of substances into which the requirement is likely to fall. This has been done by using inexpensive mixtures obtained from natural products (casein hydrolyzate, nucleic acid hydrolysates).

Secondly, if we know (as we do now) which types of auxotrophs constitute a high proportion of the total, it is economical to identify these first and carry out the further search only with the remainder.

Thirdly, there are three ways in which growth-response tests are more easily carried out: (1) spot-inoculating a number of strains on a series of differently supplemented plates (plate test); (2) spot-inoculating a number of substances on a series of plates, each inoculated all over with a particular auxotroph (auxanography), and (3) inoculating each strain in a series of test tubes with liquid minimal medium variously supplemented. We have found that the plate test is more efficient when a small number of substances (not more than five) have to be tested on a large number of strains (say twenty or more); that auxanography is more efficient in the converse case and when interactions between growth factors (inhibitions, sparing effects, etc.) are suspected; and that the tests in liquid medium are useful only as a final check.

A routine procedure of high efficiency, achieved only recently, is shown diagrammatically in Fig. 7; it was possible only after a precise knowledge of the proportion of different auxotrophs in the yield of starvation experiments became known. Before that the routine was as follows:

1. Simultaneous plate tests (20 auxotrophs per plate) on minimal medium, minimal medium plus casein-hydrolyzate (0.1 ml. of standard solution per plate), minimal medium plus nucleic acid hydrolyzate (0.06 ml. of standard solution per plate), minimal medium plus vitamins (0.02 ml. of standard solution per plate). Growth of any auxotroph on only one of the supplemented plates suggested a further search for individual amino acids; for individual purines, pyrimidines, and nucleosides; or for individual vitamins. Growth on both casein hydrolyzate and nucleic acid hydrolyzate indicated a requirement for amino groups, ammonium salts, or nitrite.

2. Auxanographic tests with all available amino acids, nucleosides, or vitamins of the strains which grew on the corresponding plates (Pontecorvo, 1949b).

The more efficient routine mentioned above and illustrated in Fig. 7 as applied to a hypothetical group of 100 auxotrophs is as follows:

1. Plate tests are made of the 100 strains on 5 dishes of minimal medium and 5 of minimal medium plus sodium thiosulphate (1 g./l.); none grows on the former, and about 60 strains grow on the latter. These are plate tested (3 dishes) on minimal medium plus sodium sulphite (1 g./l.) and about 50 grow. We have therefore classified as parathiotrophic 60 strains out of 100 by using 13 dishes. Of the 60 parathiotrophic, 50 grow on either sulphite or thiosulphate, and 10 grow only on the latter.

2. Plate test of the remaining 40 strains is done on 2 dishes of minimal medium plus adenine hydrochloride (80 mg./l.); 20 grow and are further analyzed auxanographically with the available related compounds (other purines, pyrimidines, nucleosides, and presumed precursors).

3. The 20 which did not grow on adenine are plate tested simultaneously on 1 dish each of minimal medium plus casein hydrolyzate and minimal medium plus vitamin solution; 12 strains grow on casein hydrolyzate, 7 on vitamins. The former are tested auxanographically with all available amino acids, the latter with all available B-vitamins. The negative strain is tested auxanographically for multiple requirements (see below).

a. *Classification of Requirements of the Products of Crosses.* The tests for the characterization of auxotrophs isolated from mutation experiments are also used for the classification of the products of crosses in which nutritional requirements are segregating (section V—3, 4). There are, however, some notable differences in the situation: (1) among the products of crosses, the kinds of requirements for which each isolate has to be classified are few and already known; and (2) a high proportion of isolates may show multiple requirements.

Since rarely more than three requirements are segregating in a cross, there are usually very many isolates to be tested against only few growth factors, and therefore the plate test is used extensively. If only one requirement segregates, the isolates fall into two classes; the isolates are tested simultaneously on dishes with and without the growth factor. If two requirements segregate, the isolates fall into four classes (requiring one, requiring the other, requiring both, requiring neither). They are plate tested simultaneously on two series of dishes, one with one growth factor and the other with the other: growth on both kinds of dishes indicates no requirement; growth on one indicates that requirement; growth on neither, both requirements (Fig. 13). The last are checked for growth on a medium with both growth factors.

If three requirements segregate A, B, C, the isolates fall into eight classes; they are plate tested on three series of dishes, each supplemented with two (A B; A C; B C) growth factors. Growth on all three indicates no requirement; growth on none indicates three requirements; growth on one indicates two requirements; growth on two indicates one

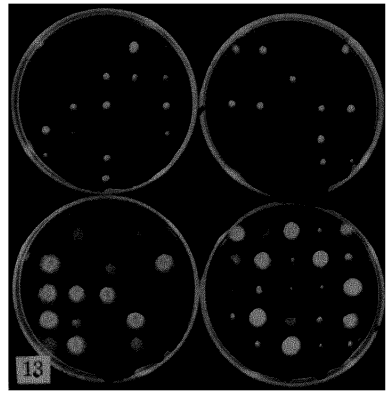


FIG. 13. Plate tests. Twenty-five segregants for P.A.B.A. and biotin requirements tested simultaneously on two plates of minimal medium, one supplemented with biotin (*left*) and one with P.A.B.A. (*right*). Growth on either, both, or neither classifies the segregants. *Above*, 25 strains photographed 24 hours after inoculation; *below*, 25 other strains photographed 48 hours after inoculation, when the biotin-requirers began to be "breast-fed."

requirement. The isolates classified as having three requirements can be tested auxanographically. Figure 14 shows the kind of auxanogram given by isolates with one, two, and three requirements, respectively.

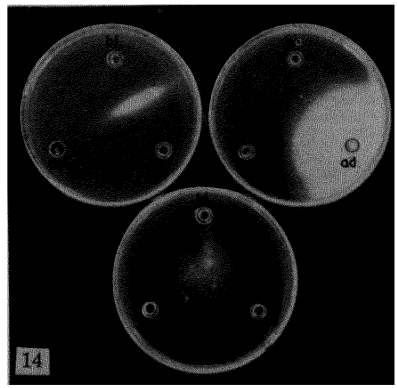


FIG. 14. Three kinds of auxanograms given by segregants of a cross in which three growth-factor requirements segregate: *ad*, adenine; *bi*, biotin; *arg*, arginine. *Top right*, single requirement. *Top left*, double requirement. *Bottom*, triple requirement.

4. *Selection of Prototrophs*
by J. A. Roper

A problem discussed in sections IV-1 and IV-2 is the selection of a small proportion of auxotrophs from a mixture of auxotrophs and prototrophs. In certain cases the reverse selection, of rare prototrophs from auxotrophs, is required.

Giles and Lederberg (1948) and Kölmark and Westergaard (1949) using *Neurospora crassa* have developed a technique for the selection of prototrophic back-mutant conidia. This technique has also been used as a tool for detecting mutagenic activities (see Westergaard, 1952). The selection of prototrophs from auxotrophs is also crucial in the isolation of heterozygous diploids (Roper, 1952), and in the section on "Pseudoallelism" (VI-3) it will be shown in operation for selecting rare prototroph recombinants.

A preliminary investigation has been made of the suitability of *A. nidulans* as material for back-mutation studies. The technique is essentially like that of Westergaard (1952); two strains of *A. nidulans* were used: $bi_1\ lys_2$ and $bi_1\ arg_1$. Double mutants were used in the tests to minimize dangers of undetected contamination and to facilitate crossing of back-mutants. Each strain was repeatedly purified by single conidium isolation. Conidial suspensions from 5-day-old cultures on minimal medium with biotin and lysine or biotin and arginine, respectively, were plated at high density (up to 3×10^7 per petri dish) on minimal medium with biotin alone. An aliquot of the suspension was diluted and plated on complete medium for estimation of the percentage of viable conidia. On minimal medium plus biotin, only the conidia independent of lysine or arginine could grow. Although the genotype of such reverted types was not tested, they will be called back-mutants. Plates were incubated for 7 days, during which time back-mutants were scored and isolated as they became visible. Results of these preliminary experiments are given in Table 10. No arginine-independent back-mutants were obtained. Lysine-independent back-mutants were recovered at a frequency of about 1 in 1.4×10^8.

In the application of the technique to quantitative work a number of potential sources of error have been anticipated (Roper, 1950b); some of these have been the object of later independent work by Grigg (1952). In the first place, it is necessary both to know the initial number of nuclei under test and to ensure no increase in this number through germination of the auxotrophic conidia on the selective medium. Such germination may also introduce errors due to the possible effects on mutation of nuclear division under restricted metabolic conditions.

TABLE 10

Selection of Back-Mutants of *A. nidulans*

Strain	*Number of conidia tested*		*Viability on complete medium*	*Biotinless back-mutants*
	Total	Per dish		
bi, lys$_2$	3.6×10^9	3×10^7	> 80%	25
bi, arg$_1$	2.1×10^9	3×10^7	About 100%	0

Since the conidia of *A. nidulans* are uninucleate and, in the tested strains, do not germinate during the test, quantitative errors from this source are avoided. This is not so for the adenineless strain of *Neurospora* used by Kölmark and Westergaard (1949) which has multinucleate conidia germinating on minimal medium.

A second suggested source of quantitative error lies in the possible loss of prototrophs when they are selected from a large mass of auxotrophs. Especially where conidia of the test strain germinate on minimal medium or are "breast-fed" (section V-3) by growing back-mutants, recovery of rare prototrophs from a mesh of germination tubes may not be complete. In the tested strains of *A. nidulans,* no germination of auxotrophic conidia occurred after 7 days of incubation and there was no "breast-feeding" of auxotrophs even among the hyphae of a back-mutant colony (Fig. 15). However, there was still indirect evidence of

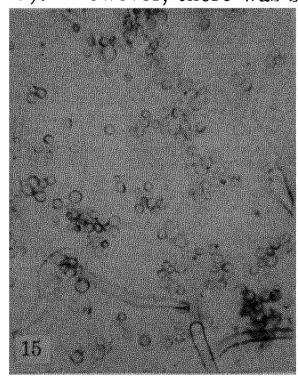


FIG. 15. Conidia of strain *bi, lys$_2$* (requiring biotin and lysine) on minimal medium plus biotin 100 hours after plating. Even near colonies of lysine-independent "back-mutants," the conidia do not germinate, though they may swell up. The hyphae are from one such back-mutant colony.

the inhibitory effects of high concentrations of auxotrophic conidia. During the first 72 hours of incubation back-mutant colonies were found only towards the edge of the inoculum, where the conidial density is lowest. Colonies appearing nearer the center of the plate were very delayed and normally became visible only after 72 to 120 hours of incubation. Since there is a considerable fall in viable count during the incubation period, some prototrophs may die before they can give rise to visible colonies. The extent of this inhibitory effect has not yet been investigated by means of reconstruction experiments.

For several strains of *Neurospora*, Grigg (1952) has found inhibition of prototrophs by auxotrophs on a sorbose minimal medium. On this medium, the efficiency of selection of prototrophs depends on the density of viable auxotrophic conidia surrounding them. Grigg has not investigated the degree to which this inhibition may depend on germination of the auxotrophic conidia: a matter which is probably of decisive importance. Kölmark and Westergaard (1952) have confirmed the inhibitory effect found by Grigg, but only for the sorbose medium. For the same conidial density range, Westergaard has shown that there is no inhibition on the glucose medium which is normally used in the *Neurospora* back-mutation technique. The inhibition may therefore be attributed mainly to competition for nutrients or energy.

A further source of error suggested by Grigg is intraconidial inhibitions involved in the use of multinucleate conidia of *Neurospora*. Even if correct for *Neurospora*, this cannot apply where uninucleate conidia, such as those of *A. nidulans*, are used.

Provided it is possible to overcome any possible inhibitory effects of auxotrophs in the selection of rare prototrophs, it seems likely that the conidia of some strains of *A. nidulans* should provide more suitable material for quantitative mutation studies than the macroconidia of *Neurospora*.

V. Methods of Genetic Analysis

1. *Heterokaryosis*

Heterokaryotic hyphae are formed when two strains are grown together on solid medium from mixed point-inoculum of conidia, ascospores, or mycelium. Usually, however, the heterokaryotic hyphae form only a small portion of the developing mycelium because, unless they grow much faster than the homokaryotic hyphae, the rate at which they are formed is balanced by that of the opposite process (Pontecorvo and Gemmell, 1944; Pontecorvo, 1947). When the growth rate of the heterokaryon is higher than that of the two homokaryons, it may be so under

any conditions or only under special conditions; these may or may not be controllable. In either case, the heterokaryon is "balanced"; i.e., once it constitutes an appreciable proportion of the mycelium, it is usually perpetuated through successive mass transfers of hyphal tips. The simplest examples of balanced heterokaryons are: (1) a heterokaryon, with growth habit approaching that of the wild type, formed between two mutant strains with stunted growth on any medium; and (2) a heterokaryon, able to grow on minimal medium, formed between two strains requiring *different* growth factors and therefore neither able to grow on that medium. In this second case it is the absence of the two growth factors which operates as a selective agent against the homokaryotic hyphae.

Three-component heterokaryons can be produced. We have obtained without difficulty heterokaryotic heads with chains of three different colors, white, yellow, and green (see further), by using a balanced heterokaryon between two strains with yellow and white conidia, respectively, and adding a third strain with green conidia. A *balanced* three-component heterokaryon undoubtedly could also be produced. It could be synthesized by using three strains in which three growth-factor requirements were shared two-by-two (A b c; a b C; a B c).

Even when the heterokaryon has a growth rate much greater than those of the two homokaryons, it does not easily become established automatically, though once established it perpetuates itself automatically on transfers of mycelium. To establish a balanced heterokaryon, two conditions are necessary. One is that primary heterokaryotic hyphae be formed in appreciable numbers from hyphal anastomosis. The other is that these primary heterokaryotic hyphae be enabled to multiply and escape from the meshes of parental mycelium in order that their higher growth rate may begin to tell. These two conditions are somewhat contradictory because to obtain many hyphal fusions a thick growth helps, but to enable the heterokaryotic hyphae to "escape" a thin growth helps.

Two techniques have been used to achieve the compromise, one or the other more appropriate to different combinations of strains. With one, conidia of the two strains, mixed in equal proportions in a suspension as dense as possible, are streaked over or stabbed into the agar medium. When the heterokaryon is to be formed between two strains with different nutritional requirements, this medium must be selective, i.e., minimal or lacking at least one growth factor for each strain. When the heterokaryon is to be formed between strains with stunted growth under any conditions, of course, no selective medium is used. In the first case, just enough supplemented medium must be carried over when inoculating to ensure a little initial growth of the two strains. Inocula-

tion is made by drawing parallel streaks about 2 cm. apart and 2 mm. wide, or a circle, or by making a series of stabs 1 cm. from the edge of the petri dish. After 5 to 10 days small patches of heterokaryotic mycelium may arise from the few points where one of the heterokaryotic hyphae formed succeeds in finding its way through the parental mycelium (Fig. 16).

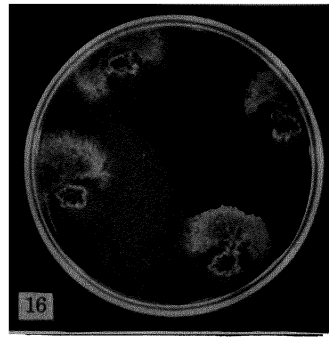


FIG. 16. Balanced heterokaryons arising from point inocula in minimal medium of two strains requiring adenine and aneurin, respectively. Note that the heterokaryon succeeds in "escaping" only at a few points.

With the other technique, the mixed inoculum is grown for 24 hours in liquid complete medium, and then the mycelium, centrifuged and washed once, is spread out on to the agar surface of the medium, teasing out the meshes. This agar medium will again be selective in the case of strains differing in nutritional requirements.

With both techniques, as soon as patches of heterokaryotic mycelium appear, as revealed by their growth habit and, if color markers were used, by the presence of heads with mixed colors, small portions of agar with hyphal tips are transferred on to fresh medium. Usually one transfer is sufficient to establish the heterokaryon.

The use of differences in color of the conidia between the component strains is most convenient in watching the progress of a heterokaryon, in guarding against its loss, and in detecting changes in the balance between the two kinds of nuclei. As markers we have extensively used (section VI-1) the genes *W/w* (colored versus white conidia) and *Y/y* (green versus yellow conidia). Both these genes have cell-localized action (Pontecorvo, 1947), though not completely so in the case of *W/w*. This means that in a combination of strains differing in color, a heterokaryotic head bears conidial chains some of one color and some of the other (Fig. 3) (Gossop, Yuill, and Yuill, 1940).

A well-balanced heterokaryon, say between a yellow and a white

strain, shows a thorough mixture of apparently pure yellow heads, apparently pure white heads, and mixed heads. In the last, the proportion of yellow to white chains varies all the way in different heads from almost all white to almost all yellow. The visibly mixed heads never constitute the major proportion, even in cases in which the ratio of yellow to white heads is not unduly lopsided (Table 11). This perhaps indicates that in

TABLE 11

Distribution of Homo- and Heterokaryotic Heads in Balanced Heterokaryons

Heterokaryon	*Heads*			
	Yellow	White	Mixed *	Total
w_a Y AD$_1$ lys + *W y ad$_1$ LYS*	45	130	46	221
w_a Y BI lys orn$_1$ + *W y bi LYS ORN$_1$*	81	179	25	285

* Certainly underestimated, because those with only very few conidial chains of the other color are likely to be misclassified as wholly yellow or wholly white.

the mycelium the nuclei of the two kinds tend to be clustered according to kind, and that the number of nuclei entering a conidiophore is small. In support of this second deduction stands the fact that in mixed heads, chains of conidia of the same color tend to be clustered. If the nuclei multiply abundantly within the vesicle, by reason of common descent those of one kind are more likely to be next to one another and therefore to enter groups of neighboring sterigmata.

Balanced heterokaryons are easily lost for a number of reasons. The most common are: back-mutation, non-genetic adaptation, recombination through sexual reproduction, formation of heterozygous diploid nuclei (section VII-1), and accumulation in the medium of metabolites enabling either or both component strains to grow independently. In any one of the first four instances, the heterokaryon is lost because of changes in the nuclei or the cells; as a consequence of these changes homokaryotic hyphae of a new type arise against which there is no selection. In the last instance, it is the medium itself which is no longer selective.

Heterokaryons, one of the components of which requires a vitamin, are those which more easily lose the balance as growth proceeds in a petri dish; presumably the heterokaryon produces the vitamin in excess

and releases it into the medium, enabling the vitamin-requiring component to grow independently. When the two component strains differ in the color of their conidia, the gradual increase in the proportion of homokaryotic hyphae of the vitamin-requiring one reveals itself in an increasing proportion of heads of the corresponding color. This process can be imitated artificially in a more extreme form by adding to the agar medium sufficient amounts of the growth factor (or factors) required by one strain after the mycelium has covered, say, one-half of the petri dish. The subsequent growth shows exclusively, or prevalently, heads of the corresponding color (Fig. 17). Whether or not the increase

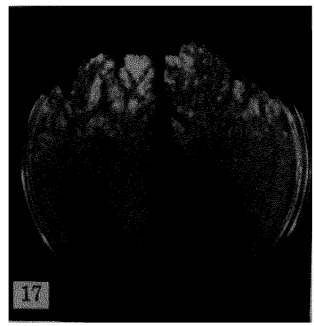


FIG. 17. Balanced heterokaryon between a yellow aneurin-requiring and a green adenine-requiring strain. After some growth, aneurin was added to the medium; the yellow component sectored out.

in the proportion of homokaryotic heads implies a change in the balance between nuclei of the two kinds *within the heterokaryotic hyphae* will be discussed presently in connection with the general problem of "nuclear ratios" in heterokaryons.

Since the conidia in *A. nidulans* are uninucleate, a sample of conidia is a sample of nuclei. If in a heterokaryon the ratio of the two kinds of nuclei in the conidia were identical to, or highly correlated with, the same ratio in the mycelium, sampling of the conidia would be equivalent to sampling of the nuclei of the hyphae. Unfortunately, we have been unable to obtain any crucial evidence for or against this possibility.

An obvious way to test it was to compare the allele ratios in a sample of conidia with the same ratios in a sample of ascospores. Out of three cases tested involving balance between adenine requirement and biotin requirement, the conidia agree with the ascospores in one, but not in the other two (Table 12). In view of relative heterothallism (section V-4) (which implies non-random karyogamy or striking differential survival *within one perithecium* of different products of karyogamy), it is now

TABLE 12

Comparison between the Allele Ratios in the Conidia and Those in the Ascospores of Three Heterokaryons Balanced between ad_1 (Adenine Requirement) and *bi* (Biotin Requirement)

	Heterokaryon					Allele ratios
			w	*W*		
1.	*w* ad_1 *y BI*	Conidia	44	395	439	1:9
	+					
	W AD_1 *Y bi*	Ascospores	12	70	82	1:6
			56	465	520	
			Y	*y*		
2.	ad_1 *y BI*	Conidia	280	16	296	17:1
	+					
	AD_1 *Y bi*	Ascospores *	489	175	664	3:1
			769	191	960	
			Y	*y*		
3.	ad_1 *Y BI*	Conidia	286	1019	1305	1:3.5
	+					
	AD_1 *y bi*	Ascospores *	1191	2333	3524	1:2
			1477	3352	4829	

* Only those not requiring adenine were classified. This might reduce the proportion of *y* in 2 and of *Y* in 3, thus tending to blur rather than emphasize the difference between ascospores and conidia.

The allele ratios in the conidia are significantly different from those in the ascospores in heterokaryons 2 and 3 but not in 1. Though the balance is between the same requirements in all three heterokaryons, the ratio of adenine-requiring to biotin-requiring nuclei (calculated from conidia) are 1:9; 1:17, and 1:3.5, respectively.

clear that there is no necessary correspondence between the allele ratios in the conidia and those *in the ascospores.* It is still an open question whether there is such correspondence in the case of the nuclear ratios *in the hyphae.*

Even though we have not carried out quantitative work by means of single hyphal-tip isolations, there is overwhelming qualitative evidence that, at least in *A. nidulans,* a balanced heterokaryon consists of a mixed population of hyphae, some heterokaryotic and some homokaryotic of either type.

As the colony grows, there are at least two types of interplaying dynamic equilibria: (1) between the two types of nuclei within the heterokaryotic hyphae, and (2) between the three types of hyphae. The latter is a form of syntrophism, though of a subtle kind: the heterokaryotic hyphae, which do not benefit as much as the homokaryotic hyphae from it, continuously break up to form the latter, but are also

continuously re-formed from the latter, and the whole process is not independent of syntrophism itself. As to (1), there is no clue in *A. nidulans* of what goes on. In the work with *Neurospora* (e.g., Ryan and Lederberg, 1946; Emerson, 1948) changes in the nuclear ratios of a heterokaryotic colony have been attributed to (1). Clearly, they could have been attributed equally well to (2) or to both. There is, in fact, no evidence of whether the nuclear ratios *within the heterokaryotic hyphae* are adjustable at all. If they were not, the selective adjustment of the nuclear ratios in a growing colony as a whole—about which there is little doubt—could involve the distribution of the total population of nuclei as between heterokaryotic and homokaryotic hyphae. The problem boils down to whether there is a differential rate of mitosis of the nuclei within a hypha, or a differential rate of multiplication of the hyphae according to the nuclear ratios which they *happen* to have.*

In many cases, balanced heterokaryons, instead of showing uniform growth of mycelium and, if color markers are present, uniform and thorough mixture of heads of the three kinds, show a patchy growth, with patches of heterokaryotic hyphae alternating with others of homokaryotic hyphae, often growing thinly. The complex dynamic equilibrium between and within hyphae mentioned above, is unstable, and growth proceeds in a see-saw way. In one case, a partial explanation of the phenomenon may be offered. Heterokaryons between an arginine-requiring strain and strains requiring some other growth factor (especially lysine) give this alternation of good and poor growth, and color markers show the areas of good growth to be mainly heterokaryotic and the others mainly homokaryotic. It is known that arginine intake or utilization is inhibited competitively by exogenous lysine and vice versa (section VI-4) (Pontecorvo, 1950, 1952c). A strain which requires arginine, and presumably synthesizes lysine, whenever growing on limiting amounts of arginine will accumulate lysine and therefore interfere with its own further utilization of arginine. In a balanced heterokaryon, whenever growth happens to slow down in any part of the heterokaryotic colony because of the available amount of arginine, it will stop altogether because of the accumulation of lysine. The other component of the heterokaryon, however, will grow and accumulate more arginine, which will, in its turn, overcome the endogenous inhibition. If the balanced heterokaryon is one between a lysine-requiring and an arginine-

* In two interesting papers, Jinks (1952, *Heredity* 6, 77-87; *Proc. roy. Soc.*, B, 140, 83-106) produces new very precise quantitative evidence of selective adjustment in the nuclear ratios of wild heterokaryons of *Penicillium*. This work, however, still does not provide the necessary crucial evidence as to the problem of how the nuclear ratios are adjusted.

requiring strain, the pendulum equilibrium is even more marked, because for each of the two components there is no compromise between optimal growth and self-poisoning.

Whether these unstable balanced heterokaryons oscillating between the two homokaryotic conditions behave in this way by adjusting the nuclear ratios within or between hyphae is, again, unknown.

The study of heterokaryons has revealed complexities not suspected at the start (Pontecorvo, 1947).

2. *Crossing*

A. nidulans being homothallic, when we grow two strains in mixed culture, the ascospores of any one ascus may derive their nuclei from a selfed zygote of one strain, from a selfed zygote of the other strain, or from a crossed zygote. For genetic analysis, we are interested only in the asci of crossed origin, and therefore we must aim at the maximum proportion of these.

Before the recent discovery of "relative heterothallism" in *A. nidulans* (Hemmons, Pontecorvo, and Bufton, 1952; see also section V-4), a reasonable working hypothesis was that to obtain the maximum proportion of crossed asci it was necessary to secure in the mycelium as thorough a mixture as possible of nuclei derived from the two strains to be crossed. With random distribution of the two kinds of nuclei in the mycelium and random karyogamy between any two of them, a mycelium in which the nuclei of the two kinds were in equal proportions would yield 50% asci of crossed origin.

Starting from these considerations (which we know now to be wrong) two techniques of crossing were developed. One made use of the fact that in a balanced heterokaryon nuclei of the two strains are present and multiply side by side. Another made use of the fact that hyphal anastomoses occur readily soon after germination of the conidia and, therefore, packing together in equal proportions conidia of two strains in non-selective agar medium ensures a high number of inter-strain anastomoses. If the inoculation is at such a high density that very little growth can take place before the formation of the perithecia, a high proportion of the heterokaryotic hyphae formed will remain heterokaryotic up to the time of the formation of the perithecia. A proportion of perithecia will thus start with nuclei of both kinds.

The first technique ("heterokaryon cross") simply consists in forming a balanced heterokaryon, as mentioned in the previous section, and waiting for at least 10 days, when mature asci begin to be present. The heterokaryon can be kept for months in a cupboard, the agar can be left to dry off, and the ascospores can be used at any later time, because

they are not ejected from the perithecia. The heterokaryon cross is applicable only to pairs of strains between which a balanced heterokaryon can be formed.

The second technique ("mixed inoculum cross") consists in mixing together two suspensions of conidia, one from each of the strains to be crossed, containing equal numbers of viable conidia of each kind. The mixed suspension is then plated, aiming at not less than 5 million conidia per petri dish. A bottom layer of complete medium is first poured; the mixed suspension is spread onto the surface of this layer, and then a second layer of not more than 5 ml. of complete medium is poured on top. The mixed inoculum cross has the advantage of being applicable to any combination of strains, irrespective of whether either, both, or neither requires any growth factor or has a reduced growth rate.

Which one of the two techniques is to be preferred in any one case it is not easy to decide. On the one hand, with complete homothallism, the heterokaryon technique could give as optimal results not more than 50% asci of crossed origin, and this only in the ideal case of a balanced heterokaryon in which all the mycelium were heterokaryotic and the ratio of the nuclei of two kinds were selectively adjusted to 1:1. This is certainly an uncommon occurrence (Beadle and Coonradt, 1944; Pontecorvo, 1947) and therefore one could have expected the heterokaryon technique to yield usually well below the maximum theoretical 50% crossed asci. The discovery of relative heterothallism, however, makes this inference groundless, because a self-fertile strain may yield almost 100% crossed asci when in combination with a second one, and only a few per cent when in combination with a third one. Clearly, the nuclear ratios in the heterokaryon cannot account for proportions of crossed asci in excess of 50%.

The mixed inoculum technique, on the other hand, still gives up to 100% hybrid asci in certain combinations of strains, and in others it gives proportions of hybrid asci which may approximate or fall short of the theoretical maximum for complete homothallism.

Whichever the technique used to obtain asci of crossed origin, once these are available they must be used for genetic analysis. Three techniques of genetic analysis have been developed: (1) recombinant selection from random samples of ascospores, (2) perithecium analysis, and (3) ascus analysis. The first two have been used extensively in our work; ascus analysis has not.

Ascus analysis in *A. nidulans* does not differ in principle from ascus analysis in yeast or in any other species in which the spores are not linearly arranged. In practice, some technical modifications were neces-

sary to adapt it to *A. nidulans;* they have been developed by Miss Hemmons, and they will be published fully at a later date. The data from ascus analysis in *A. nidulans* require the treatment developed by Lindegren (1949) and Whitehouse (1950, and personal communication for linked loci) involving the use of three loci, two by two.

Another quite novel technique of genetic analysis has been developed; it is based on mitotic recombination in artificially produced heterozygous diploids. Since it does not make use of sexual reproduction, it will be dealt with separately in sections VII-1 and VII-2.

3. *Recombinant Selection from Random Samples of Ascospores*

This was the first technique used for genetic analysis in *A. nidulans,* and indeed, in any homothallic fungus (Pontecorvo 1949a). It is still widely used in our work, especially for detecting and estimating close linkage.

In a cross of two strains of a heterothallic organism, a random sample of ascospores is a sample of the products of meiosis of a large number of zygotes, all of which were of crossed origin. In a homothallic organism, it is a sample of the products of meiosis of zygotes, some of which were of crossed origin and some of selfed origin of either type. Plating of such a sample can yield genetic information only if we have some means of distinguishing between the ascospores derived from selfed asci and those derived from crossed asci. This can be done by using two pairs of genetic markers for identifying and selecting recombinants; and of course, recombinants can only result from crossed karyogamy. Segregations at other loci are then studied only among the selected recombinants. The principle underlying this technique is the same as that underlying Lederberg's (1947) technique for crossing bacteria.

Notations

Italic capitals: wild type or dominant alleles.

Italic lower case: mutant or recessive alleles.

Loci determining nutritional requirements: the symbols use the first two or three letters of the growth factor required; e.g., *LYS/lys,* locus at which a mutant allele determines lysine requirement; *BI/bi,* locus at which a mutant allele determines biotin requirement. Different loci at which mutant alleles determine apparently the same requirement ("mimics") are distinguished by a subscript; e.g. AD_1/ad_1, AD_2/ad_2, etc., different loci at which mutant alleles determine a requirement for adenine.

Crosses: the two genotypes of the strains crossed are separated by the symbol //.

Zygotes: the genotype of the heterozygotes from a cross is indicated by separating the symbols of the alleles by a fraction sign, loci known to be linked having a common fraction sign. All the alleles contributed by one strain are above the signs,

those contributed by the other strain below. Thus, the heterozygotes from a cross *lys Y BI//LYS y bi* will be represented by $\frac{lys}{LYS}\ \frac{Y\ BI}{y\ bi}$, the two loci *Y/y* and *BI/bi* being known to be linked.

A full list of identified loci is on p. 202, Tab. 17.

The way in which recombinants for the two pairs of desired markers are selected in any one case may be (1) *visual,* when differences classifiable by inspection are used, e.g., colors of conidia; (2) *automatic,* when, for example, differences in nutritional requirements are used and plating is on selective media which permit the growth of only certain types of recombinants; (3) *a combination of both;* or (4) *based on testing individual isolates,* and using for Mendelian analysis any one or more recombinant types.

For example, a mutant allele at the *Y/y* locus gives light yellow conidia instead of green as in the wild type. Three mutant alleles at the *W/w* locus give colorless (white) conidia and are epistatic to *Y/y*. From a cross between two strains, one with yellow (*W y*) and one with white conidia (*w Y*), recombinant ascospores (*W Y*) give origin to colonies with green conidia. We can select green colonies and classify them for other segregating genes. Alternatively, we may use, say, a mutant allele at the *LYS/lys* locus, which determines a requirement for lysine, and one at the *BI/bi* locus, which determines a requirement for biotin. From a cross between two strains, one lysine-requiring (*lys BI*) and the other biotin-requiring (*LYS bi*), recombinant ascospores (*LYS BI*) are the only ones capable of giving origin to colonies on a medium lacking both growth factors. We can isolate these and classify them for other segregating genes.

From a cross between two strains differing at all of the four loci just mentioned (e.g. *lys* w_a *Y BI//LYS W y bi*), we can select in three ways: color alone, requirements alone, and one color and one requirement. In the first case we plate a random sample of ascospores on medium containing both lysine and biotin; we identify visually the *W Y* recombinant colonies because of their green color; we isolate them and classify them (section IV-3) as to their requirements: *lys BI; LYS bi; LYS BI;* or *lys bi.* In the second case we plate on medium devoid of lysine and biotin, and we classify as to their colors the colonies which came up (all necessarily *LYS BI*): w_a *Y* (white); *W y* (yellow); *W Y* (green); w_a *y* (white) (w_a is epistatic to *Y/y*). In the third case, we plate either on medium containing biotin but not lysine or on medium containing lysine but not biotin. On these partially selective media one of the two parental types is capable of growing (the *lys* w_a *Y BI* type on lysine medium, and the *LYS W y bi* type on biotin medium). W[illegible]

select, therefore, the recombinants between colors and requirements. Thus, on biotin medium, we discard the yellow, because they will include a proportion of parental combinations (*LYS W y bi*) derived both from selfed and from crossed asci, but we isolate and classify all the white and, of course, the green. On the other hand, on lysine medium, we discard the white but isolate and classify the yellow and the green.

In the examples of completely or partially selective plating given above, at least one of the criteria for selection is based on the automatic sieve of the medium. When, however, the proportion of crossed asci in a cross is high, the automatic sieve of the medium is not essential. All the colonies from a non-selective medium are isolated and typed, and those which are recombinant in a desired way are retained for further classification as to other segregating loci. Thus, for example, we may plate on medium containing biotin and lysine, isolate all the yellow, and use for classification as to *BI* or *bi* all those which are lysine requiring, and therefore certainly of crossed origin, or we may use for classification as to colors all the double requirers *lys bi*, etc.

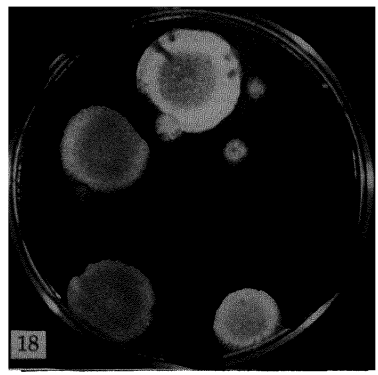


FIG. 18. "Breast feeding." When the prototrophs from a cross segregating for biotin requirement have formed large colonies, they release biotin in the medium in quantity sufficient for growth of biotin requirers (small colonies).

Clearly, in each cross a number of selections are possible. The choice of the most efficient ones is a matter of experimental design, but also of experience. For instance, the requirement for a growth factor is sometimes satisfied by syntrophism; thus, biotin-requiring colonies do grow on biotinless medium if a number of non-requiring colonies are growing in the same petri dish and presumably secreting biotin ("breast feeding") (Fig. 18). Furthermore, certain genotypes determine a considerable delay in germination. This means that, in a plating where

colonies of these genotypes should come up, this delay may lead to the loss of a proportion of them because of smothering by others. These two examples of the kinds of practical difficulties encountered show where experience helps.

We must now discuss the question of how to identify and measure linkage. In the cross given above, as an example, if the four loci segregated independently, no matter which two were used for selection, barring differential viability, the other two should give 1:1:1:1 ratios or the modified 1:2:1 ratio due to epistasis of w_a. On the other hand, if there were linkage and/or differential viability, the ratios would be distorted in certain characteristic directions.

In this cross, the workable types of selection of recombinants at two loci permitting the classification of segregants at both the other two loci, are the following:

Method of selection	*Selected recombinants*	*Classifiable combinations as to the other two loci*			
Visual	*W Y*	*lys BI*	*LYS bi*	*lys bi*	*LYS BI*
Nutritional, automatic	*LYS BI*	*W y*	$w_a Y$	$w_a y$	*W Y*
			indistinguishable		
Nutritional, requiring complete testing	*lys bi*	*W y*	$w_a Y$	$w_a y$	*W Y*
			indistinguishable		

The number of useful selections is restricted by the epistasis of w_a over Y/y.

The following data (kindly supplied by Miss L. Hemmons) can illustrate the kind of results obtained:

Cross: *lys* w_a *Y BI//LYS W y bi*

Selected recombinants	*Segregations at other loci*			
	Parental		Recombinant	
	lys BI	*LYS bi*	*lys bi*	*LYS BI*
W Y	64	8	5	110
	W y	$w_a Y$	$w_a y$	*W Y*
LYS BI	2	39		40
lys bi	22	28		3

Crosses Involving the *Y/y* and *BI/bi* Loci in Coupling: $\frac{y\ bi}{Y\ BI}$

Cross	Selection	Segregations at other loci			
		Unusable classes	Cross-overs	Non-cross-overs	Recombination fraction
1. $\frac{lys\ \ w_a\ \ Y\ BI_1\ \ THI}{LYS\ \ W\ \ y\ \ bi_1\ \ thi}$	a. *LYS BI₁ THI*	$w_a y$ and $w_a Y$ 177	*W y* 14	*W Y* 194	$\frac{14}{208}$
	b. *LYS W y THI*	.....	*BI* 4	*bi* 37	$\frac{4}{41}$
	c. *LYS W Y THI*	.....	*bi* 0	*BI* 46	$\frac{0}{46}$
2. $\frac{lys\ \ w_a\ \ Y\ BI_1}{LYS\ \ W\ \ y\ \ bi_1}$	a. *LYS BI₁*	$w_a y$ and $w_a Y$ 39	*W y* 2	*W Y* 40	$\frac{2}{42}$
	b. *lys bi₁*	$w_a y$ and $w_a Y$ 28	*W y* 22	*W Y* 3	$\frac{3}{25}$
	c. *lys W y*	.....	*BI* 4	*bi* 46	$\frac{4}{50}$
	d. *W Y*	.....	*LYS bi₁* 8 *lys bi₁* 5	*LYS BI₁* 110 *lys BI₁* 64	$\frac{13}{187}$
3. $\frac{Y\ BI_1\ \ thi}{y\ \ bi_1\ \ THI}$	*BI₁ THI*	.....	*y* 3	*Y* 82	$\frac{3}{85}$
4. $\frac{ad_1\ \ Y\ BI_1}{AD_1\ \ y\ \ bi_1}$	a. *AD₁ BI₁*	.....	*y* 49	*Y* 974	$\frac{49}{1023}$
	b. *AD₁ Y*	.....	*bi₁* 3	*BI₁* 104	$\frac{3}{107}$
				Total	$\frac{95}{1814}$

Recombination fraction: 0.052 ± 0.005
Homogeneity (pooling 1b, 1c: 2a, 2b; and 2c, 3, 4b): $\chi^2_4 = 3.12$; P ≈ 0.65

TABLE 14

Crosses Involving the Y/y and BI/bi Loci in Repulsion: $Y\ bi/y\ BI$

Cross	Selection	Segregations at other loci			
		Unusable classes	Cross-overs	Non-crossovers	Recombination fraction
1. $\frac{AD_1}{ad_1}\ \frac{Y\ bi}{y\ BI}$	a. $AD_1\ BI$	.....	$\frac{Y}{8}$	$\frac{y}{153}$	$\frac{8}{161}$
	b. $AD_1\ y$	.....	$\frac{bi}{8}$	$\frac{BI}{142}$	$\frac{8}{150}$
2. $\frac{ad_1}{AD_1}\ \frac{W}{w}\ \frac{Y\ bi}{y\ BI}\ \frac{pyro}{PYRO}$	a. $AD_1\ BI\ PYRO$	$w\ y$ and $w\ Y$ 38	$W\ Y$ 1	$W\ y$ 35	$\frac{1}{36}$
	b. $AD_1\ BI$ *	$w\ y$ and $w\ Y$ 46	$W\ Y$ 3	$W\ y$ 46	$\frac{3}{49}$
3. $\frac{S_d}{s_d}\ \frac{AD_1}{ad_1}\ \frac{Y\ bi}{y\ BI}$	a. $S_d\ AD_1\ y$	.....	$\frac{bi}{3}$	$\frac{BI}{72}$	$\frac{3}{75}$
	b. $AD_1\ y$	.....	$\frac{bi}{s_d\ \ S_d}$ 4 2	$\frac{BI}{s_d\ \ S_d}$ 44 47	$\frac{6}{97}$
4. $\frac{AD_1}{ad_1}\ \frac{W}{w}\ \frac{y\ BI}{Y\ bi}$	a. $AD_1\ BI$	$w\ y$ and $w\ Y$ 59	$W\ Y$ 3	$W\ y$ 69	$\frac{3}{72}$
	b. $ad_1\ W$	.....	$Y\ BI\ \ y\ bi$ 0 0	$Y\ bi\ \ y\ BI$ 6 4	$\frac{0}{10}$
				Total	$\frac{32}{650}$

Recombination fraction: 0.049 ± 0.008

Homogeneity (pooling 2a, 2b, 3a, 4a, 4b): $\chi^2_3 = 0.68$; $P \approx 0.85$.

* Not classified as to $PYRO/pyro$.

Clearly, neither are the four combinations at the *lys* and *bi* loci equally frequent when we select the color recombinants, nor are the color combinations in 1:2:1 ratios when we select the prototrophs or the double requirers. Two departures are obvious: first, among the *W Y*, the *lys* are less frequent than the *LYS* (69 to 118); this could be due to lower viability or to linkage in coupling of *lys* and w_a. Linkage, however, must be excluded because the other two selections show no shortage of w_a when we select the *LYS*, nor of *W* when we select the *lys*. Second, selection for *Y* brings about a shortage of *bi* (13 *bi*:174 *BI*), selection for *BI* brings about a shortage *y* (2 *W y*:40 *W Y*), and selection for *bi* brings about a shortage of *Y* (3 *W Y*:22 *W y*). The parental combinations being complementary to those which are short (*Y* with *BI*, and *y* with *bi*), this shortage can be taken as an indication of linkage between the *Y/y* and *BI/bi* loci.

Data are available from three more crosses with the loci *Y/y* and *BI/bi* again in coupling. They are collected in Table 13 together with those already given. Not all the workable selections have been carried out in each cross. The results all support linkage. A test of homogeneity over all the sets of results in coupling gives $P \approx 0.65$, and the pooled recombination fraction is $95/1814 = 0.052 \pm 0.005$.

TABLE 15

Detection, by Means of Double Crossovers, of Linkage Between Loci 50 or More Units Apart

	Cross	*Selection*	*Double crossovers*	*Single crossovers*	*Recombination fraction* *
1.	$\frac{w\ ad_1\ AD_2\ y \quad THI}{W\ AD_1\ ad_2\ y \quad thi}$	$AD_1\ AD_2\ THI$	w 9	W 29	$\frac{9}{38}$
2.	$\frac{W\ AD_1\ ad_2\ y \quad thi}{W\ ad_1\ AD_2\ Y \quad THI}$	$AD_1\ AD_2\ THI$	y 14	Y 35	$\frac{14}{49}$
3.	$\frac{w\ ad_1\ y\ BI}{W\ AD_1\ Y\ bi}$	$W\ ad_1$	Y and $Y\ bi$ 21	y and $y\ bi$ 28	$\frac{47}{107}$
		$w\ AD_1$	BI 26	bi 32	

* Double crossovers/doubles + singles.

ad_1 and ad_2 are known to be closely linked.

Crosses 2 and 3 suggest linkage in coupling between ad_2 and *y*, and ad_1 and *y*, respectively (results of cross 3 non-significant). Cross 1 suggests linkage in coupling between *w* and ad_1.

The next step is that of testing the *Y/y* and *BI/bi* loci in repulsion. Clearly, it would be best to compare the results from crosses in which the same other genes are segregating in the same parental combinations as in the coupling crosses. These results, however, are not available except in one case (cross 4 in coupling and cross 1 in repulsion). The data from the four available crosses in repulsion are collected in Table 14. A test of homogeneity on these sets gives $P \approx 0.85$. The recombination fraction from these crosses in repulsion is $32/650 = 0.049 \pm 0.008$.

A test of homogeneity over the coupling and repulsion data combined gives $\chi^2_8 = 4.5$ and $P \approx 0.80$. The results can therefore be pooled and the recombination fraction is $127/2464 = 0.0515 \pm 0.0045$.

In the present case, the conclusion is unequivocal, since qualitatively consistent and quantitatively homogeneous results have been obtained despite the fact that the linkage of *Y/y* and *BI/bi* has been tested in crosses involving six other loci. As will be shown (Tables 15 and 18), there is some evidence that two of these other loci—*W/w* and AD_1/ad_1—are on the same chromosome as *Y/y* and *BI/bi* but more than 50 c. Morgan away. This would require the special mathematical treatment (Bailey, 1951) for the case of selection based on two loci including the segment over which recombination is measured. If the evidence as to these further linkages were confirmed, a situation like this would occur in cross 4, selection a in coupling; and in crosses 1, selection a; 2, selections a and b; and 4, selection a; all in repulsion (Tables 13 and 14).

Whenever viability effects occur, the treatment of the data from any one or more types of selection could be carried out following Mather (1951, Chapter VIII, 21) provided data were available from both coupling and repulsion crosses. The problem, of course, has to be explored by the statistician for some of the new aspects which selection of recombinants introduces. When the loci used for selection are not linked with those the linkage of which has to be estimated, the situation is simple. But it is not simple when there is linkage between the two groups of loci. Bailey (1951) has started to deal with the case of selection for two loci *including* the segment over which an estimate of linkage is wanted. There are, however, other cases for which the help of the statistician would be most welcome. Some will be considered presently.

In any series of crosses segregating at least at three loci—A/a, B/b, C/c—there are three possible ways of changing the procedure. The first is that of changing the distribution of alleles between the strains to be crossed: e.g., A B C//a b c, a B C//A b c, a b C//A B c, or A b C //a B c ("phase" permutations). The second is that of using for selection different pairs of loci: e.g., A and B, A and C, or B and

("marker" permutations). The third is that of using for selection in any one cross one or the other or both of the two reciprocal recombinations of alleles at the two chosen loci: e.g., in cross A B c//a b C when selecting on loci A and C, we may use either or both recombinant classes A C and a c ("allele" permutations).

If we carried out on a sufficiently large scale all possible crosses differing in "phase," and in each cross we used all two-by-two "marker" permutations for selection, and in each of these we selected both "allele" permutations, we should have complete information for detecting and estimating any linkage and any viability.

Clearly it is necessary to decide how far we can reduce the necessary number of permutations. Bailey's (1951) analysis has dealt only with a special case, that of a single linkage group, using two "phase" permutations, one "marker" combination, and one "allele" combination. Bailey has not considered the information to be gained by the use of both "allele" permutations at any pair of loci, and/or more than one "marker" permutations. For the spade-work in *Aspergillus,* where a large number of loci are ready for analysis, and in many other microorganisms, a generalized mathematical treatment of this kind is badly needed.

There are, in addition, innumerable special problems. For instance, linkage between three loci showing 50% recombination two-by-two may be detected by making use of interference in double crossovers as in the examples of Table 15. In this case again, the problem is one of valid detection of linkage, as distinct from viability effects, and of its estimation.

Another problem, which has been considered by Roper (section VI-3), is that of estimating linkage between loci determining nutritional requirements by comparing the proportion of spores giving origin to colonies on a medium which selects for two given loci with the proportion on a medium which selects for two others. In cases of extremely close linkage, this is a very convenient way of estimating it, though the precise statistical treatment has to be developed.

Pending the development of correct methods of treatment for crosses based on recombinant selection, we shall have to use treatments undoubtedly questionable, but sufficient for the immediate purpose of building tentative chromosome maps.

4. *Perithecium Analysis and Relative Heterothallism*
by L. M. Hemmons, G. Pontecorvo, and A. W. J. Bufton

Homothallism may be defined as the ability of a strain whose nuclei are derived from a single haploid nucleus to go through a complete sexual cycle. Heterothallism can then be defined as the inability to complete the sexual cycle without karyogamy between nuclei of different origin. On the basis of these definitions, the three strains of *Aspergillus nidulans* (section III-1) from which all the others were derived in our work are unquestionably homothallic.

The evidence is as follows:

1. Fully fertile strains can be derived by isolation of single ascospores from each of these three strains and from a yellow spore mutant (y), obtained by irradiation of the wild type, from which most of our yellow spore strains have been derived.

2. The eight spores of one ascus from each of these four strains (wild type, w_a, s_d, and y) were dissected and single-ascospore cultures established. Each of the eight spores from one ascus gave origin to fertile strains.

3. The eight spores of an ascus from one of the single-ascospore strains of (2) were again dissected, and single-ascospore cultures established (seven out of eight in the case of the wild type). All cultures were fertile.

4. The majority of the mutant strains derived from the four above-mentioned are still self-fertile and remain so after single-ascospore isolation. The majority of recombinant strains obtained from crossing are also self-fertile after single-ascospore or single-conidium isolation.

Nobody has investigated hitherto what happens in a homothallic species when nuclei of different origins are confronted within the hyphae. Clearly, this could not be done before the development of the technique for genetic analysis in homothallic species (section V-3). The investigation of this problem has revealed the existence of what we call "relative heterothallism," namely, the formation of crossed asci in excess of 50% in certain combinations of strains (Hemmons, Pontecorvo, and Bufton, 1952). The technique which has led to the accidental discovery of relative heterothallism ("perithecium analysis") is valuable *per se* as an additional means for genetic analysis in *A. nidulans* and undoubtedly in other homothallic species.

The first point investigated was: What kind or kinds of asci are to be found in individual perithecia in a cross made by mixed inoculum technique? By using color markers (y/Y) it is not necessary to dissect the eight spores of each ascus to see whether the ascus was derived fro

a selfed or from a crossed zygote. It is sufficient to crush the whole ascus on a small square of cellophane placed over the surface of agar medium. The colony resulting from each crushed ascus will be either uniformly of one color (yellow *or* green), if the ascus was of selfed origin, or show sectors of both colors, if the ascus was of crossed origin (Fig. 19). Even if the viability of the ascospores were as low as 50%,

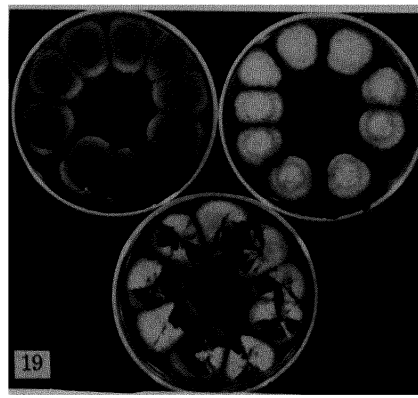


FIG. 19. Each colony originated from a whole ascus from a cross *y bi*, //+, the asci in each petri dish coming from one perithecium. *Top left*, all asci selfed green; *top right*, all asci selfed yellow; *below*, all asci of crossed origin.

provided it were not strongly differential, misclassification of an ascus due to the failure of all the spores of one type to develop would be unimportant.

To ensure high viability of ascospores, the perithecia must be fully ripe. Asci from such perithecia are liable to burst when the perithecium is teased open with needles in order to liberate the asci. Hence, rarely can more than 10 unbroken asci be extracted from each perithecium.

From a cross (mixed inoculum technique) of wild type and yellow (*Y//y*) 52 perithecia yielded 7 or more unbroken asci, and a test of viability of the ascospores gave viable counts of more than 80%. Each ascus, when crushed as mentioned above, gave a colony either uniformly green or uniformly yellow or sectored yellow-green, revealing the selfed (*Y//Y* or *y//y*) or crossed (*Y//y*) origin of the ascus. The seven or more asci from each perithecium were crushed on separate dishes (Fig. 19), and each perithecium was then classified according to the types of colonies which its asci yielded. The results were as follows:

18 perithecia gave asci all alike, producing only yellow colonies
14 perithecia gave asci all alike, producing only green colonies

13 perithecia gave asci all alike, producing only sectored colonies
7 perithecia gave asci of more than one kind
52

Thus, each of 18 perithecia yielded 7 or more asci all of *y//y* origin, each of 14 perithecia yielded asci all of *Y//Y* origin, each of the 13 perithecia *yielded asci all of Y//y* origin, and each of the remaining 7 perithecia yielded asci of more than one origin. In detail:

4 yielded asci some of *y//y* and some of *Y//y* origin
2 yielded asci some of *Y//Y* and some of *Y//y* origin
1 yielded asci some of *Y//Y* and some of *y//y* origin
0 yielded asci of all three origins

A random sample of 1793 ascospores from this cross gave 55% *y* and 45% *Y*. Taking these proportions to represent the proportions of the two types of nuclei in the mycelium, we may calculate the distributions of asci of the three possible origins to be expected on each of the three following hypotheses.

First, we may make the hypothesis that the nuclei are distributed at random in the mycelium and that karyogamy takes place between any two nuclei at random. In this case, the selfed yellow and the crossed and selfed green asci should be in proportions $p^2 : 2pq : q^2$, where $p = 1 - q = 0.55$ represents the proportion of *y* nuclei in the mycelium. The actual distribution of the asci from the above 52 perithecia was: 189 selfed yellow, 136 crossed, 133 selfed green. There is thus a shortage of crossed asci, and the first hypothesis is untenable.

Second, we may make the hypothesis that the nuclei of the two kinds are not distributed at random in the mycelium (say, somewhat clustered according to kind), but within one perithecium karyogamy is at random. In this case, any one perithecium which yielded crossed asci at all should yield the three kinds of ascus in binomial distribution, with *p* and *q* having different values for each of these perithecia. This is not the case: out of 19 perithecia which yielded crossed asci, 13 yielded *only* crossed asci, 4 yielded crossed asci and selfed *y//y* but no selfed *Y//Y*, and 2 yielded crossed asci and selfed *Y//Y* but no selfed *y//y*. The second hypothesis must also be rejected.

We are therefore left with the hypothesis discussed in section II-2 that very few, usually two, nuclei give origin to all nuclei of the ascus primordia in one perithecium, presumably by some system of conjugate divisions.

A further analysis of the yield of individual perithecia from the cross already mentioned was carried out by sampling 200 or more ascospores from each perithecium. Individual perithecia were stripped of

mycelium, Hülle cells, and conidia by rolling them repeatedly with a needle over the surface of agar medium under the dissecting microscope. Each perithecium cleaned in this way was then crushed in 0.1 to 0.2 ml. of saline, and the resulting suspension, diluted as necessary, was plated on three to four dishes to give 200 to 300 colonies. The hard integument of the perithecium of *A. nidulans* makes it withstand the rolling well. The results were as follows:

Plating of Random Sample of More Than 200 Ascospores from Each of 105 Perithecia. Cross: *Y//y*

Types of colonies produced by each perithecium

	Only yellow	Only green	Yellow and green	
			In ratio * of 1:1	In ratios * different from 1:1
No. of perithecia	43	30	18	14

* Significant level taken as 0.05.

These results agree with the previous ones, based on isolation of whole asci, in showing that: (1) there is a tendency for individual perithecia to produce asci all of one kind, i.e., either all selfed yellow, or all selfed green, or *all crossed,* and (2) less than 15% of the perithecia in this cross contain asci of more than one kind. A part of this 15% is accounted for by the occasional development of two perithecia, one inside the other or one fused to the other ("twins"), as can be verified microscopically.

The finding that a high proportion of perithecia which contain crossed asci contains only, or almost only, asci of this kind, opened the way to a new method of genetic analysis. If perithecia of this kind (for short: "crossed" perithecia) could be identified, random samples of ascospores from them could be used *without selection* for Mendelian analysis. A sample of ascospores from one or more "crossed" perithecia is, in fact, equivalent to a sample of gametes from a cross in higher organisms.

Clearly, one cannot identify a "crossed" perithecium without sampling the ascospores to find whether they give 1:1 allele ratios for certain markers. In a cross like the one exemplified above, these perithecia constituted about one-sixth of the total. It was necessary, therefore, to find some way of avoiding extensive platings from the five-sixths of the wrong kind. This was done by making small assay platings of aliquots of the spore suspension from each of a number of perithecia and preserving the major part of each suspension in the refrigerator. After 2 days, one or more of the suspensions, which in the assay plating

had given the correct allele ratios, were further plated on non-selective medium on a scale sufficient for the complete analysis of all segregating genes. Later, the discovery that in certain crosses the proportion of "crossed" perithecia could approach 100% made assay platings unnecessary in these cases.

This technique—which we call "perithecium analysis"—was further refined by a quick method of estimating the upper proportion of "crossed" perithecia in any cross involving visible markers. A number of perithecia were cleaned as mentioned above, crushed on the surface of a non-selective agar medium, and the ascospores streaked out. Up to 10 perithecia could be streaked on a petri dish. After growth, if the cross involved one pair of color alleles (e.g., Y//y), each perithecium would produce one of three kinds of streak: yellow, green, or bicolor. If two pairs of color alleles were segregating (e.g., *w Y//W y*), the streaks would be yellow, white, or tricolor (yellow, white, green) (Fig. 20). The proportion of bicolor (or tricolor) streaks indicates the highest

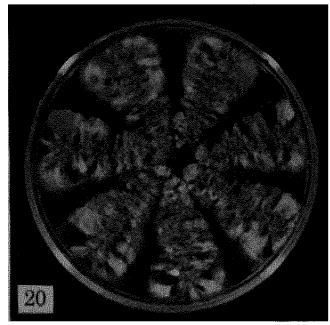


FIG. 20. Each streak from the ascospores of one perithecium from a cross segregating for *Wy* and *wY*; all perithecia contain exclusively, or mainly, crossed asci.

possible proportion of perithecia suitable for perithecium analysis.

An example of perithecium analysis is given in Table 16. The cross involved three linked loci, and the samples of ascospores were taken from two perithecia, each giving for the color markers (*Y/y*) allele ratios not significantly different from 1:1. Full classification for the other two segregating genes gave good allele ratios also for these. The results from the two perithecia were statistically homogeneous and when pooled could be treated as those from a three-point backcross in higher organisms.

The limitations and the usefulness of perithecium analysis as compared with the method of recombinant selection (section V-3) are apparent from the example given. As for limitations, first, perithecium

analysis is inefficient for accurate estimates of linkage when linkage is close. In fact, 721 colonies had to be isolated and fully tested in order to obtain 54 recombinants between *y* and *bi;* with recombinant selection (see, e.g., Table 14, cross 4), classification by inspection would have given the same results. When it comes to obtaining recombinants and estimating recombination fractions as low as 1/1000, let alone the extremely low ones characteristic of pseudo-alleles (section VI-3), perithecium analysis is out of the question.

TABLE 16

Perithecium Analysis in the Three-Point Cross: *paba$_1$ y BI$_1$//PABA$_1$ Y bi$_1$*

		paba$_1$ Bi$_1$	*PABA$_1$ bi$_1$*	*paba$_1$ bi$_1$*	*PABA$_1$ BI$_1$*	Totals
Perithecium I						
	y	144	2	11	39	196
	Y	0	156	42	10	208
	Totals	144	158	53	49	404
Perithecium II						
	y	117	3	5	24	149
	Y	1	125	20	22	168
	Totals	118	128	25	46	317

Allele ratios: *Y/y* 376/345 ($P = 0.25$); *PABA$_1$/paba$_1$* 381/340 ($P = 0.15$); *BI$_1$/bi$_1$* 357/364 ($P = 0.75$)

Crossover values: *paba$_1$ — y* 0.18 ± 0.02
y — bi 0.075 ± 0.009

Combinations:

Parental	*paba$_1$ y BI*	261	542
	PABA$_1$ Y bi	281	
Crossovers in I	*paba$_1$ Y bi*	62	125
	PABA$_1$ y BI	63	
Crossovers in II	*paba$_1$ y bi*	16	48
	PABA$_1$ Y BI	32	
Doubles	*paba$_1$ Y BI*	1	6
	PABA$_1$ y bi	5	
			721

Secondly, the validity of perithecium analysis rests on the choice of perithecia which yield only, or practically only, crossed asci. The

preliminary identification of such perithecia is based on their giving 1:1 allele ratios at the loci used as markers. These ratios may not be obtained when there is close linkage between an allele lowering viability and one of the marker loci. To avoid this difficulty, it is helpful to use more than one locus as marker; the two color loci W/w and Y/y are obviously convenient.

As for the advantages, perithecium analysis utilizes all products of meiosis, and therefore all the usual treatments developed in classical genetics for genetic ratios can be applied to it unmodified. There is no need to wait for the development of special statistical treatments, as mentioned in section V-3. Furthermore, the fact that platings are made on non-selective media avoids the common complication that the viability of certain types of segregants is different on different media.

One of us (A.W.J.B.), while carrying out perithecium analysis with a certain cross, found that all the perithecia tested (30) contained crossed asci. This was quite unexpected, and a quick search was started, by means of the streaked perithecium technique, among all other available crosses. For this search, at least 8 perithecia from each cross were streaked. In some of the crosses the plates available had been prepared as mixed inocula, in other crosses as balanced heterokaryons, and in others as both. It worked out that the method of crossing was not crucial. The results showed that a proportion of "crossed" perithecia in excess of 50%, or even up to 100%, was by no means uncommon; in the 27 crosses examined to date, 16 were of this kind. The 27 crosses included 21 different strains and 17 identified loci.

Although the analysis of the mechanism of relative heterothallism is still far from complete, the following tentative conclusions can be drawn so far:

(1) Relative heterothallism has arisen in the laboratory, because it occurs between certain pairs of strains derived exclusively by successive mutations from one and the same original fully self-fertile strain (wild type).

(2) It cannot be clearly associated with any one identified locus, because from a cross giving 100% crossed asci one can recover pairs of recombinants differing from each other at precisely the same loci and yet yielding less than 50% crossed asci.

(3) It is found in crosses in which either, both, or neither parent is fully self-fertile; unlike absolute heterothallism, it is not associated with self-sterility.

(4) It is not obviously connected with the ease with which two strains form balanced heterokaryons.

Apart from being technically convenient for genetic analysis, rela-

tive heterothallism is certainly a phenomenon deserving full investigation. Its occurrence in natural populations of homothallic species is likely, to say the least, and it may have to be considered together with heterokaryosis as one of the factors in the genetic systems of such species.

VI. Formal Genetics

1. *Identification of Loci*

Of the 600 mutant strains available, only very few have been used so far in genetic tests. The 27 used are listed in Table 17; they are all due to independent mutation and there are several groups of "mimics," i.e., phenotypically indistinguishable in the relevant respect. The mimics include: 3 independent cases of mutation to colorless conidia; 4 to adenine/hypoxanthine requirement (out of more than 100 found); 4 to biotin/desthiobiotin requirement; 2 to P.A.B.A. requirement (out of 6 found); 4 to arginine/ornithine requirement (out of 5 found). There are other groups of "mimics" as yet untapped, among which are the overwhelming one of about 300 independent mutations to thiosulphate/sulphite requirement and about 20 to thiosulphate requirement. The only groups at present under systematic investigation are those of the adenine, P.A.B.A., and pyridoxine requirements.

The genetic tests carried out included the three methods described in section V, i.e., recombinant selection, perithecium analysis, and balanced heterokaryosis. The last is dependable only in very clear-cut cases, since in the others failure to obtain a balanced heterokaryon is not significant and success may be simulated by adaptation of one or both strains.

The detailed results of the crosses made cannot be given here. Those for the study of the *bi* and the *paba* pseudo-alleles are given in section VI-3. Those which have led to the detection and estimation of linkage between BI_1/bi_1 and Y/y were given in section V-3. In section VI-2 more data will be given on the 11 linked loci (or 7, if each series of pseudo-alleles is considered as one locus) belonging to the *bi* linkage group, as we call it. The only other probable example of linkage so far includes the two loci pr_1 and *co;* but the work on these, in collaboration with Mr. A. W. J. Bufton, is still incomplete.

Of the loci not belonging to either of these linkage groups, *LYS/lys* and *THI/thi* have been used most extensively. The *lys* allele has a viability of about 60%. The other mutants have been tested in various combinations, and for each there is at least *prima facie* evidence that

TABLE 17

Mutant Alleles Used in Crosses up to April, 1952

Alleles			*Strain and mode of origin*	*Linkage and allelism* *
Symbol	Designation	Phenotype		
	Visible			
w_a	White alba	Colorless conidia	+: spontaneous, 1936 †	All alleles of one another and of *W*, and epistatic to *Y*/*y*. "*bi* group" (¶)
w	White		*y*: X-rays, 1946	
w_n	White new		*paba*$_1$ *bi*$_1$: spontaneous, 1951	
y	Yellow	Yellow conidia	+: X-rays, 1946	"*bi* group"
co	Compact	Compact colony	*paba*$_1$ *y*: unknown, 1951	Not in "*bi* group," probably linked with *pr*$_1$
pr$_1$	Poorly proteolytic-1	Reduced proteolytic activity	*bi*$_1$: U.V., 1951	See *co*
	Nutritional			
ad$_1$	Adenine-1	Adenine/hypoxanthine	*y*: X-rays, 1946	"*bi group*"; all not allelic
ad$_2$	Adenine-2		*y*: X-rays, 1946	
ad$_3$	Adenine-3		*y thi*: X-rays, 1948	
ad$_4$	Adenine-4		*bi*$_1$: X-rays, 1948	
bi$_1$	Biotin-1	Biotin/desthiobiotin	+: X-rays, 1947	"*bi* group"; *bi*$_1$, *bi*$_2$, and *bi*$_3$, pseudo-alleles; *bi*$_4$, incompletely tested
bi$_2$	Biotin-2		*y thi*: X-rays, 1948	
bi$_3$	Biotin-3		*y thi*: X-rays, 1948	
bi$_4$	Biotin-4		*y*: X-rays, 1948	

paba$_1$	P.A.B.A.-1	*p*-Aminobenzoic acid	*bi*$_1$: X-rays, starvation, 1951	"*bi* group," pseudo-alleles
paba$_6$	P.A.B.A.-6	*p*-Aminobenzoic acid	*bi*$_1$: U.V., starvation, 1951	
pyro$_4$	Pyridoxine-4	Pyridoxine	*bi*$_1$: U.V., starvation, 1951	Not in "*bi* group"
s$_6$	S-delta	Sulphite/thiosulphate	+: N-mustard, 1948 ‡	Not in "*bi* group"
thi	Thiazole	Aneurin/thiazole	*y*: X-rays, 1946	No linkage with *lys* or with "*bi* group"
nic$_2$	Nicotinic-2	Anthranilic/nicotinic acid	+: X-rays, 1947	Not allelic; no linkage with "*bi* group" or *thi*
nic$_3$	Nicotinic-3	Anthranilic/tryptophan/ nicotinic acid	*y thi*: X-rays, 1948	
orn$_1$	Ornithine-1	Arginine/ornithine	*y thi*: X-rays, 1949	At least two different loci; no linkage with "*bi* group"
orn$_2$	Ornithine-2	Arginine/ornithine	*bi*$_1$: X-rays, 1949	
orn$_3$	Ornithine-3	Arginine/ornithine	*y thi*: X-rays, 1948	
orn$_4$	Ornithine-4	Arginine/ornithine	*bi*$_1$: U.V. starvation, 1950	
lys	Lysine	Lysine	*w*$_6$: X-rays, 1947	No linkage with "*bi* group," *thi*, *orn*$_1$, or *panto*
panto	Pantothenate	Pantothenate	*y thi*: X-rays, 1948	No linkage with "*bi* group," *lys*, *thi*, or *orn*$_1$

* The linkage group from *w* to *ad*$_4$ will be referred to as the "bi group."

† From Mr. E. Yuill.

‡ From Dr. Hockenhull.

TABLE 18

Summary of Tests for Detecting Linkage in the *bi* Linkage Group

Region	*Cross* *	*Selection*	*Segregations*			*Tentative recombination fractions* †
y–bi₁	See Tables 13 and 14 pooled results of crosses in coupling and repulsion					$\frac{127}{2464} = 0.0515 \pm 0.0045$
y–ad₂			***y***	***Y***	**Total**	
	1. $\frac{ad_2\ y\ BI_1}{AD_2\ Y\ bi_1}$	$AD_2\ BI_1$	50	42	92	$\frac{50 \times 0.05}{42} = 0.06$
	2. $\frac{PABA_1\ ad_2\ y\ BI_1}{paba_1\ AD_2\ Y\ bi_1}$	$AD_2\ BI_1$	84	56	140	$\frac{84 \times 0.05}{56} = 0.075$
	3.‡ $\frac{ad_2\ Y}{AD_2\ y}\ \frac{THI}{thi}$	$AD_2\ THI$	130	11	141	$\frac{11}{141} = 0.078$
paba₁–y						
	4. $\frac{PABA_1\ y\ bi_1}{paba_1\ Y\ BI_1}$	$PABA_1\ BI_1$	67	198	265	$\frac{198 \times 0.05}{67} = 0.148$
	5. $\frac{W\ ad_1\ PABA_1\ y\ BI_1}{w_n\ AD_1\ paba_1\ Y\ bi_1}$	$W\ AD_1\ PABA_1$	82	20	102	$\frac{20}{102} = 0.196$
paba₁–bi₁			***BI₁***	***bi₁***		
	5. $\frac{W\ ad_1\ PABA_1\ y\ BI_1}{w_n\ AD_1\ paba_1\ Y\ bi_1}$	$w_n\ PABA_1$	20	3	23	$\frac{6}{38} = 0.158$
			PABA₁	***paba₁***		
		$w_n\ BI_1$	12	3	15	
y–ad₄			***y***	***Y***		
	6. $\frac{ad_2\ y\ BI_1\ AD_4}{AD_2\ Y\ bi_1\ ad_4}\ \frac{thi}{THI}$	$AD_2\ AD_4\ THI$	21	2	23	$\frac{5}{48} = 0.104$
		$AD_2\ AD_4$	22	3	25	

bi_1–ad_3				y		Y		
				BI_1	bi_1	BI_1	bi_1	
	7. $\frac{ad_3\ y\ BI_1\ AD_4}{AD_3\ Y\ bi_1\ ad_4}$		$AD_3\ AD_4$	11	1	15	2	29
w–ad_1				Wy	WY	wy and wY		
	5. $\frac{W\ ad_1\ PABA_1\ y\ BI_1}{w_n\ AD_1\ paba_1\ Y\ bi_1}$		$AD_1\ PABA_1$	82	20	139		241
			$AD_1\ BI_1$	113	14	148		275
			$AD_1\ PABA_1\ BI_1$	102	1	120		223
				W		w		
	8. $\frac{w\ ad_1\ AD_3\ y}{W\ AD_1\ ad_3\ Y}\ \frac{THI}{thi}$		$AD_1\ AD_3\ THI$	41		14		55
ad_3–y				y		Y		
	9. $\frac{ad_1\ AD_3\ Y}{AD_1\ ad_3\ y}\ \frac{THI}{thi}$		$AD_1\ AD_3\ THI$	14		35		49
			$AD_1\ AD_3$	20		55		75
ad_1–ad_3						ad_1 or ad_3		
	9. $\frac{ad_1\ AD_3\ Y}{AD_1\ ad_3\ y}\ \frac{THI}{thi}$		Perithecium analysis	$AD_1\ AD_3$		or $ad_1\ ad_3$		
				1		703		704

$\frac{2 \times 0.05}{15} = 0.007$	
$\frac{102}{241} = 0.42$	
$\frac{127}{275} = 0.46$	
$\frac{103}{223} = 0.46$	
$\frac{14}{55} = 0.26$	
$\frac{34}{124} = 0.28$	
$2 \times \frac{1}{704} = 0.003$ §	

* Data of crosses 2, 3, 4, and 5 from Dr. J. A. Roper. Part of the data of cross 9 from Mr. R. H. Pritchard.

† Some of the recombination fractions have been calculated from the comparison of recombination in a given region with that of the *y*–*bi* region taken as 0.05.

‡ Cross 3 (see Table 23) was also segregating for $BI_2\ bi_1/bi_2\ BI_1$, which are irrelevant here.

§ The recombination fraction was also estimated to be about 0.003 from the ratio of $AD_1\ AD_2$ among the viable ascospores, this cross yielding almost exclusively crossed asci.

the mutant phenotype is due to a single locus not belonging to the *bi* linkage group just mentioned and not linked with other tested loci.

Clearly, to advance quickly in the mapping of the chromosomes, we need to rationalize the procedure for testing against one another the enormous number of mutants available.

2. *The bi Linkage Group*

The first linkage detected was that between the Y/y and the BI_1/bi_1 loci; the data are summarized in Tables 13 and 14. The pooled recombination fraction from coupling and repulsion is 0.0515 ± 0.004. The second linkage found was between AD_2/ad_2 and the two just mentioned. It was detected as follows (Table 18). Plating on minimal medium a random sample of ascospores from the cross $ad_2\ y\ BI_1//AD_2\ Y\ bi_1$, 50 yellow and 42 green colonies were obtained. Without linkage of ad_2, such a plating should have yielded about 87 yellow and 5 green. The conclusion was that Y/y was located about midway between AD_2/ad_2 and BI_1/bi_1. Further crosses confirmed this conclusion. The available information on crosses involving ad_2 and y in coupling and repulsion gives a recombination fraction of between 0.06 and 0.08.

The third linkage—between $paba_1$ and y—was detected by Mr. A. W. J. Bufton. The recombination fractions (data by Dr. J. A. Roper) are 0.148 and 0.198, respectively, in two crosses in repulsion. The $paba_1$–bi_1 recombination fraction (repulsion) in one of these crosses is 0.158 (Table 18).

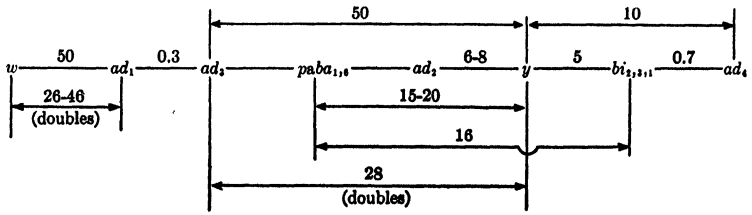
The fourth linkage detected was between ad_4 and bi_1. Crosses 6 and 7 in Table 18 indicate the kind of evidence. In cross 6, if ad_4 segregated independently, selection of $AD_3\ AD_4$ recombinants should yield a small excess of Y because of the loose linkage between ad_3 and y (see cross 9). Instead of this, the Y constitute only 0.10 of the total. This can only be due to close linkage. The AD_4/ad_4 locus could be either beyond bi_1 or between y and bi_1. The next cross shows that it is beyond bi_1. Thus the calculation of the recombination fraction requires Bailey's (1951) treatment, since the segment over which it has to be calculated lies between the two loci used for selection. Unfortunately, our data are not suitable for this treatment. But cross 7 permits a crude indirect estimate of recombination between bi_1 and ad_4. The recombination fraction between bi_1 and y has been estimated independently with good precision as 0.05. In cross 7 there are 15 crossovers between bi_1 and y and 2 between bi_1 and ad_4; thus $2/x = 15/0.05$ gives 0.007 as the desired estimate. This admittedly questionable method has been used repeatedly in Table 18.

Finally we come to three loci—W/w, AD_1/ad_1, and AD_3/ad_3—

which are better considered together. In the first place, ad_1 and ad_3 are very closely linked. This has been shown in two ways; firstly, with perithecium analysis, only one AD_1 AD_3 crossover was obtained among 703 colonies from a cross in repulsion: the recombination fraction is thus $2 \times 1/703 = 0.003$. Secondly, with recombinant selection, the proportion of AD_1 AD_3 recombinants among viable ascospores was found to be again about 0.003. Since cross 9 gave almost exclusively crossed asci, this proportion can be taken to be as practically identical to the recombination fraction. Recent work by Mr. R. H. Pritchard with heterozygous diploids shows that ad_1 and ad_3 are not pseudo-alleles.

That ad_1 and ad_3 as well as *w* belonged probably to the *bi* linkage group was found by making use of interference in multiple crossovers as mentioned on p. 193 and shown in Table 15. We can select *crossovers* in the *bi* group and see how other markers in the same group segregate. Crosses 5, 8, and 9 show the results: when we select a crossover between ad_1 and ad_3, both *w* (cross 8) and *y* (cross 9) show linkage with the ad_1–ad_3 region; the recombination fractions so obtained (0.26 and 0.28) are, as expected in the case of linkage, smaller than when calculated on single crossovers. Many other crosses in which only single crossovers between the ad_1–ad_3 region and either *w* or *y* were measured showed free recombination. Slightly more stringent conditions—such as making one crossover compulsory over a long region (cross 5)—yield recombination fractions (about 0.46) smaller than 0.50 but considerably greater than those obtained when the compulsory crossover is in the ad_1–ad_3 region.

We can now construct a tentative linkage map of the *bi* chromosome. The recombination fractions, crude and questionably derived as they are, leave very little doubt as to the sequence of loci and give a rough idea of the distances:



In the course of the work on the *bi* linkage group we have come across cases of disturbed segregations which can be attributed only to chromosomal rearrangements. One case in Table 12 determines spurious close linkage between ad_1 and *y* in a particular strain. Another determines absence of single crossovers in the region $paba_1$–ad_3 when a strain ad_3 *y* is used.

3. *Pseudo-Allelism*

by J. A. Roper

A number of cases have been reported of closely linked loci mutant alleles at which determine similar phenotypes. For example "lozenge" (Green and Green, 1949), "star-asteriod," "bithorax" (Lewis, 1945, 1950), "singed" (Ives and Noyes, 1951) and "white" (MacKendrick and Pontecorvo, 1952) in *Drosophila;* "brachyury" (Dunn and Caspari, 1943) in the mouse; perhaps *Rh* in man (Fisher, 1946); inositol (Giles, 1952) and nicotinic acid requirement in *Neurospora* (Bonner, 1950); biotin requirement in *Aspergillus* (Roper, 1950a); adenine requirement in *Aspergillus* (Pontecorvo, 1952c).

In some of these cases, whether or not the close linkage is a matter of chance is unknown. In other instances it has been shown that the investigated alleles constitute a pseudo-allelic series. Pseudo-alleles are, for all general purposes, alleles of one gene at one locus; closer investigation, however, reveals crossing-over between different pseudo-alleles. Further, such alleles show an effect which has been described as a position effect in that the genotypes $m_1\, m_2/+\ +$ and $m_1+/+m_2$ give different phenotypes. The former is wild type or nearer to wild type than the latter, which is mutant, or more extreme.

There have been several approaches to the study of pseudo-alleles. Probably the most completely investigated pseudo-allelic series is the case of "lozenge" (Green and Green, 1949). The study of "lozenge" followed the observation (Oliver, 1940; Oliver and Green, 1944) of wild-type progeny from females of genotype lz^g/lz^e. Lewis (see 1950) has found cases of pseudo-allelism associated with repeats and deduces a connection between repeats and pseudo-alleles. In the present instances of pseudo-allelism found in *A. nidulans,* the working hypothesis which prompted the investigation has already been published (Pontecorvo, 1950) and can briefly be stated as follows. Close linkage might be expected between some of the genes acting on any one series of biochemical reactions where the intermediates are non-diffusible, labile, or present in very low concentration. It seems more likely, however, that the cases of close linkage which have so far resulted from the research prompted by this working hypothesis are concerned with intra-genic rather than inter-genic organization (Pontecorvo, 1952b; 1952c).

For the initial investigation three biotin-requiring strains of *A. nidulans,* independently obtained by X-ray treatment, were used. In their responses to known and possible intermediates in biotin synthesis the three strains were identical (Table 19).

Despite the failure to distinguish the strains biochemically, crosses

TABLE 19

Responses of Three Biotin-Requiring Strains of *A. nidulans* to Intermediates in Biotin Synthesis

	bi$_1$	*bi*$_2$	*bi*$_3$
Pimelic acid	—	—	—
7,8-Diamino pelargonic acid *	+	+	+
Desthiobiotin	+	+	+
Biotin	+	+	+

* Kindly supplied by Professor B.C.J.G. Knight.

of the strains, two by two, gave in every case, though very infrequently, biotin-independent types. If these biotin-independent types arose as a result of recombination, and not, say, mutation, the requirements would be conditioned by alleles at three different closely linked loci. The steps taken to establish that this was the case were as follows:

Pontecorvo, Forbes, and Adam (1949) had already shown that a locus for biotin requirement in one strain (*bi*$_1$) was linked with and approximately 5 c. Morgan distant from the *y* locus (See section V-2 and V-3 for meaning of symbols). The mutant alleles determining the biotin requirements in the other two strains were provisionally designated *bi*$_2$ and *bi*$_3$. The locus of each of these was tested for linkage with the *y* locus. Only one cross, involving the *bi*$_2$ locus, is exemplified (Table 20).

This cross showed that mutation in a single gene determines the biotin requirement and that the locus of this gene is linked some 4 to 5 c.

TABLE 20

Cross to Locate the *bi*$_2$ Locus with Respect to the *y* Locus:

$$\frac{ad_1\ Y\ BI_2}{AD_1\ y\ bi_2} \quad \frac{THI}{thi}$$

Ascospores plated on	*Recombinants selected by medium*	*Colonies*	
		Yellow	Green
Minimal medium with biotin	*THI AD*$_1$	182 (77 tested: 74*bi*$_2$, 3*BI*$_2$)	192 (60 tested: 57*BI*$_2$, 3*bi*$_2$)
Minimal medium with aneurin	*BI*$_2$ *AD*$_1$	10	227 (32 tested: 18*THI*, 14*thi*)

Morgan from the *y* locus. A similar cross was made to test the bi_3 locus. Again, it was shown that mutation in a single gene determines the biotin requirement and that the locus bi_3 was some 4 to 5 c. Morgan from the *y* locus.

A series of crosses, details of three of which are given in Table 21,

TABLE 21

Crosses to Determine the Order of the Loci bi_1, bi_2, and bi_3 with Respect to One Another and the *y* Locus:

1. $\frac{y \quad BI_3\, bi_1}{Y \quad bi_3\, BI_1}$ 2. $\frac{y \quad bi_1\, BI_1}{Y \quad BI_3\, bi_1}$ 3. $\frac{y \quad bi_3\, BI_1}{Y \quad BI_3\, bi_1}$

Recombinants selected from platings on minimal medium	*Colonies*	
	Green	Yellow
Cross 1: $BI_3\, BI_1$	0	9
Cross 2: $BI_3\, BI_1$	27	0
Cross 3: $BI_3\, BI_3$	48	3

showed two facts: (1) that all three biotin loci were on the same side of the *y* locus, and (2) that if the biotin-independent types were the result of recombination, and not of some other process, then the order of the loci with respect to one another and the *y* locus was *y* bi_3 bi_2 bi_1.

The results so far did not unambiguously prove recombination as an explanation of the biotin-independent types. Further, if recombination did in fact occur, it was also necessary to obtain an estimate of the recombination frequencies between the biotin loci.

To test for recombination as an explanation of these results, a series of crosses was made of which one example is given in Table 22. The crosses were designed in such a way that: (*a*) if the biotin independent types arose as a result of recombination, the recombinants would show the expected segregations for linked and freely recombining markers; (*b*) more than half of the recombinants would differ from either parent by at least two alleles; and (*c*) freely recombining nutritional markers used in addition to the biotin markers would allow, as shown below, an easy estimation of the recombination frequencies between the closely linked loci. Both *a* and *b* would give unambiguous proof of crossing-over as opposed to mutation.

The results of these crosses showed, for the reasons given above, that recombination between the biotin loci did take place. A crude calculation of the recombination frequencies was made as follows: colonies obtained on minimal medium with biotin (Table 22) were from ascospores

TABLE 22

Cross to Detect and Estimate the Frequency of Recombination Between Two *bi* Loci:

W AD_1 y bi_2 BI_1 thi/w ad_1 Y BI_2 bi_1 THI

Ascospores plated on	*Total number of ascospores plated*	*Recombinants selected*	*Colonies*		
			White	Yellow	Green
Minimal medium with biotin	62.7×10^3	*AD_1 THI*	312 133 tested, all *bi*	318 140 tested, all *bi*	6 5 tested, all *bi*
Minimal medium with aneurin and adenine	3.48×10^6	*BI_2 BI_1*	39 21 *ad_1 THI* 16 *ad_1 thi* 2 *AD_1 thi*	4 1 *AD_1 thi* 3 *AD_1 THI*	33 18 *ad_1 THI* 15 *ad_1 thi*

The results show that strain *w ad_1 Y BI_2 bi_1 THI* has a chromosome rearrangement such that the locus *ad_1*, instead of segregating independently of the *y* locus, is linked about 2 c. Morgan from it.

necessarily recombinant for the freely recombining alleles *AD* and *THI*. On this medium, 62.7×10^3 ascospores gave 636 colonies. On minimal medium with aneurin and adenine the colonies were from ascospores recombinant for the alleles BI_2 and BI_1. If the biotin alleles recombined freely, then 62.7×10^3 ascospores should have given 636 colonies, which was not the case. In fact, 3.48×10^6 ascospores gave only 76 colonies. These figures allow estimation of the recombination frequency by the following formula:

$$\text{Recombination fraction } (\%) = \frac{p}{q} \times \frac{50}{1}$$

where p and q are, respectively, the number of recombinant colonies obtained for the closely linked loci and the number expected for free recombination. For the bi_2 and bi_1 loci, the estimated recombination frequency was approximately 0.1%. A similar cross using the loci bi_2 and bi_1 gave a recombination frequency of 0.04%.

The accuracy of this method of estimation may be open to criticism mainly for the reasons discussed in section IV-4, where it is suggested that high concentrations of auxotrophs may inhibit growth of rare prototrophs. However, in the first place the density of ascospores used in this work was not usually higher than 10^6 per plate. In the second place, in one cross investigated for this specific purpose no inhibitory effect was found over a range of densities from 10^3 to 10^6 per plate (Table 23). Further, estimations were made of one recombination frequency with ascospores from heterokaryons having widely different percentages of hybrids and, therefore, different proportions of prototrophs. No serious variation in the estimated values was found. It seems likely that any error from this source is less than errors due to variations in viability on different media and experimental errors involved in serial dilutions and platings. For these reasons it is impossible, at present, to give standard errors for these very low recombination frequencies. It can only be said that estimations of any one recombination frequency by a series of different crosses gave values in which the extremes differed by less than 50% of the mean of all the estimations.

Since a chromosome rearrangement was detected in crosses of the type shown in Table 22, it was thought necessary to repeat part of the work with strains having no known chromosomal abnormalities. Two such crosses are shown in Table 23. In one of these the critical arrangement of two markers, one on each side of the *bi* loci, was used. These crosses entirely confirmed the previous results.

Finally, one cross was made between two strains having the same biotin allele, bi_1. This cross (Table 24) gave no results in any way resembling recombination of the bi_1 alleles. This was so, although the high number of ascospores plated on the biotinless medium would have detected a "recombination" frequency much smaller than those measured when different biotin alleles were used. This again confirms that the three biotin alleles bi_1, bi_2, and bi_3 differ and that their loci do recombine.

Further tests were then made in an attempt to differentiate, biochemically, the phenotypes determined by each of the three mutant biotin alleles. All tests, including cross-feeding experiments, failed to show biochemical differences in the effects of the three mutant alleles. Finally, for some combinations of biotin alleles, diploid strains were prepared (see section VII-1). The phenotypes of these diploids and of some heterokaryons are listed in Table 29, section VII-1. A number of critical genotypes, particularly heterozygotes with two mutant biotin alleles in coupling (e.g., $^{+}bi_2\ bi_1/BI_2\ BI_1$) have not yet been tested because of the difficulty of obtaining strains with two mutant biotin alleles in coupling.

TABLE 23

Crosses To Estimate the Recombination Frequency between the bi_2 and bi_1 Loci Using Linked Markers on One Side or on Both Sides of These Loci

Ascospores plated on	Recombinants selected	Number of ascospores plated per dish	Total number of ascospores plated	Colonies	
				Yellow	*Green*
Cross: $\frac{AD_2\ y\ bi_2\ BI_1}{ad_2\ Y\ BI_2\ bi_1}$	$\frac{thi}{THI}$				
Minimal medium with biotin	$AD_2\ THI$		18×10^8	130 (all *bi*)	11 (all *bi*)
Minimal medium with aneurin and adenine	$BI_2\ BI_1$		1.8×10^6	2 (both AD^2 *THI*)	31 (17 ad_2 *thi*, 13 ad_2 *THI*, 1 AD_2 *THI*)
Cross: $\frac{y\ bi_2\ BI_1\ AD_1}{Y\ BI_2\ bi_1\ ad_1}$	$\frac{thi}{THI}$				
Minimal medium with biotin	$AD_1\ THI$	35	70	0	0
		350	700	0	0
		3.5×10^3	7×10^3	2	0
		4×10^4	16×10^4	41	6
		8×10^4	32×10^4	97	9
		Total	4.88×10^5	138 (all *bi*)	15 (all *bi*)
Minimal medium with aneurin and adenine	$BI_2\ BI_1$	3.5×10^3	7×10^3	0	0
		3.5×10^4	7×10^4	0	0
		5.7×10^5	17.1×10^5	0	0
		3.2×10^6	9.6×10^6	0	4
		6.4×10^6	19.2×10^6	0	11
		Total	3.06×10^7	0	15 (6 AD_1 *thi* 9 AD_1 *THI*)

By the calculation used on p. 211 the recombination frequency between the bi_2 and bi_1 loci on the basis of the above data is 0.12% in the first cross and 0.08% in the second.

TABLE 24

Cross Involving Identical Biotin Alleles in the Two Strains:

$$\frac{AD_1\ \ y\ \ bi_1\ \ thi}{ad_1\ \ Y\ \ bi_1\ \ THI}$$

Ascospores plated on	*Number of ascospores plated*	*Recombinants selected*	*Colonies*	
			Yellow	Green
Minimal medium with biotin	9×10^8	*AD, THI*	175	14
Minimal medium with aneurin and adenine	54×10^6	*BI*	0	0

By analogy with the lozenge loci, it is expected that a genotype such as that above would give a wild-type phenotype.

Preliminary investigations have also been made on two *p*-aminobenzoic acid-requiring strains of *A. nidulans*. The results so far indicate that in each strain a single allele determines the difference from wild type and that the loci of the two mutant alleles recombine with a frequency of about 0.002%. It is not yet known whether the wild-type alleles at the two *paba* loci determine different biochemical reactions.

Any hypothesis to explain the results obtained for the *bi* loci must account for two facts: (1) the extremely close linkage of three loci, mutant alleles at which determine similar and probably identical phenotypes; such close linkage for three loci is hardly likely to be a matter of chance; and (2) the fact that the phenotoype of the diploid, $bi_2\ BI_1/BI_2\ bi_1$, and the heterokaryon, $bi_2\ BI_1 + BI_2\ bi_1$, are mutant and not wild type as expected from the fact that each mutant *bi* allele tested separately is recessive.

Two explanations at present seem possible. The first, and less likely, is that the results are convalidating the working hypothesis which prompted the investigation. The wild-type alleles at the three *bi* loci would then control different biochemical reactions, the differences not being detected by the tests used. The wild-type alleles would function normally only when they were all three on one and the same chromosome. This would be the case if the intermediates in the reactions controlled by the wild-type alleles were labile, or non-diffusible, or present in very low concentration. Thus the unexpected phenotypes would be explained.

The essentials of the second and more plausible hypothesis have been considered by Muller (1947; see also Raffell and Muller, 1940), Goldschmidt (1944, 1946, 1950) and Pontecorvo (1952b, 1952c). That is, that the gene as a working unit in physiological action is based on a chromosome segment larger than either the unit of mutation or recombination. Mutation at different sites in the *bi* gene gives at least three, and possibly many, mutant alleles, any one of which inactivates the gene. In some cases recombination between alleles will be possible. The phenotype of the double heterozygote in repulsion is then as expected, since, in this diploid, both biotin genes are inactivated by mutations at different sites. Assembly of all the normal parts of the gene on a single chromosome is necessary for the normal functioning of the gene. Bonner (1950) has suggested that a similar situation may apply to the *Q*-locus in *Neurospora*. Stephens (1951) has made a critical analysis of the possible association between repeats and pseudo-alleles and finds no case in which the association is satisfactorily proved. As Goldschmidt (1950) has pointed out, an hypothesis such as the second outlined above requires no such association. However, a variety of cases have been labeled as pseudo-allelomorphism, and it may be that they do not constitute a homogeneous group.

4. *Physiological Genetics*

The non-systematic observations reported in the present section are by-products of the main genetic work. We have deliberately not investigated sequences of biochemical reactions except where this work was necessary for the approach to a genetic problem, as, for example, the study of pseudo-alleles.

These observations fall under two headings: types of nutritional mutants, and novel properties of some, i.e., properties not found or not described in other microorganisms.

The detailed classification of 578 auxotrophs was given in Table 6. This table did not include: (*a*) 5 mutants (3 requiring sulphite, 1 adenine, and 1 unknown factor) out of 203 isolates obtained after X-rays and starvation for less than 96 hours: (*b*) 29 mutants (1 requiring guanosine, 1 tryptophan, 1 adenine, and 26 sulphite or thiosulphate) obtained out of 1346 isolates in the selective experiment summarized in Table 7; and (*c*) 52 mutants (25 parathiotrophic, 5 requiring amino acids, 16 nucleic-acid components, and 7 vitamins, but not further classified) obtained out of 800 isolates in the selective experiment summarized in Table 8. Adding on to Table 6 the auxotrophs under *a* and *b*, but not those under *c* because they are incompletely classified, we have a grand total of 612 auxotrophs. For only 33 of these have we failed to identify a

single growth factor capable of satisfying the requirements of each strain; but for 24 the tests have not been complete or decisive. Even if all 33 were cases of genuine multiple requirements, this would constitute only about 5% of the total.

A glance at the list of auxotrophs in Table 6 and a comparison with corresponding lists for *Penicillium notatum-chrysogenum* (Bonner, 1946), *Ophiostoma* (Fries, 1945), and *Neurospora* (Tatum, Barratt, Fries, and Bonner, 1950) shows certain similarities and certain striking differences. In the first place, the high proportions of arginine, lysine, and adenine requirers is common in all four species. The high proportion of parathiotrophic mutants found in *Aspergillus* and *Ophiostoma,* however, does not seem to be paralleled in *Neurospora* or *Penicillium,* where there is a correspondingly high proportion of methionine/cystine requirers. Whether this is a real difference or simply due to the array of compounds used in the tests, is not clear from the published accounts.

In *Aspergillus,* inositol and histidine auxotrophs have not been obtained, not even among 1346 isolates in a selective technique experiment (Table 7) which yielded the two previously unobtained types requiring guanosine and tryptophan. In view of the considerable proportion of inositol requirers in the three other species, the failure in *Aspergillus* seems to be of some interest.

Two groups of auxotrophs in *Aspergillus* show novel properties: the arginine requirers and the nicotinic acid requirers. Twenty-four mutants responding to arginine or to related compounds have been obtained (Table 25). Citrulline is ineffective in all strains, whether they

TABLE 25

Growth Response of 24 Mutants Requiring Arginine or Related Substances

No.	*Arginine*	*Ornithine*	*Proline*	*Glutamic Acid*
1	+	−	−	−
5	+	+	−	−
14	+	+	+	−
1	+	+	+	+
2	−	−	+	−
1	−	+	−	−
Tot. 24				

None responds to citrulline. Lysine inhibits competitively the utilization of arginine or ornithine but has a sparing effect on proline.

DL-α-Amino-δ-OH-valeric acid (kindly supplied by Dr. J.R.S. Fincham) has shown no growth-promoting activity when tested on one proline-requiring strain, on two arginine/ornithine/proline-requiring strains, on three arginine/ornithine-requiring strains, and on the one arginine-requiring strain. It has an inhibitory effect, not competitive, on the utilization of proline by the proline-requiring strain.

are able to utilize ornithine or proline or not. Other sources of guanidino and ureido groups are also ineffective (e.g., guanidino acetic acid, creatine, etc.) alone or in combination with aspartic acid. Most of the strains have been tested for interactions with lysine; lysine inhibits competitively growth on exogeneous arginine or ornithine, with complete inhibition at molar ratios of about 2:1. It has, however, a striking sparing effect (Fig. 21) on exogeneous proline, also for a strain responding

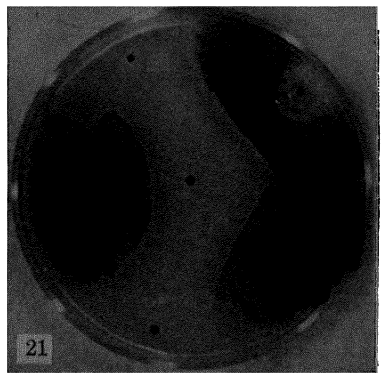


FIG. 21. Auxanography of an arginine/ornithine/proline-requiring strain. Spots clockwise from top: glutamic acid, arginine, ornithine, citrulline, proline; in the center: lysine. Note inhibition by lysine of growth on arginine or ornithine and sparing effect of lysine on proline.

only to proline. α-Amino-δ-OH-valeric acid is ineffective.

Eight of the ten lysine-requiring strains have been tested for response to α-aminoadipic acid, kindly supplied by Dr. Neuberger, and to α, ε-diaminopimelic acid, kindly supplied by Mrs. Work: neither is effective. The same strains are competitively inhibited by arginine and by ornithine similarly to what Doermann (1944) found in *Neurospora.*

By crossing, a recombinant has been obtained which requires both arginine/ornithine and lysine; it grows only within very finely balanced limits of concentrations of arginine and lysine.

Another interesting group of nine mutants obtained in *Aspergillus,* and apparently not yet described in other molds, responds to anthranilic acid, or nicotinic acid. Their growth responses are summarized in Table 26. They are essentially of two kinds: those which respond to anthranilic acid and 3-OH-anthranilic acid, but not to compounds in the tryptophan pathway; and those which, like the *Neurospora* mutants, respond to the whole series. It is significant that all these mutants are "adaptable"; i.e., they show a lag phase of several days but will eventually

TABLE 26

Growth Responses of 9 Mutants Requiring Nicotinic Acid or Related Substances

	Mutants	
	nic$_1$, *nic*$_3$, *nic*$_5$, *nic*$_9$	*nic*$_2$, *nic*$_4$, *nic*$_6$, *nic*$_7$,
Anthranilic acid	+	+
Indole	+	—
DL-Tryptophan	+	—
3-OH-Tryptophan	—	—
L-Kynurenine sulphate	+	—
3-OH-kynurenine	+	+
3-OH-anthranilic acid	+	+
Quinolinic acid	+	+
Nicotinic acid	+	+

All the strains grow without supplement after a lag of 3 to 4 days DL-phenylalanine delays this adaptation, which is not transmitted through the conidia. The efficiency of DL-tryptophan for the four strains responding to it is about 1/200 of that of nicotinic acid.

Dr. Neuberger, Dr. Weidel and Dr. Thorpe have kindly supplied the above 3-OH analogues.

grow at an almost normal rate without the growth factor. This adaptation is not transmitted through the conidia.

These results suggest (Pontecorvo, 1950) that in *Aspergillus*, besides the route of biosynthesis of nicotinic acid *via* tryptophan, there must be an alternative short-cut from anthranilic acid to 3-OH-anthranilic acid.

VII. Diploids and Mitotic Recombination

by G. Pontecorvo and J. A. Roper

1. *Isolation and Properties of Heterozygous Diploids*

Attempts at obtaining diploid nuclei in the vegetative cells of filamentous fungi have been made repeatedly (review Beneke and Wilson, 1950). Most were based on the use of polyploidogenic agents and aimed at doubling the chromosome number of individual nuclei. In no case, however, was crucial genetic or cytological evidence of polyploidy obtained, though Sansome with *Penicillium* (1949) and with *Neurospora* (1950) went farthest in this direction. In the light of what will be discussed here, it seems also possible that certain results of Quintanilha (1938) and Papazian (1950), i.e., the occasional occurrence of recombinant genotypes in heterokaryons, may well be due to formation of diploid nuclei followed by mitotic recombination (section VII-2).

The technique with which diploids were obtained in *A. nidulans* (Roper, 1952) was based on a different approach: instead of looking for *diploid nuclei originated from individual haploid nuclei the chromosome complement of which had been doubled,* the attempt was made to obtain them *as a consequence of fusions between haploid nuclei.* The two haploids could then be different in genetic markers and the resulting diploid heterozygous. Thus heterozygosis itself could be used for detecting and isolating the diploid strains, at the same time making sure that their nuclei were actually diploid.

The technique based on this approach has been fully successful in *A. nidulans* (Roper, 1952) and has been applied to *A. niger* (Pontecorvo, 1952a), where sexual reproduction does not occur. It is now being applied to other imperfect fungi. An unexpected consequence of diploidy in the vegetative cells of both these species worked out to be the high frequency with which segregation and recombination occur at mitosis, a fact of far-reaching theoretical and practical interest (section VII-2).

Roper's technique in its present routine version is as follows. The conidia of a balanced heterokaryon, when plated on non-supplemented medium, give origin to no colonies at all or to rare delayed heterokaryons arising from new anastomoses. If, however, by fusion between nuclei, *one of each parental kind,* heterozygous diploid nuclei have arisen, the conidia carrying these will give origin to colonies usually able to grow on non-supplemented medium. The use of color markers makes the isolation of diploids even easier: e.g., if the balanced heterokaryon is between a white and yellow strain, the diploid will be green, besides being able to grow on a medium which is inadequate for the parent strains. When using color markers, it is not even necessary to plate the conidia; occasionally a green sector, or small patch of mycelium, arises in a growing heterokaryon and a green (diploid) strain can be established from it. A proportion of our diploids has been isolated in this last way (Table 27).

To obtain the diploids, Roper (1952) treated with *d*-camphor vapor the balanced heterokaryon, on the reasonable assumption that it might stimulate either the coalescence of pairs of resting, or prophase nuclei next to one another within a heterokaryotic hypha, or the coalescence of pairs of spindles in metaphase or anaphase. However, we know now that very rarely heterozygous diploid nuclei do arise in the hyphae even without camphor treatment (Table 28). It remains to be seen whether camphor increases the frequency of fusions, or selects the diploids, or simply helps them to become established by a dilution effect, which might help the rare diploid hyphae to "escape" from the meshes of the heterokaryon. In the same way (section V-1), dilution by teas-

TABLE 27

Heterozygous Diploids Obtained in *A. nidulans* and in *A. niger*

	How obtained	*How isolated*
A. nidulans		
$\frac{w\ ad_1\ Y\ BI_2\ bi_1\quad THI}{W\ AD_1\ y\ bi_2\ BI_1\quad thi}$	Camphor	Sector
$\frac{w_a Y\ BI_1\quad lys\quad orn_1}{W\ y\ bi_1\quad LYS\quad ORN_1}$	Camphor	Sector
$\frac{w_a\ AD_2\ Y\quad lys}{W\ ad_2\ y\quad LYS}$	Camphor	Plating
$\frac{w_a\ AD_2\ Y\ BI_1\quad lys}{W\ ad_2\ y\ bi_1\quad LYS}$	Spontaneous	Sector
$\frac{w_a\ Y\ BI_1\quad LYS\quad panto\quad orn_1}{W\ y\ bi_1\quad lys\quad PANTO\quad ORN_1}$	Camphor	Plating
$\frac{w_n\ paba_1\quad AD_2\ Y\ bi_1}{W\ PABA_1\ ad_2\ y\ BI_1}$	Spontaneous	Sector
$\frac{y\ bi_2\ BI_1\ AD_4\quad thi}{Y\ BI_2\ bi_1\ ad_4\quad THI}$	Camphor	Plating
$\frac{w\ ad_1\ y\quad LYS}{w_a\ AD_1\ Y\quad lys}$	Spontaneous	Plating
A. niger *		
(A1) Fawn, aneurin requiring / (F92) Olive, histidine requiring	Spontaneous	Sector
(A33) Fawn, arginine requiring / (F104) Olive, casein digest requiring	Spontaneous	Sector
(A35) Fawn, guanosine requiring / (F92) Olive, histidine requiring	Spontaneous	Sector

* The code numbers of the two strains from which the diploid is derived are in brackets above and below the fraction sign.

ing out the mycelium is known to help rare heterokaryotic hyphae to escape from the parental mycelium when an attempt is made to form balanced heterokaryons. That the dilution is drastic is shown by the fact that, after camphor treatment of a colony, growth restarts from isolated points along the hyphal tips and in the body of the colony; only a small proportion of the mycelium seems to survive the treatment.

TABLE 28

Frequency of Heterozygous Diploid Conidia in Balanced Heterokaryons

		Conidia plated on selective medium (no.)	*Diploid colonies* (no.)	*Diploid colonies* (per 10^6 conidia)
A. nidulans				
y bi$_1$ *BI*$_1$ *AD*$_4$ *thi* + *Y BI*$_1$ *bi*$_1$ *ad*$_4$ *THI*	Camphor	4,200,000	1141	271
	Control	487,000,000	122	0.25
w$_a$ *AD*$_2$ *Y lys* + *W ad*$_2$ *y LYS*	Camphor	12,000,000	41	3.4
A. niger				
A35 + F92	Camphor	1,760,000	8	4.6
	Control	8,700,000	3	0.4

The ease with which the technique yields diploids is shown in Table 28. So far, with the eight balanced heterokaryons of *A. nidulans* to which the technique has been applied, the diploid has been obtained in every case at the first attempt. The same success has been obtained in *A. niger* in all three combinations attempted.

The question of how, as a rare accident, two nuclei happen to fuse in these filamentous fungi might have a general interest. It is known that binucleate cells occur often in animals, including mammals, especially in the male gonads. Usually the two nuclei divide synchronously but form separate spindles; one case, however, (Pontecorvo, 1943, p. 34), has been recorded of almost certain formation of a fused spindle. If this were the mechanism operating in the formation of our diploids, it would not be so interesting. But if the fusion of the nuclei took place otherwise than by coalescence of two spindles, then we would have a valuable tool for investigating substances and conditions which affect the fusion of two nuclei. We may well have a new way of approaching the problem of regulated nuclear fusion as it occurs at fertilization in all organisms with a sexual cycle.

The crucial point in Roper's technique is the ability to select the diploid conidia which, even after camphor treatment, form an exceedingly small proportion of the total (Table 28), and to recognize the diploid strains by their phenotype. The selection is based on the assumption that most nutritional requirements which behave as recessives

in heterokaryons would behave as recessives in diploids as well, and therefore a diploid heterozygous for two requirements would show neither. This has proved to be the case for all nine requirements tested so far in *A. nidulans* (Table 29) and for all five tested in *A. niger*. There

TABLE 29

Dominance in Heterokaryons and in Heterozygotes of *A. nidulans*

	Phenotypes			*Phenotypes*	
	Heterokaryon	Heterozygote	Heterokaryon		Heterozygote
$\frac{y}{Y}$	Yellow or green	Green	Pseudo-alleles		
$\frac{w}{W}$	White or colored	Colored			
$\frac{w}{w_a}$	White	White	$\frac{bi_1\ BI_2}{BI_1\ BI_2}$	Non-requirer	Non-requirer
$\frac{lys}{LYS}$	Non-requirer	Non-requirer	$\frac{BI_1\ bi_2}{BI_1\ BI_2}$	Non-requirer	?
$\frac{orn_1}{ORN_1}$	Non-requirer	Non-requirer	$\frac{bi_1\ BI_2}{BI_1\ bi_2}$	Requirer	Requirer
$\frac{ad_1}{AD_1}$	Non-requirer	Non-requirer			
$\frac{ad_2}{AD_2}$	Non-requirer	Non-requirer			
$\frac{ad_4}{AD_4}$	Non-requirer	Non-requirer			
$\frac{panto}{PANTO}$	Non-requirer	Non-requirer			

is an exception which confirms the rule. In the case of the pseudo-alleles bi_1 and bi_2 (section VI-3), the phenotype of the diploid doubly heterozygous *in repulsion* is biotin-requiring, like that of the corresponding heterokaryon.

The use of conidial differences as additional markers to identify diploids was based again on the assumption that the mutant alleles would be recessive. There was, in this case, no information to go by from the heterokaryons because both color markers used in *A. nidulans* (*y* and *w*) are autonomous in action; the color of the conidia formed by heterokaryons is determined by the type of nucleus segregated in each chain. However, the guess that the two mutant alleles would be recessive in the double heterozygote was correct; the conidia of heterozygotes of consti-

tion *W/w Y/y* are green. On the other hand, the color markers used in *A. niger* (fawn and olive, the wild type being black), are not autonomous; i.e., each heterokaryotic head has chains uniform in color and this color varies from one head to another, ranging from fawn to olive through black, presumably according to the nuclear ratios; the diploids have uniformly black heads.

Apart from being phenotypically as expected, the heterozygous diploids of *A. nidulans* are unequivocably identifiable also on the basis of other properties: diameter of conidia (Table 30); meiosis (section II-3); low viability of the ascospores; degeneration of the majority of asci after meiosis; presence of 16-spore asci; segregation in the asci; mitotic segregation and recombination.

TABLE 30

Diameter of Conidia of Haploid and Diploid Strains of *A. nidulans* and *A. niger*

	Conidia measured (no.)	*Mean diameter* (μ)
A. nidulans		
Diploids		
$\frac{w\ ad_1\ Y\ BI_2\ bi_1}{W\ AD_1\ y\ bi_2\ BI_1}\ \frac{THI}{thi}$	55	4.3
$\frac{w_a\ AD_2\ Y\ BI}{W\ ad_2\ y\ bi}\ \frac{lys}{LYS}$	82	3.8
$\frac{w_a\ AD_2\ y}{W\ ad_2\ y}\ \frac{lys}{LYS}$ (yellow conidia)*	79	3.9
Haploids		
Wild type	85	3.1
y (yellow conidia)	89	3.2
A. niger		
Diploids		
A1/F92 (black conidia)	53	5.4
A35/F92 (black conidia)	58	5.3
II/33d (olive conidia)†	62	5.5
Haploids		
Wild type (black conidia)	52	4.3
680F (olive conidia)	64	4.7

* Mitotic recombinant from $\frac{w_a\ AD_2\ Y}{W\ ad_2\ y}\ \frac{lys}{LYS}$

† Mitotic recombinant from A1/F92.

Measurements with eyepiece micrometer of pieces of chains of conidia in suspension.

As to the diameter of the conidia, the data of Table 30 need no comment. About meiosis, the little which can be said is that it is certainly tetraploid (section II-3). As to the low viability of the ascospores (less than 1 in 50) and the degeneration of the majority of the asci after meiosis, they were expected, of course. However, they are far too severe to be accounted for entirely by the formation of unbalanced products as a consequence of tetraploid meiosis. These disturbances may be in part an additional manifestation of whatever causes the formation of 16-spore asci. Crosses between haploids and diploids will probably tell whether or not a "maternal effect" of the diploid mycelium plays a part.

As to the 16-spore asci (Fig. 22), they came, of course, as a surprise;

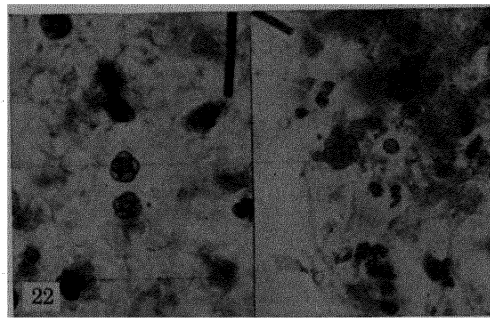


FIG. 22. *Left:* 8-spore asci of a haploid strain. *Right:* 16-spore ascus of a diploid strain, with debris of many others degenerating.

so far they have never been found in haploid strains and they have been found in every diploid strain. In the diploid strains, individual perithecia with 16-spore asci carry these almost exclusively with an occasional 8-spore ascus and rare asci with any number of spores from a single gigantic one to 16. In certain diploids, however, one finds side by side typical perithecia, as those just described, and perithecia packed with asci, all 8-spored, as in haploid strains. The analysis of these perithecia has not started. If the asci arise parthenogenetically in a diploid heterozygous for known markers, these asci should show ordinary diplo-haploid segregation; if they arise from patches of mycelium which have reverted to the haploid condition, usually they should not segregate at all. It will have to be seen whether or not the formation of perithecia with 8-spore asci in certain diploids is in any way connected with relative heterothallism (section V-4).

As to segregation in the 16-spore asci, the low viability of the ascospores is a serious obstacle. Ascus dissection is clearly out of the question, but random sampling of ascospores is possible. We have so far

carried out only qualitative analysis, mainly to ascertain whether a diploid was segregating for the markers for which it was supposed to be heterozygous. This has usually proved to be the case, with two exceptions: a diploid originally heterozygous for *lys* lost this mutant allele within the course of a few sub-cultures, and a diploid supposed to be heterozygous for *panto* did not segregate for it. Evidently *lys*, and presumably *panto*, reduce the fitness in heterozygous condition and are therefore supplanted by their normal alleles as soon as mitotic recombination (see below) produces homozygotes for these.

Of the ascospores produced by diploids, some are haploid and some diploid. To ascertain whether triploid ascospores also are produced, as expected, would require a special type of genetic analysis or substantial improvements in the cytological examination of meiosis; neither has been attempted.

As to mitotic segregation and recombination, they occur in every diploid strain of *A. nidulans* and *A. niger* so-far examined, i.e., in the eleven strains listed in Table 27 and in a large number of strains derived from the above.

Prompted by the classical work of Stern (1936) on somatic crossing-over in *Drosophila* we deliberately looked for the occurrence of mitotic segregation and recombination as soon as diploids were obtained: we did not expect however, to find this process occurring as regularly and frequently as it does.

2. *Mitotic Recombination and Its Use in Genetic Analysis*

Mitotic segregation manifests itself in heterozygous diploids of *A. nidulans* in the following way. Colonies of a green-spore diploid strain (heterozygous for *w Y/W y* and purified by single conidium micromanipulation) started from point inoculum on agar medium show a number of single heads, or spots with a few heads or small sectors bearing white or yellow conidia (Fig. 23). Alternatively, plating of green conidia from such a strain yields green colonies, most of which show a few single heads, small patches of heads, or sectors with white or yellow conidia.

Isolation and purification, by single conidium micromanipulation, of yellow segregants ("first order" segregants) gives strains most of which again produce spots with white heads ("second order" segregants). The converse, of course, cannot be tested because of the epistasis of *w*. The segregants, both of first and second order, are usually still diploid, as shown by their producing 16-spore asci and segregating *via* ascospores for other markers for which the parent strain was heterozygous. In some cases the diameter of the conidia of the segregants has been measured and found to fall within the diploid range (Table 30).

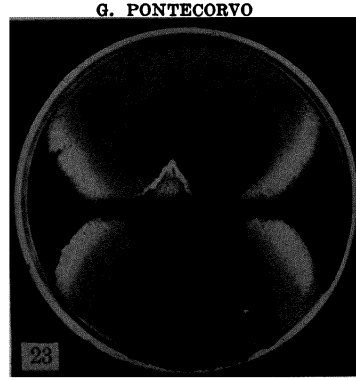


FIG. 23. Colonies of a diploid heterozygous $\frac{w\ Y}{W\ y}$ (green) showing yellow or white spots and one white sector, due to mitotic recombination.

Besides segregation, the process may involve also recombination. For instance, a diploid derived from haploids *W ad₂ y LYS* and *w₀ AD₂ Y lys* yielded yellow segregants, some of which were lysine-requiring, and white segregants, some of which adenine-requiring (Table 31), besides other types of recombinants. Mitotic recombination of this kind has been obtained consistently from all diploids analyzed both in *A. nidulans* and *niger*. The problems raised by the occurrence of mitotic segregation and recombination in the vegetative cells, and the possibilities opened by it, are somewhat overwhelming. We shall try to visualize some and illustrate the kind of preliminary attack opened on a few.

In the first place, there is the question of the rate of occurrence and of the regularity of the process. The difficulty here is the same as in the measurement of mutation rates; i.e., the clonal distribution of the segregant nuclei. If we sample the conidia of a heterozygous diploid, the proportion among them of homozygotes for any one marker would permit an estimation of the segregation rate only if it were known that the segregant nuclei did not multiply differentially: in fact, it is certain that in most cases they do. In one sample of plated conidia from each of four diploids, all heterozygous at the *w/W* locus, the following proportions of homozygotes for the recessive were obtained:

From diploid: $\frac{w_0\ AD_2\ Y\ BI}{W\ ad_2\ y\ bi}\ \frac{lys}{LYS}$: 3 out of 763

From diploid: $\frac{w_0\ AD_2\ y}{W\ ad_2\ y}\ \frac{lys}{LYS}$: 0 out of 70

From diploid: $\frac{w_0\ AD_2\ Y}{W\ ad_2\ y}\ \frac{lys}{LYS}$: 0 out of 236

From diploid: $\frac{w_n\ paba\ AD_2\ Y\ bi}{W\ PABA\ ad_2\ y\ BI}$: 3 out of 531

TABLE 31

An Example of Mitotic Recombination

Diploid, green conidia prototroph, purified by single conidium micromanipulation:

$$\frac{w_+ AD_+ Y}{W\ ad_+ y} \quad \frac{lys}{LYS}$$

→ 16-spore asci, segregating for yellow, white, and green and for the requirements.

↓

"First order" mitotic segregants and recombinants isolated by inspection

White, requiring:				Yellow, requiring:			
Adenine	Adenine and lysine	Neither	Lysine	Adenine	Adenine and lysine	Neither	Lysine
2	1	7	0	6	1	1	0

Purified by single conidium micromanipulation → 5 out of 7 give "second-order" white mitotic recombinants

Purified by single conidium micromanipulation → 16-spore asci segregating for lysine and adenine requirements, for yellow and white

Purified by single conidium micromanipulation → "Second order" mitotic segregation for lysine and adenine requirements, for yellow and white

Taken at their face value, these results indicate a proportion of homozygotes of about 1 in 300. Clearly, mitotic segregation is not a very rare event. Furthermore, 20 colonies from plating of conidia of the first of the above strains were carefully scanned when about 1 cm. in diameter: a total of 21 white spots and 17 yellow spots were identified in them. This gives roughly an average of one white or yellow visible segregant spot per 35 sq. mm. of colony surface. But to be visible, a segregant spot must include at least one head; for each such spot identified there must be many hundreds carrying only one or few white or yellow conidia which escape detection.

A second problem is that of the mechanism of mitotic segregation and recombination. Any interpretation of the results must account for the fact that the majority of segregants are still diploid and heterozygous for some of the markers present in the parent strain. Almost certainly, however, somatic reduction to the haploid condition, with or without recombination between non-homologous chromosomes, also occurs. For instance, a proportion of biotin-independent mitotic recombinants from diploid *y* bi_2 BI_1 AD_4 *thi/Y* BI_2 bi_1 ad_4 *THI* (see section VI-3) were probably haploid because: (1) they failed to segregate further, (2) they abundantly produced 8-spore asci only; and (3) they had conidia of haploid size. If confirmed and extended, results of this kind will provide excellent material for testing Huskins' ideas on reduction in somatic tissues. Haploidization, however, could not possibly account for more than a minor fraction of the segregants and recombinants obtained, which, it must be emphasized again, are mainly diploid.

As an interpretation of the results, we are therefore left with the theory of somatic crossing-over as developed by Stern (1936) for *Drosophila* and applied by Demerec (1936) to the investigation of cell-localized lethal gene action. The theory is that somatic crossing-over occurs in a small proportion of diploid nuclei in mitosis, that it takes place at the four-strand stage and that, barring non-disjunction and multiple crossovers, it must inevitably lead to segregation at heterozygous loci distal to any point of exchange.

In trying to test the theory of somatic crossing-over in *A. nidulans*, we have one marked advantage and one minor limitation. The former is that we can isolate segregant cells and analyze their genotypes, whereas the work in *Drosophila* is limited almost exclusively to the identification of the phenotypes of these cells. True, nuclei originated from mitotic crossing-over have been recovered in certain cases in the pollen in higher plants and in the sperm in *Drosophila*, but this is not as satisfactory as the possibility of recovery from cells only a few *mitotic* divisions removed from the one in which the process took place. The minor limita-

tion is that in *A. nidulans* we do not have a pair of closely linked markers, both identifiable by inspection, such as the classical *y* used by Stern and by Demerec in *Drosophila*.

The results to date with *A. nidulans* (and with *A. niger* within the limits imposed by the absence of cross-checking *via* sexual reproduction) seem to substantiate fully Stern's theory of somatic crossing-over. However, we have not yet recovered in one and the same diploid nucleus the two complementary products of somatic crossing-over. Until this is done, we feel that only tentatively can mitotic recombination be taken to result from a crossing-over-like process.

As markers, we have used more extensively the two color loci W/w_a and Y/y, two "nutritional" loci on the same chromosome (AD_2/ad_2 and BI_1/bi_1), and the LYS/lys locus, which segregates independently of all

TABLE 32

Mitotic Recombinants from Diploid Strains of *A. nidulans*

	Diploids Green	*Recombinants* * White	Yellow	*Total*
1.	$\frac{w_a\ Y\ BI_1}{W\ y\ bi_1}$ †	bi_1 BI_1 5 6 11	bi_1 BI_1 20 0 20	31
2.	$\frac{w_a\ AD_2\ Y}{W\ ad_2\ y}\ \frac{lys}{LYS}$	$\frac{ad_2}{lys}$ $\frac{}{LYS}$ $\frac{AD_2}{lys}$ $\frac{}{LYS}$ 1 4 1 46 5 47 52	$\frac{ad_2}{lys}$ $\frac{}{LYS}$ $\frac{AD_2}{lys}$ $\frac{}{LYS}$ 2 13 0 1 15 1 16	68
3.	$\frac{w_a\ AD_2\ Y\ BI_1}{W\ ad_2\ y\ bi_1}\ \frac{lys}{LYS}$	$\frac{ad_2\ bi_1}{lys\ LYS}$ $\frac{AD_2\ BI_1}{lys\ LYS}$ 3 7 11 10 10 21 31	$\frac{ad_2\ bi_1}{lys\ LYS}$ $\frac{AD_2\ BI_1}{lys\ LYS}$ 7 13 0 0 20 0 20	51

* The symbols of the alleles are used to indicate the phenotypes of the recombinants.

† This strain, originally heterozygous for *LYS/lys*, *ORN/orn*, and *PANTO/panto*, was mixed in these respects when analyzed. The sample of white and yellow is not a random sample in this case.

the others. Experiments with seven more markers (five of which are in the linkage group just mentioned) have not yet gone far enough to be reported here.

Since mitotic segregants constitute only a small proportion of a growing colony, they have to be selected out. The selection is visual as to the conidial color markers, but enrichment is necessary as to the nutritional markers. A technique to this end is now available (Forbes, 1952); it is analogous to the penicillin technique for isolating bacterial mutants since it is based on the preferential killing by SO_2 of prototrophs pregerminated in minimal medium, where auxotrophs do not germinate.

With three strains the results of visual selection of mitotic segregants differing from the green parent, heterozygous diploid (W/w_a Y/y), in being white or yellow, are shown in Table 32. For strains 1 and 2 the selection of color segregants was carried out exclusively in large colonies grown from point inoculum of conidia by picking out of each segregant spot a single head (white or yellow). For strain 3 (see also Table 33), some of the color segregants were also picked from small colonies obtained by plating conidia. In this case, picking not more than one segregant per colony ensures that the same segregant clone is not isolated more than once. In view of the methods of selection, the proportions of yellow to white segregants tested are not representative of the actual proportions in the colonies.

Keeping in mind (section V-1, 2) that the loci AD_2/ad_2, Y/y, and BI_1/bi_1 are closely linked and in this order, that the W/w_a locus is probably on the same chromosome, but more than 50 units beyond AD_2/ad_2 (Table 18), and that the locus LYS/lys segregates independently of these four, the following points of interest arise from Tables 32 and 33.

1. Simultaneous segregation at more than one locus does not occur at random; the alleles in coupling at closely linked loci tend to segregate together. Thus all the yellow from diploid 1 are also homozygous for bi_1; all but one of the yellow from diploid 2 are also homozygous for ad_2; and all the yellow from diploid 3 are also homozygous for ad_2 and bi_1. Homozygosis at the freely segregating locus LYS/lys occurs in 2 out of 52 white and 2 out of 16 yellow in diploid 2, and in 14 out of 31 white and 7 out of 20 yellow in diploid 3. Simultaneous homozygosis for w_a and the recessive alleles in the y region, which if on the same chromosome is more than 50 units away, occurs in 5 out of 11 white in 1, in 5 out of 47 in 2 and in 10 out of 31 in 3. It is to be noted that w_a is in repulsion relative to these other recessive alleles in all three cases.

2. Simultaneous segregation at two non-linked loci occurs far in excess of what would be expected from the frequency of segregation at each

TABLE 33 *

Mitotic Recombinants from Diploid:

$$\frac{w_a\ AD_2\ Y\ BI_1}{W\ ad_2\ y\ bi_1} \quad \frac{lys}{LYS}$$

	$AD_2\ BI_1$		$ad_2\ bi_1$		
1. Unselected **	*lys*	*LYS*	*lys*	*LYS*	*Total*
Green	0	240	0	0	240
White	0	0	0	0	0
Yellow	0	0	0	0	0
Total	0	240	0	0	240
2. Selected only for color †					
White	11	10	3	7	31
Yellow	0	0	7	13	20
Total	11	10	10	20	51
3. Selected only for requirements ‡					
Green	2	195	0	0	197
White	0	1	0	0	1
Yellow	0	0	1	1	2
Total	2	196	1	1	200
4. Selected for color and requirements ‖					
White	2	0	1	3	6
Yellow	0	0	0	15	15
Total	2	0	1	18	21

* The symbols of the alleles are used to indicate the phenotypes of the recombinants.

** Random sample of 240 colonies out of 763 (of which 3 white, no yellow) from plated conidia.

† Some by isolation of heads from mosaic spots; some from white colonies obtained in 1.

‡ Random sample of 200 colonies out of 1716 (of which 7 were white and 17 yellow) from pregerminated conidia treated with SO_2

‖ The white and the yellow out of the 1516 colonies not included in the random sample in 3.

locus. For instance, simultaneous segregation for *lys* occurs in 21 out of 119 yellow or white in diploids 2 and 3. We know (p. 226, and Table 33) that homozygosis for *y* or *lys* did not occur once among 236 conidia of diploid 2 and among 763 conidia of diploid 3. Among the latter, homozygosis for w_a occurred three times. Yet we find now that about 1 in 6 of the color segregants are also homozygous for *lys*. Even though the data are very limited, the converse is also true: Table 33 shows that in selecting for *lys*, 1 out of the 3 *lys* obtained was yellow. Clearly (Pontecorvo, 1952a), somatic segregation occurs in a small pro-

portion of nuclei, but in those in which it occurs it tends to involve more than one chromosome and (if W/w were really on the same chromosome as Y/y) more than one region of a chromosome.

3. If the tentative location of W/w_a on the same chromosome as AD_2/ad_2, Y/y, and BI_1/bi_1 were confirmed, the results of Table 33 would leave no doubt as to recombination of linked genes. All the cases in which homozygosis for w_a and for one or more of the recessive alleles in the ad_2–bi_1 region occurred would imply multiple exchanges in one chromosome. Precisely, if the centromere were either beyond W/w_a or beyond BI_1/bi_1, three exchanges involving chromatids 1,3 and 4,2; 2,4 would be necessary. If the centromere were between W/w_a and AD_2/ad_2, two disparate exchanges would be required, involving one arm each. Barring these as examples of crossing-over, there is only one other case in Table 33 of recombination between unquestionably linked loci, i.e., the yellow adenine-independent recombinant from diploid 2. This recombinant was fully tested (Table 31) and its genotype is as expected. However, a diploid of this genotype could also have arisen by $Y \rightarrow y$ mutation in the parent strain.*

As mentioned before, a technique by Forbes (1952) makes it possible to select the auxotrophic segregants from the mass of parental prototrophic conidia. This technique is still being improved, but the results of Table 33 (obtained in collaboration with Mr. E. C. Forbes) show that it is no longer impossible to isolate these segregants: a yield of 4 auxotrophs out of 200 tested colonies was obtained in a sample enriched by this technique as compared with 0 out of 240 in the untreated control. The number of auxotrophs obtained is unfortunately too small to expect any recombinant for the linked markers among them. We hope to get these as soon as the technique will be perfected.

Other examples of what can be only *either* mitotic crossing-over between closely linked markers *or* mutation have been obtained: e.g., biotin-requiring green from diploids of constitution $\frac{Y\ BI_1}{y\ bi_1}$, and biotin and adenine-independent green from diploids of constitution $\frac{y\ bi_2\ BI_1\ AD_4}{Y\ BI_2\ bi_1\ ad_4}$.

* Unquestionable evidence of mitotic recombination between loci which are certainly linked has now been obtained. From a diploid with $paba_1$ and y in coupling, out of 51 yellow recombinants visually selected, 39 were $paba_1$ and 12 $PABA_1$. The two loci show 15-20% recombination at meiosis. These results suggest that the $PABA_1/paba_1$ locus is proximal and that, in terms of *mitotic* recombination, the 'distance' between the centromere and this locus is about three times that between this locus and Y/y.

Though every detail of our results is in agreement with the theory of mitotic crossing-over, we shall not take it as proven until we can recover the complementary products of one exchange. Work to this end is in progress. It may be mentioned, for instance, that in *A. niger* the diploid doubly heterozygous for fawn and olive has conidia considerably lighter than the wild type. Often in a growing colony near to spots segregant for fawn, spots darker than the diploid are found; when isolated and purified, these give origin to strains segregating for olive but not for fawn. We deduce that the darker types are "twin" products, homozygous for the wild-type allele of fawn. Though we have no such convenient situation in *A. nidulans,* this finding suggests that in the vicinity of a recessive segregant spot detectable by inspection we should find the corresponding dominant homozygote not distinguishable by inspection from the heterozygous parent.

The fact that at mitosis the alleles of closely linked loci tend to segregate together and to recombine as a group with other non-linked loci makes it possible to carry out genetic analysis *via* mitotic recombination. In *A. nidulans,* for instance, out of a total of 101 segregants tested which could have shown recombination between two of the three closely linked loci in the ad_2–bi_1 region, only one showed it. On the other hand, between 7 and 50% of those which could have shown recombination between non-linked loci did show it. In a species without sexual reproduction, results of this kind can be used to detect linkage groups.

The analysis, however, may go further. If mitotic crossing-over will be proved to be the cause of segregation (and all seems to point this way), then we shall be able to locate the centromere in a sequence of linked genes as the point at which the *direction* of linked segregation is inverted. If we have the sequence a-b-c-centromere-d-e-f, homozygosis for d will usually carry with it homozygosis for e and f, but not so often for a, b or c. On the other hand, homozygosis for c will carry with it homozygosis for b and a, but not so often for d, e, or f. On the basis of this reasoning and of certain results with mitotic recombination in *A. nidulans,* we have tentatively located the centromere in the *bi* linkage group between ad_1 and $paba_1$. Unfortunately this is a region where an independent check by ascus analysis is not easy.

The production of heterozygous diploids and the use of mitotic recombination has opened the way to genetic analysis and "breeding" in asexual species of filamentous fungi. The first results in applying our technique to *A. niger* show that this way is relatively easy and extremely promising both in fundamental research and practical applications.

VIII. Summary and Conclusions

The work reported in this paper is obviously spadework in extension rather than in depth. In the process of this spadework, results of general implication have been obtained, and some have been followed up even if outside our main line of interest. A summary of the salient points will make the general picture clearer.

1. The genetic analysis of a homothallic fungus has been carried out for the first time and shown to be perfectly manageable.

2. The formal genetics of *A. nidulans* has gone as far as the identification of at least 18 loci, 5 of which, and probably 7, are in one linkage group.

3. Two of the regions investigated for pseudo-allelism (the *bi* and the *paba* loci) show it. A third investigated region (ad_1 and ad_3) is not of this kind. Taken together with results in organisms ranging from *Drosophila* to maize, where pseudo-allelism has been found almost invariably whenever looked for, this may suggest that recombination between some of the members of one allelic series is the rule rather than the exception.

4. The detailed investigation of the asci of individual perithecia has led to the discovery of relative heterothallism, i.e., the fact that a self-fertile strain may take part preferentially in outcrossing, if given the opportunity.

5. The biochemical genetics of *A. nidulans*, though similar to that of *Neurospora* and *Ophiostoma*, has revealed certain interesting differences in detail: e.g., the inability of citrulline to replace ornithine for strains responding to ornithine or arginine; the inability of tryptophan to replace anthranilic acid for certain strains responding to anthranilic acid or nicotinic acid; the competitive inhibition by lysine of exogenous arginine or ornithine and its sparing effect on exogenous proline; etc.

6. The production of strains carrying in their vegetative cells diploid nuclei heterozygous for known markers has opened the way to a more thorough study of somatic segregation and recombination than could be possible in higher animals or plants. Genetic recombination can now be obtained outside the sexual cycle, and this has already been done with the asexual species *A. niger*.

7. The comparative physiological genetics of heterozygotes and heterokaryons in one and the same species is now possible. This was one of the missing links in the study of the relations between spatial distribution and action of genes which prompted the present work (Pontecorvo, 1947, 1950, 1952b, 1952c).

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Genetics of the Silkworm, *Bombyx mori* *

* Contributions from the National Institute of Genetics, Japan, No. 33.

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CONTENTS

I. Introduction

The domestic silkworm, *Bombyx mori,* is one of the best-suited animals for genetic research. The reasons are as follows. (*a*) It has many easily distinguishable characteristics in various stages of the life history —egg, larva, cocoon, pupa, and moth. (*b*) The eggs hatch out several times a year with artificial stimulation. Four generations can be reared without difficulty in southern Japan. A life cycle of this insect covers an average of 50 days; therefore, it is possible to observe seven generations a year, if there are proper accommodations for winter culture. (*c*) The raising of the silkworm is easy and amusing. It does not require much space or cost. (*d*) The female lays numerous (about 500) eggs, and the male is polygamous, so one male may, if necessary, be mated to eight females. (*e*) The silkworm is an economic insect, and its genetic investigation may have some practical applications.

As the pioneer in the field of genetics of the silkworm, I should like to mention the name of a French investigator, G. Coutagne (1902), though he is little known to geneticists. Although his results were published after the rediscovery of Mendel's papers, his work had been done entirely in pre-Mendelian era. Nevertheless, his description of results is wonderfully exact, clear, and interesting from the standpoint of Mendelism as was pointed out by Tanaka (1913a). He actually observed the law of complete as well as incomplete dominance in F_1, and phenotypic segregation in F_2 of monohybrids (in 3:1 ratio), dihybrids (9:3:3:1), and trihybrids (27:9:9:9:3:3:3:1). In certain cases, diheterozygotes were accidentally mated with double recessives, and the segregation of four phenotypes in equal numbers was observed. It was known to him that the white cocoon color of many races is inherited as a recessive, whereas that of Bagdad race is a dominant. Though he did not express

his experiments in Mendelian terms, his work should be highly esteemed.

The first author who investigated silkworm genetics from the Mendelian point of view is Toyama (1906), as is well known. He studied colors and other characters of the cocoon, larval markings, and egg colors (yolk colors, speaking strictly) and came to the conclusions that most of these characters are inherited in the Mendelian fashion. His theoretical analysis was somewhat peculiar, as shown by so-called "modified dihybrids" (cf. Tanaka, 1913a). An example of a gynandromorph whose origin had been exactly known was reported by him and attracted much attention in those days. Later he published papers on inheritance of the chocolate-colored larvae (first instar) (Toyama, 1909) and of the dominant white cocoon (Toyama, 1912a, 1913), proving that both of them strictly follow Mendelian laws. His most important contribution must be the discovery of the phenomenon of the maternal inheritance of certain egg colors and voltinism and the establishment of its Mendelian nature (Toyama, 1913).

Kellogg (1908) mentioned that larval markings and cocoon characters are inherited approximately after the Mendelian manner but that other characters are not. He adopted the term "individual idiosyncrasy" which Luther Burbank used for plants and invented a new phrase, "race or strain idiosyncrasy," to explain the varying dominance of the white cocoon color in different races. His results were criticized by Toyama (1909). Toyama (1913) also denied so-called non-Mendelian inheritance of voltinism advocated by McCracken (1908).

In the next year, when Morgan (1912) found a peculiar mode of linkage in *Drosophila* in which no crossing over occurs in the male, Tanaka (1913b), reported that crossing over does not take place in the female silkworm. These two remain as the only cases in which the occurrence of crossing-over is limited to the homogametic sex. Tanaka (1916, 1917, 1918, 1922, 1925, 1926) found, for the first time in the silkworm, multiple allelism, polymery, equistasis, sex linkage, non-disjunction, lethal factors, somatic mutations, and hereditary mosaicism. He observed three linkage groups and published the first chromosome map of the silkworm (Tanaka, 1927b).

Since 1916, Tanaka has described numerous natural mutations and mosaics obtained in his cultures and has bred several new strains from bilateral or one-fourth mosaics. In 1917, Tanaka got an artificial mutation which had some translucent areas of skin on the right side of the dorsal median line in a culture hatched from eggs of a pure non-translucent race kept in a cold storage and then centrifuged. He resumed the experiment in cooperation with one of his students, Hasimoto, on a more extended scale. The results (1928) were strikingly positive, and nu-

merous mosaics, gynandromorphs, malformations, and exceptions to the sex linkage were obtained.

This is a sketch of silkworm genetics in its early days. Since 1930, rapid strides were made, chiefly because of an increased number of students of genetics in Japan. Every field of experimental, cytological, physiological, biochemical, embryologic or developmental, and applied genetics was covered. Advances in the last twenty years will be discussed in the following pages.

1. *Revised Gene Symbols*

While this article was in press, certain changes in gene names and symbols were made by the Committee of Japan Science Council. All those that occur in the text were checked and revised in proof, but it was not possible to make corresponding changes in the figures. Therefore, a list of revised symbols that appear in chromosome maps and text figures is given below:

Former Symbol	New symbol
Es (*E*-small egg)	*Sme* (Dominant small egg)
Grl (Light gray)	*Lg* (Light gray)
E^{Cr} (Crescent C_7)	E^{Ds} (Double stars)
G-16 (*E16* gray egg)	*Gr-16* (*E16* gray egg)
m_3 or M_3 (Trimolting)	M^3 (Trimolting)
$+^m$ or $+^{M_4}$ (Tetramolting)	$+^M$ (Tetramolting)
m_5 (Pentamolting)	M^5 (Pentamolting)

II. Heritable Characters in the Silkworm

In this section, the names of characters, symbols of genes, and short descriptions of characteristics are given.

1. *Egg Characters*

a. *Egg Shapes.* The egg shape is entirely dependent on the shape of the chorion which is formed in the maternal body before fertilization.

(i). *Normal shape, +.* The egg is elliptic in circumference, slightly narrowed on the anterior end, where the micropyle is situated. The newly layed egg is oval in shape, but it becomes dorso-ventrally flattened with time, owing to the evaporation of the water content of the yolk, and a round depression appears on the center of the dorsal surface.

(ii). *Spindle shaped, sp.* The egg is long and narrow, pointed on both ends, dorsally convex. According to Takasaki (in press) there are three different genes which have very similar phenotypic effects but pro-

duce the normal shape when crossed with each other. Kei (1934) found four abnormal egg shapes in a Cantung race (Daizo), but their hereditary behavior has not been thoroughly worked out.

(iii). *Kidney shaped, ki.* The egg is kidney or bean shaped, lethal. The embryo develops only ectodermal organs, such as skin, mouth parts,

FIG. 1. Lethal embryo from the kidney-shaped egg (from Suzuki, unpublished).

bristles, etc., but no other internal organs, and dies early within the egg. This egg shape is determined in the body of the mother with the genotype *ki/ki.* The fate of the embryo depends solely on the shape of the egg, or it is, in turn, controlled by the maternal genotype. The embryos formed within the kidney-shaped eggs inevitably die in early embryonal development, no matter if the embryonal genotypes are *ki/+* or *ki/ki.* Thus the lethality in this case might be considered as an example of maternal inheritance. On the other hand, if we assume that the development and non-development of definite germinal layers are predetermined in the egg cytoplasm before fertilization, the inheritance of the kidney lethal must be nothing else than an ordinary one.

(iv). *Giant egg, Ge.* The length and width of the normal egg being taken as 1, those of the giant egg are 1.26 and 1.11, respectively. There are some shrinkages on the dorsal surface. Though it resembles a tetraploid egg, it proved to be diploid cytologically. The hatched larva is

bigger and heavier than the normal; the female moth lays only a small number of eggs; sex linked (Aruga, 1943).

(v) *Small egg, sm.* A natural mutant was obtained by Tanaka (1932), the length and width of which measure about 75% of the normal. Although the number of eggs in the female body is about the same as in the normal, only two-fifths of them are deposited, the rest being retained within the body. Neither fertilization nor blastoderm formation takes place in the small egg. This strain often segregates "scanty type," in which eggs are as big as normal, but are formed in a small number, about one-half of the small egg type. Some eggs of the "scanty type" are fertilized and embryos form within them, but no larvae hatch out. The mechanism of degeneration of yolk and eggs in this strain was followed by Hayashi (1937, 1940) histologically.

(vi) *Dominant small egg, Sme.* The egg is fertilizable, although very small. It is an X-ray product with a deficiency, dominant. Homozygotes die within the eggs (Takasaki, 1947).

(vii) *Clumpy, rd.* The shape is irregular and highly variable; the ratio of length to breadth is larger than normal; ovarioles and eggs are partly degenerated (Kei, 1939, 1943).

b. *Egg-Shell Colors.* (i) *Normal egg color, +.* Egg colors are influenced by both the colors of the egg shell and the serosa. In the normal egg, the chorion is colorless and transparent, while the serosa is dark purplish brown in the hibernating egg. Such a color is sometimes called black.

(ii) *Yellow egg shell, ye.* Toyama (1913) described the maternal inheritance of this color, but Tanaka (1919) pointed out that the green color depends on the yellow tint of the shell, which optically looks greenish when combined with the normal coloration of the serosa, so that its inheritance cannot be called maternal. Aruga (1943) found that this gene is located on the sex chromosome.

(iii) *Gray egg, Gr.* The shell is milky white, which makes the egg gray-colored in optical combination with the dark serosa pigment. When *Gr* is combined with the red serosa, the egg shows a pink color. The dorsal surface of the gray egg does not sink and is marked with several longitudinal ridges. Tanaka (1943) published its locus in the chromosome II. Takasaki (1947) mentioned other gray egg genes, Gr_{16} and *Lg* Gr_{16} is dominant and allelic to *Gr*, while *Lg* (light gray) is incompletely dominant to the normal. Kei (1943) discovered two gray egg genes, *Ae* and *Se*. *Ae* is probably a synonym or an allele of *Gr*. The *Se* egg is characterized by numerous small ridges running in various directions on the egg surface, and degenerated pores and micropyle.

Aruga (1944) got a gray egg strain from a mosaic egg. Chikushi

(1948) obtained a gray strain as an induced mutation, and Sasaki (1949) got another gray egg strain as a natural mutation. These three grays much resemble with each other and might possibly be the same as *Lg*.

c. *Yolk Colors.* Yolk colors are easily observable through the egg shell in newly laid eggs before the dark serosa pigment is deposited. The yolk colors are coincident with blood colors, namely, light yellow (or "white" as is often called) in white cocoon races, and deep yellow or brownish yellow in yellow cocoon races, the former being a simple recessive to the latter. Therefore, the same symbol, *Y*, can be used both for the yellow yolk and yellow blood, and $+^{Y}$ for white yolk and blood. The cocoon color, on the other hand, does not necessarily accord with yolk and blood colors, as is the case in the yellow-blooded white cocoon strain.

d. *Egg Colors Due to Serosa Pigment.* Serosa pigments are produced about 96 hours after egg laying when eggs are kept at 25°C. Egg colors are influenced by the translucent genes for the larval skin. For instance, eggs of two genotypes, *od*/W and *od*/+, are laid side by side in the cross $+^{od}$/W × *od*/*od*. From *od*/W eggs, translucent female larvae emerge, whereas *od*/+ eggs give normal males only. These two sorts of eggs are distinguishable by colors, *od*/W being lighter than *od*/+ eggs.

(i) *White egg 1, w_1.* Serosa contains no pigment; the compound eyes of the moth are white. The character is inherited maternally.

(ii) *White egg 2, w_2.* With the elapse of time after laying, the egg gradually changes from white to a light reddish color. Some reddish pigment granules are produced in the serosa cells, but the number is few and their distribution is irregular. The compound eyes of the adult are white. The inheritance is ordinary.

(iii) *White egg 3, w_3.* This new gene was discovered by Suzuki (1944). It is not really white but intermediate between normal and white, i.e., light purplish brown because of the light brownish pigment scattered in the serosa. The eyes of the moth are black. The three whites mentioned above are linked together.

(iv) *Brown egg 2, b_2.* This egg is sometimes called Uda's brown egg, which might have originated from Toyama's brown. Grayish-brown pigment particles are scattered in the serosa cells; the compound eyes are dark. A peculiar combined type of maternal and ordinary inheritance is seen in this strain, as analyzed by Tanaka (1924b).

(v) *Pink-eyed white egg, pe.* Pigment is absent from the serosa, so the egg is white. No pigment granules are seen in the compound eyes, but the crystal cones are homogeneously tinted light reddish brown, hence the pink eye. It is linked with *re* and is inherited ordinarily.

(vi) *Red egg, re.* The serosa of the red egg contains as many pig-

ment granules as the serosa of the normal egg, but the pigment is reddish brown instead of the dark brown of the normal. The facet eyes are dark red. Inheritance is ordinary.

(vii) *No glue, Ng.* Normal eggs are firmly attached to the substrate by some glutinous substance secreted from the mucous glands connected with the oviduct, whereas eggs of the Bagdad race which are homozygous for *Ng* are only lightly attached to the paper, cloth, etc., and easily get free and slip down from the substrate. This is due to the poor development of the mucous glands in *Ng* females.

(viii) *Female sterile, fs.* In this strain, a high percentage, often up to 100%, of the eggs is unfertilized. The cause of sterility was attributed to abnormal positions, inverted or transverse, of eggs caused by excessive peristalsis of the oviduct so that the entrance of sperm is hindered (Fujimoto, 1943).

FIG. 2. Normal marking (+^{p}), full-grown larva (dorsal view). *ch*, caudal horn; *e*, eye-spot; *h*, head; *l*, lunule or crescent; *s*, star spot.

2. *Larval Characters*

a. *Color Patterns.* Larval color patterns chiefly depend on the nature and distribution of pigments in the dermal cells and the primary cuticle.

(i) *Normal marking,* $+^{p}$. The normal pattern is composed of three pairs of spots, i.e., the eye-spots on the second thoracic segment, the crescents or lunules, and the star-spots on the second and fifth abdominal segment, respectively. With regard to the intensity of these spots, Tanaka (1916, 1919) distinguished four classes in this marking, $+^{p1}$ $+^{p2}$, $+^{p3}$, and $+^{p4}$ in order from the lightest to the darkest. They are multiple alleles, the darker being epistatic to the lighter. The standard is $+^{p3}$.

(ii) *Plain, p.* This is the basic gene of the largest allelic group in the silkworm. When full-grown, the larva is white all over the body, except for some linear traces of crescents and stars which are often observable.

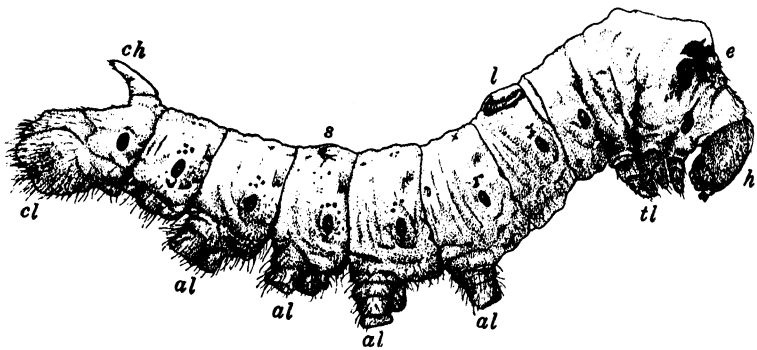


FIG. 3. Normal marking of the larva immediately after the last molt (lateral view) (after Tsujita's photograph). *al,* abdominal legs; *ch,* caudal horn; *h,* head; *l,* lunule or crescent; *s,* star spot; *tl,* thoracic legs.

(iii) *Moricaud,* p^{M}. This name came from the French and was adopted by Coutagne; it means sambo. The marking consists of innumerable dark lines and dots, but not solid black as in the striped. In the striped-moricaud heterozygote, both markings appear superimposed, as in a double-printed photograph. The moricaud marking is fully developed first in the fourth instar, as most other markings are. The larval marking of the wild silkworm, *Bombyx mandarina,* the supposed ancestor of the domestic silkworm, is moricaud, though it is not exactly the same as the moricaud of *B. mori.* There are several classes distin-

guishable with regard to the intensity of pigmentation in the moricaud of the domestic silkworm.

(iv) *Striped*, p^S. In the full-grown larva, the whole body surface is solid black except the posterior margin of each segment, where a white stripe is left. It is allelic to plain, normal, and moricaud; they are

FIG. 4. Plain, full-grown larvae (dorsal and lateral views). The two on the left have cheek and tail spots; the others have no spots.

epistatic in the order $p^S > p^M > +^p > p$. The heterozygous striped is an intermediate of the parents in coloration, although it resembles the striped parent more.

(v) *Black*, p^B. This spontaneous mutation was discovered by Sakai and studied by Kawaguchi (1933). The whole body, including the ventral side, is black, with no white stripe on the posterior margin of each segment. This is a member of the *p*-allelic group.

(vi) *Dorsal spot*, p^D. This marking is practically normal, but with triangle-shaped dorsal spots which are more distinct in several posterior segments (Shimodaira, 1947).

(vii) *Light crescent*, p^L. This marking was obtained by Chikushi (1938) in a temperature-shock experiment. The crescents are light in the heterozygote but leave only traces in the homozygote.

(viii) *Crescent suppressor, I-cr.* This mutation is induced by X-rays; in co-existence with $+^{p}$, it suppresses the development of crescents and stars, leaving the eye-spots unaffected (Tazima, 1943).

(ix) *Eye-spotted, Pl.* This marking was obtained in an X-ray experiment; all spots but eye-spots are erased. *Pl* exerts a suppressing action against all members of the *E*-allelic group (Takasaki, 1947).

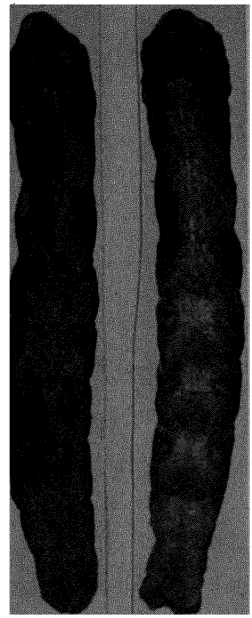


FIG. 5. Moricaud form (dorsal and ventral views).

(x) *Semi-plain, p'.* By itself no different from *p*, but it reduces the pigment in the thorax only when it co-exists with *q* in contrast to *p*, which reduces pigment in the whole body surface in *p q* (Kawaguchi, 1942).

(xi) *Sable,* p^{Sa}. This strain is induced by X-rays (Tazima, 1938). It is accompanied by a chromosomal aberration. The entire body has a sooty appearance, with a white region in the posterior margin of each segment. The dominance relation is $p < +^{p} = p^{Sa} < p^{S}$.

(xii) *Sable 2,* p^{Sa2}. This is another example of an X-ray induced mutant produced by Tazima (1938). The pigmentation is darker than in p^{Sa}; the ventral side of the thorax is uniformly dark colored, and the chitinized bands of the thoracic legs are black as coal. It is lethal when homozygous.

(xiii) *New striped, S.* This darkest striped strain was discovered by Tanaka (1933); the heterozygote is almost as dark as the homozygote. The gene is situated on chromosome II, but in a different locus than p^s.

(xiv) *Dilute striped, S^d.* This natural mutation occurred in *S* strain (Tanaka, 1933). The coloration is exceedingly light, especially when heterozygous ($S^d/+$).

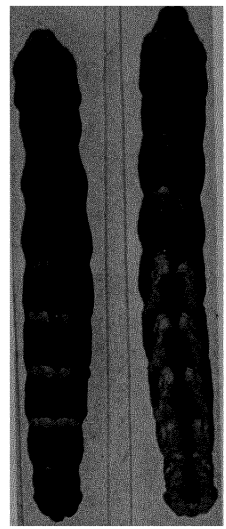


FIG. 6. Striped form (dorsal and ventral views).

(xv) *Whitish striped, p^{sw}.* This was produced by a temperature shock from *S* strain (Chikushi, 1938). When homozygous, it approaches in appearance heterozygous striped p^s/p, while the pigment becomes extremely light in the heterozygote, especially in the thoracic region.

(xvi) *Zebra, Ze.* This marking was called "striped" by Toyama, but we prefer to call it zebra according to Coutagne's nomenclature. The characteristics of this marking are as follows: a black band on the anterior end of each segment; a pair of black spots on the ventral side of each segment; dark-brown cheek spots on both sides of the head case. Zebra is equistatic to normal, moricaud, striped, quail, multilunar, and some other markings; i.e., it fully develops in F_1 without suppressing the development of the other markings enumerated above. Tanaka (1917) called zebra and multilunar "additional" markings contrasted to "fundamental" markings which include plain, normal, moricaud,

striped, quail, and pale quail. Zebra is linked with lemon and is located on chromosome III.

(xvii) *Multilunar, L.* Some pairs of large brownish or yellowish round spots appear in thoracic and abdominal segments. The number of pairs of spots differs by strains and also fluctuates by individuals

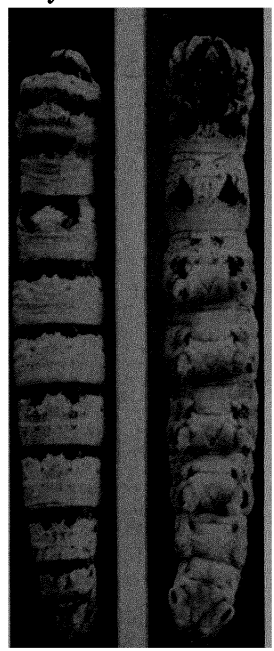


FIG. 7. Zebra combined with normal larva, *Ze*, $+^{p}$ (dorsal and ventral views).

among the sibs. Inheritance of *L* is similar to that of *Ze* with the exception that *L* pattern is scarcely visible in *p L*, whereas *Ze* is fully developed in *p Ze*. *L* is linked with *sk* (stick) in chromosome IV.

(xviii) *Quail, q.* In *q/q* larvae, the body is tinted reddish purple, and is covered with shredlike lines. The pattern is light colored only when combined with *p*. This gene is located on the left end of chromosome VII, and is linked with a green cocoon gene *Gb* and a translucent *obt*.

(xix) *No lunule, Nl.* This spontaneous mutation found by Tanaka (1925) backs the lunules and stars in spite of the presence of eye-spots. *Nl/Nl* zygotes mostly die within the egg, others in the larval stage.

(xx) *Plain supernumerary legs, E.* Supernumerary legs are present in the first and second abdominal segments. These are small in size

and morphologically resemble abdominal legs, but move like thoracic legs. *E* has no lethal action (Sasaki, 1930).

(xxi) *Extra crescents and legs, E^{El}.* Extra crescents are present in the first abdominal segment, and supernumerary legs in the first and second abdominal segments. Supernumerary legs vary considerably in size and shape. This character is partly dependent on the temperature

FIG. 8. Multilunar combined with normal larvae, *L*, $+^{p}$ (dorsal and ventral views).

to which the eggs are exposed. Supernumerary legs grow larger and more complete at 15°C., while they are markedly reduced in size at a high temperature (27°). Homozygotes are weak and tend to die during the period from the later embryonal stage to the end of larval life, and often in the pupal stage too (Kogure, 1929; Sasaki, 1932).

(xxii) *No crescent supernumerary legs, E^{Nc}.* This strain was discovered by Ichikawa (1943). The crescents are absent; the stars, light or absent; the small spots in the third thoracic segment, absent; the eye-spots, lighter than normal. The thorax is elongated before molting; by this characteristic, it is distinguishable from the normal type even in the first molt. It is lethal when homozygous.

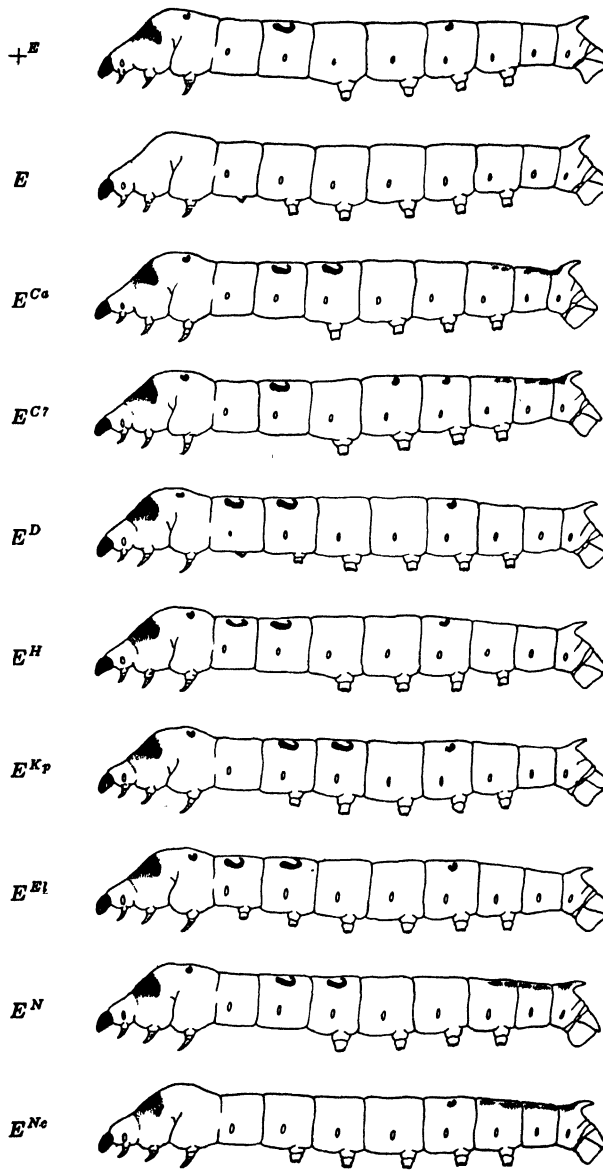


FIG. 9. Markings and leg characteristics manifested by *E*-allelic genes (from Takasaki, in press; modified).

(xxiii) *Double stars,* E^{Ds}. Extra stars are present in the fourth abdominal segment. The larva dies in the embryonal stage when the gene is homozygous (Takasaki, 1947).

(xxiv) *Additional crescents,* E^{Cs}. There are extra crescents in the third abdominal segment; stars are absent. Homozygotes die just before hatching (Suzuki, 1929).

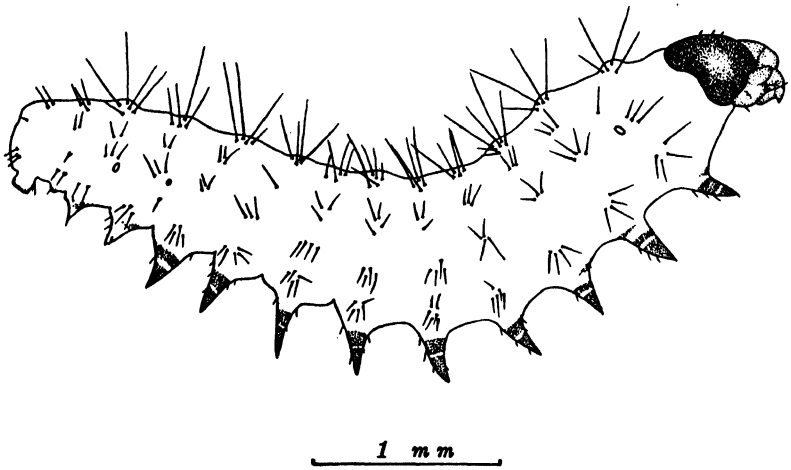


FIG. 10. An E^N/E^N embryo, which does not live further than this stage (from Ichikawa, 1943).

(xxv) *Double crescents,* E^D. Extra crescents are present in the first abdominal segment; sometimes there are supernumary legs in the first and second abdominal segments. The supernumerary legs in the first segment approach rudimentary thoracic legs, and those in the second segment resemble rudimentary abdominal legs. The spiracles in the first abdominal segment are more or less degenerated; the third thoracic and first abdominal segments are often partly fused together. The hind wings of moth are small and malformed. Almost all homozygotes and the majority of heterozygotes die as embryos (Tanaka, 1926; Hasimoto, 1941).

(xxvi) *H-extra crescents,* E^H. The extra crescents, often imperfect, appear in the first abdominal segment; no supernumerary legs. Homozygotes are weak and sometimes die within the eggs (Hasimoto, 1941a).

(xxvii) *Kp supernumerary legs,* E^{Kp}. Supernumerary legs are present in the second (sometimes the first, too) abdominal segment and extra crescents in the third abdominal segment when homozygous (Hasimoto, 1930).

(xxviii) *New additional crescents, E^N.* The extra crescents are in the third abdominal segment; stars are always absent. All homozygotes die before passing the blastokinesis; thoracic leglike appendages develop in each of eleven embryonal segments (Ichikawa, 1943).

(xxix) *No crescent supernumerary legs, E^{No}.* The larva is provided with comparatively large supernumerary legs in the second abdominal segment, and the crescents are absent from the same segment. Supernumerary leg is dominant and no-crescent is recessive to the normal.

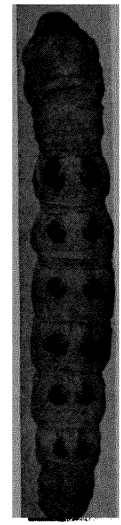


FIG. 11. Multistar form (dorsal view).

Homozygotes are weaker and less fertile than heterozygotes (Sasaki, 1940).

(xxx) *Np-supernumerary, E^{Np}.* Homozygotes perish just before hatching; a few heterozygotes hatch out, but their growth is irregular. Sometimes supernumerary legs are present in the first abdominal segment of the larva and moth. A pair of the crescent-like depressions are present in the mesothorax of the pupa (Ichikawa, 1948).

(xxxi) *Supernumerary legs, Sl.* Expressivity of the supernumerary legs on the second abdominal segment is very variable; crescents are underdeveloped and irregular in shape. This strain is induced by X-ray treatment (Aruga, 1943).

(xxxii) *Multistars, ms.* When well developed, star-spots are seen in each segment from the third to the seventh abdominal segments. This is a simple recessive to the normal (Tanaka, 1927b).

(xxxiii) *Dilute black, bd.* The whole body, the lateral and ventral sides inclusive, is dilute black; the frontal spot is on the head; there is high mortality in the larval stage. The female moth is completely sterile (Sasaki, 1941).

(xxxiv) *Ursa, U.* Dorsal and lateral sides of the body are covered by dark-brown pigments, leaving a white longitudinal band above the dorsal vessel; the ventral side is especially dark in the anterior segments down to the third abdominal segment; the posterior segments are nearly white (Hasimoto, 1941a).

(xxxv) *Brown ursa,* U^{Br}. This is allelic to ursa. There are numerous dark-brown dots on the reddish-brown ground color; the dorsal median white band is absent. The intersegmental membrane and the sternum are colored; it has a dark-brown ring around the spiracle. This is a recessive lethal, but the lethal action does not operate in the embryonal stage; it appears in fourth molt, when homozygotes are totally destroyed because they are not able to cast off the old skin (Tsujita, 1946).

(xxxvi) *Cheek and tail spots, cts.* Conspicuous chocolate-colored spots are present on both sides of cranium, and there is a pair of large triangular spots of the same color on the anal legs. Two pairs of these spots always accompany each other. This is a simple recessive (Tanaka, 1935a). Zebra marking is similarly provided with dark-brown cheek spots, which are inherited dominantly, but has no tail spots. The cheek spots in these two strains are quite different genetically.

(xxxvii) *Brown head and tail spots, bts.* The head is uniformly reddish brown; the tail spots are more reddish than those in *cts.* The outside of the thoracic legs and the sieve plate of the spiracle are brown. This is a simple recessive (Tanaka, 1948).

(xxxviii) *Mustache a, ma.* This mutation is obtained by phenyl acid treatment. A pair of black mustache-like spots is present on the first thoracic segment. These spots appear even in the plain individual in which all other spots are absent (Sakata, 1943).

(xxxix) *Mustache b, mb.* This strain is segregated from the same strain as *ma,* but it is lighter in coloration than *ma.* It is epistatic to *ma* and recessive to the normal (Sakata, 1943).

(xl) *Dirty, Di.* Irregular black lines and dots cover the dorsal surface; the eye spots are distinct; the regions of crescents and stars lack pigment when homozygous. In *ms/ms, Di/Di,* the multistars are represented as white areas in the dark ground color; in *p/p, Di/Di,* the body is as white as in *p/p,* +/+ (Chikushi, 1948).

(xli) *Dark striped 1,* Ds_1. The intensely colored striping is due to the presence of a duplicated fragment of chromosome II. This and the

following seven markings are all chromosome aberrations obtained by X-ray treatment by Aruga (1939, 1940).

(xlii) *Dark striped 2, Ds_2.* The two chromosomes II are attached, but the attachment is very unstable, dissociation occurring both in the somatic and germ tracts.

(xliii) *Dark striped 3, Ds_3.* This strain is segregated from Ds_1. There is translocation of the duplicated chromosome II to another autosome.

(xliv) *Dark striped 4, Ds_4.* The black parts are intensely pigmented so the eye-spots, crescents, and stars are not distinguishable. The heterozygotes are as dark as homozygotes. The larvae are weak; many cannot pass through the molting normally. Crossing-over between p^s and Y is more or less suppressed, as is the case in the above three strains.

(xlv) *Pale striped 1, Pas_1.* This is a translocation and duplication of chromosome II; translocation is stable. It is lethal when homozygous, partially lethal when heterozygous. The suppression of crossing over is not perceptible.

(xlvi) *Pale striped 2, Pas_2.* This probably is a chromosomal aberration; no lethal action.

(xlvii) *Pale moricaud 1, Pam_1.* This strain is a very light moricaud. Crescents and stars are imperfect or not present.

(xlviii) *Pale moricaud 2, Pam_2.* This is a light moricaud with distinct crescents and stars; frontal spots are light and imperfect.

(xlix) *Brown, Br.* This dominant gene mutation is induced by X-rays. It modifies the black pigment into brown. Phenotypes are variable according to which of the *p*-allelic markings are present with *Br*. *Ze* and *L* are not affected by this gene (Aruga, 1939).

(l) *Ventral striped, p^G.* This is induced by X-rays. The dorsal side is lightly, and the ventral stripes intensely, colored. It is a translocation of the p^s region to another chromosome (Takasaki, 1947).

(li) *Swollen, Swl.* This mutation is induced by X-rays. The larval body is plump and dotted all over. It suppresses the pigment formation of the markings of the *p*-allelic group, sometimes reducing the striped phenotypically to the plain (Takasaki, 1947).

b. *Body Shapes.* (i) *Elongate, e.* The first and second abdominal segments of the larva are unusually elongated; the intersegmental folds are stretched out in that region. This characteristic is also visible in the pupa and the moth; it is sex linked (Tanaka, 1923).

(ii) *Stick, sk.* The larval body is slender and hard to the touch, less flexible, reminding one of a withered twig. The development is somewhat slow, and mortality is slightly higher than in the normal. It is linked with multilunar (Tanaka, 1917).

(iii) *Knobbed, K.* Dermal protuberances appear on several segments and are visible also in the pupa and moth. When it coexists with *L,* the distribution and size of knobs and multilunar spots exactly coincide. The striped marking markedly reduces the size of knobs, and almost entirely suppresses it when homozygous (Tanaka, 1916, 1943).

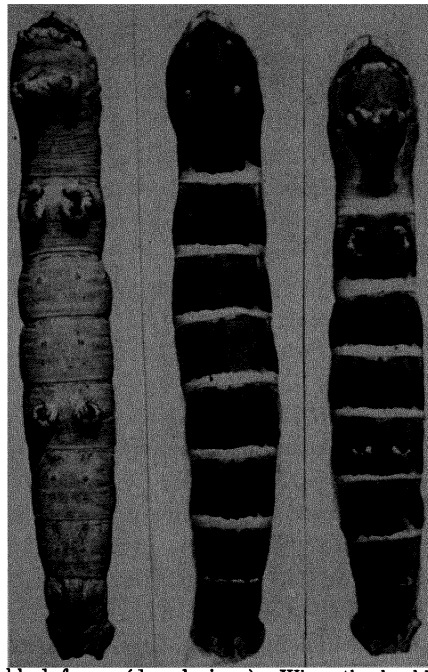


FIG. 12. Knobbed forms (dorsal views). When the knobbed gene is combined with any marking other than striped, knobs develop fully. *Left,* knobbed (*K*) combined with normal marking *K*, $+^{p}/+^{p}$; *center, K* combined with homozygous striped, *K*, p^{s}/p^{s}; *right,* knobbed combined with heterozygous striped, *K*, $p^{s}/+^{p}$. Knobs are fully developed in the form on the left, partially suppressed in that on the right, and almost entirely suppressed in the center form.

(iv) *Stony, st.* The larval body is compact and hard; each segment is marked with several transverse wrinkles. The whole body, especially the first and second segments is stretched anterio-posteriorly; the head protrudes forward. Intersegmental joints resemble bamboo. The crescents and star-spots are small and rounded. The character is distinguishable as early as the first instar; it is completely suppressed in the homozygous striped form (Tanaka, 1919, 1928).

(v) *Constricted, co.* This is manifested only in the larva with

striped marking, especially when it is homozygous for chocolate (*ch*). A few transverse grooves or wrinkles are formed on the dorsal side, and the pigment is sedimented in grooves, the remaining portion of skin being almost entirely free of the pigment. The body is slender, stretched and bamboo-like in appearance. The character is distinguishable only in the full-grown larva; it is a simple recessive to the normal. A modified type, so-called "washed," in which the pigment is missing from the whole body surface, appears often in this strain (Tanaka, 1919).

(vi) *Dominant constricted, Ct.* This spontaneous mutation occurred in a normal strain. It is dominant to the normal morphologically, and is recessive in lethality (Ogawa, 1949).

(vii) *Geometrid, ge.* In this spontaneous mutation the body is long and slender in the larva, pupa, and moth. In the larva, the second abdominal segment is remarkably tightened so that the crescents are stretched anterio-posteriorly. It is a simple recessive (Tanaka, 1936).

(viii) *Narrow breast, nb.* This spontaneous mutation was discovered by Tanaka in 1947. The larval body is nearly spindle-shaped owing to the narrowed thorax. The larva are weak and liable to be lost during rearing. It is a simple recessive. Chikushi (1951) found a linkage between *nb* and *ms*.

(ix) *Compressed, cp.* All segments are wide and short in the full-grown larva; the alimentary canal is zigzag shaped as a result of the shortening of the body. Feeding is inactive; growth is retarded; and mortality is high. It is a simple recessive (Asano, 1947).

(x) *Non-molting, nm.* Homozygotes live about two weeks without passing through any molt, and then perish. If they pass through the molt, as sometimes occurs, they grow normally afterwards (Umeya and Karasawa, 1930). According to Yokoyama (1936), the lethal action of this gene is perhaps due to the inhibition of the physiological function of oenocytes in the first instar.

(xi) *Dwarf, d.* The body length in the full-grown larva is about three-fourths that of the normal; movement is inactive; mortality is high (Suda and Hashida, 1931).

(xii) *Lethal dwarf, dw.* The larva manifests a translucent appearance in the first instar; this disappears in the third instar. The majority die on about the twenty-fourth day after hatching. The number of molts which the larvae pass through before death may be anywhere from one to four (Morohoshi, 1949).

(xiii) *Dominant dwarf, Df.* In this X-ray mutation growth is retarded, crescents are sometimes imperfect, eye-spots are often very light. Occasionally, multistar-like spots appear in the fourth instar. It is lethal when homozygous (Aruga, 1943).

(xiv) *Burnt, Bu.* Along the dorsal median line, the skin of the second to the fifth segments shows a burn-like scar; the dorsal vessel of that region is extended laterally. It is lethal when homozygous and semi-lethal when heterozygous. The lethal action takes place in the period between the "longest" stage to the blastokinesis of the embryo when the navel remains open owing to the poor development of the amnion. It is induced by X-rays (Aruga, 1938, 1939).

c. *Body Colors.* (i) *Chocolate, ch.* The newly hatched larva is reddish brown in contrast to the wild-type black. The characteristic becomes less conspicuous with age, but the eye-spots, the crescents, and the stars are still reddish brown in the full-grown normal, other parts being white. In *p/p, ch/ch* larva, no *ch* character is recognizable on the body surface, while the chocolate moricaud and striped are beautifully reddish colored. It is a simple recessive (Toyama, 1909).

(ii) *Dominant chocolate, I-a.* This is phenotypically like the chocolate, but the head is black in contrast to the dark brown of *ch/ch*. It is dominant to the normal. Three phenotypes are segregated in the F_2 of the cross *ch/ch* × *I-a/I-a*—namely, 9 dominant chocolate, 3 normal (black), 4 recessive chocolate (Endo, 1940).

(iii) *Lemon, lem.* A greenish-yellow coloring all over the skin is visible from the second instar. It is a simple recessive linked with the zebra marking (Nozaki, 1917; Takahashi, 1917).

(iv) *Yellow molting, Ym.* Immediately after a molt, especially after the second molt, the skin is covered with yellow powder secreted by the Malpighian vessels. It is dominant to the normal, in which the skin has a white powder after a molt (Shimizu, 1936; Hirobe, 1947).

(v) *Yellow lethal, l-y.* The first instar is normal, but the body becomes yellowish after the first molt. The larva cannot eat mulberry leaves, and it dies in a few days from hunger. It is a simple recessive (Suzuki, 1948; Umeya and Tsujita, 1951).

(vi) *Albino, al.* In the first instar it is indistinguishable from the normal; after the first molt, the body becomes whitish and the larva perishes without eating leaves (Tanaka, 1943).

(vii) *Sooty, so.* The head is dark colored; the thorax and abdomen are smoky in the larva as well as in the moth. It is a simple recessive (Tanaka, 1943).

(viii) *Brown body lethal, l-Bb.* This is lethal when homozygous. The heterozygotes exhibit the brown color in the skin, especially before and during the second molt; after the third instar the brown tint becomes lighter (Shimodaira, 1928).

(ix) *Conditional factor to l-Bb, Bb.* The existence of this gene is

indispensable for the production of brown color by *l-Bb* (Shimodaira, 1928).

(x) *Reddish hemolymph, Rs.* This characteristic is distinctly seen only just before the mounting, when the body, or probably the hemolymph, becomes reddish in transmitted light, in contrast to the normal greenish color. Suda and Kawaguchi (1922) thought this a single recessive to the normal, but Tanaka (1943) considered it an incomplete dominant.

d. *Translucent skin.* The translucent character is unique in silkworm genetics in that it is caused by many different genes. The skin of the translucent larva is more or less transparent depending on the small quantity of urate crystals in the epidermal cells (Jucci, 1932a; Shimizu, 1943; Hatamura, 1943; Aruga, 1943). Some translucents have been known for a long time, some appeared in hybrids, and others occurred as spontaneous mutations.

Besides these, there are known several mottled translucent strains in which translucent and opaque areas are intermingled in the skin.

The translucent characteristic is exhibited not only in the skin but also in the serosa of the egg, in the compound eye of the moth, and in the trachea, ganglions, fore-gut, silk glands, Malpighian vessels, oenocytes, salivary glands, prothoracic glands, corpora allata, and ectodermal portions of the reproductive system. Translucent epidermal cells stain deep purple by neutral red, in contrast to the opaque cells, which remain unstained. It is interesting that these staining reactions are just reversed in the glandular cells (Aruga, 1942, 1943).

As is seen in the list, the translucent genes are all recessive and are distributed in various loci of different chromosomes, any of them resulting in more or less translucency of the skin. Some are lethal in various degrees, but others are not. The mottled translucents are dominant to uniform translucents but recessive to the normal. Translucent genes partially suppress pigment formation in the serosa and make the egg colors lighter.

e. *Blood Colors.* (i) *Yellow blood, Y.* The hemolymph is deep yellow; this is simple dominant to the normal "white" (light yellow or colorless) blood (Toyama, 1906).

(ii) *Yellow inhibitor, I.* This gene completely suppresses the yellow blood and yellow cocoon. The white cocoon races which contain *I* are called dominant whites. European (Asia Minor included) white races are mostly dominant whites, whereas Oriental white races are all recessive (Toyama and Mori, 1913; Tanaka, 1913a).

(iii) *Mandarina yellow blood, Y^a.* This is a very light-yellow blood, characteristic of *Bombyx mandarina;* it becomes somewhat more dis-

A List of Some Translucent Types

Name	*Symbol*	*Linkage group (locus)*	*Degree of translucency*	*Lethality and characteristics*	*Authorities*
Sex-linked	*os*	I (0)	Low	Somewhat weak	Tanaka, 1917
Distinct	*od*	I (49.6)	High	Development retarded; mortality in young larvae high	Tanaka, 1926
Chinese	*oc*	V (35.9)	Rather high; from moderate to low in Japanese	No lethality	Tanaka, 1916, 1917; Yokoyama, 1939
Waxy	*ow*	–	Moderate	15% mortality; higher than +	Tanaka and Matsuno, 1929
Tanaka	*ot*	–	High	Mortality *ca.* 22% in larvae; majority die after pupation; moth scarcely copulate and lay eggs	Tanaka and Matsuno, 1929
Aojiku	*oa*	–	Moderate	Slightly lethal	Tanaka and Matsuno, 1929
Kinshiryu	*ok*	V (4.7)	High	When eggs incubated at high temperature, mortality 46%; at low temperature, mortality in first instar much higher	Tanaka and Matsuno, 1929; Yokoyama, 1939
Matamukashi	*om*	–	Low, more distinct when younger	Weak; larval mortality 20%; many die before emergence; numerous unfertilized eggs	Tanaka and Matsuno, 1929
White Egg	*oew*	–	High	Unseparably combined with white egg	Tanaka and Matsuno, 1929
Aojiku White Egg	*ol*	X (6.9)	High	100% lethal when reared below 23°C.; critical period is fourth molt; not lethal when reared at high temperature	Nakano, 1931, 1936

Name	*Symbol*	*Linkage group (locus)*	*Degree of translucency*	*Lethality and characteristics*	*Authorities*
Giallo Ascoli	*og*	IX (7.4)	High	No difference from the normal in larval stage; high mortality just before emergence of moth; female almost entirely sterile, male fertile; Malpighian vessels swollen and filled with dark-brown substance	Shimizu, 1931; Sasaki, 1938
Translucent white egg	*owe*	–	High	Lethality not affected by temperature; linked with green cocoon color	Sakata, 1941
Black	*obl*	–	Low	Frontal spots on head; body dark gray; larva and pupa normal; moths of both sexes highly sterile	Kei, 1942
Bione	*ob*	–	Moderate	Not lethal	Tanaka, 1943
N-115	*o-115*	–	Rather high	High mortality after third instar; possibly linked with constricted	Takasaki, 1943
B_e-Mottled	*obt*	VII (11.6)	Moderate	Not lethal	Hasimoto, 1934d
od-Mottled	*odm*	–	High	Mutable to normal; a modifier *Mo* affects the mutability	Hatamura, 1939
Tanaka's Mottled	*otm*	–	High	Not lethal	Tanaka and Chiang, 1939
o-Alpha Mottled	*oal*	–	High	Translucent area variable in size; the larger the area, the higher the lethality; an inhibitor, *I-od*, present in one of autosomes other than chromosome II	Takasaki, 1940
E-15 Mottled	*odk*	XIV (8.0)	Low	Not lethal	Hasimoto, 1941a

tinctly colored just before spinning. It is allelic to *Y* (Kawaguchi, 1934).

f. *Genes Related to Enzyme Formation.* (i) *Digestive juice amylase negative, ae.* A rich content of amylase has been found in the digestive juice of the normal larva, while its reaction is very weak in the *ae* strain, notwithstanding that the amylase content in the body fluid of the same individual is as high as in the normal (Matsumura, 1933).

(ii) *Body fluid amylase negative, be.* Amylase reaction is weak in the hemolymph and strong in the digestive fluid. This is closely linked with *ae* in chromosome VIII. Indigenous Japanese univoltines are all normal ($+^{ae}+^{be}$); all European races are either *ae be* or *ae* $+^{be}$; four possible genotypes ($+^{ae}+^{be}$, $+^{ae}$ *be*, *ae* $+^{be}$, and *ae be*) are met with in Chinese races (Matsumura, 1933).

3. *Cocoon Characters*

a. *Cocoon Colors.* (i) *Golden yellow, C.* As described previously, *Y* controls yellow blood and yellow cocoon. *Y C* cocoon is golden yellow on the outside and nearly white in the inside. The blood is yellow and the cocoon white in *Y* $+^{C}$, and both blood and cocoon are white in $+^{Y}$ *C* and $+^{Y}+^{C}$. *C* is the same gene as Uda's *Y* (Tanaka, 1913a; Uda, 1919; Kei, 1943).

(ii) *Yellow inner layer,* C^{i}. This is allelic to *C;* it makes the cocoon golden outside and yellow inside. Ogura's *X* is a synonym of C^{i} (Uda, 1919; Ogura, 1931a; Kei, 1943).

(iii) *White cocoon,* $+^{C}$. The cocoon is white outside and inside. Ogura's *x* and Kei's *c* are synonyms of this gene (Uda, 1919; Ogura, 1931a; Kei, 1943).

(iv) *Green a, Ga.* The cocoon is colored light green by the interaction of *Ga* and *Gb* (Hasimoto, 1941a).

(v) *Green b, Gb.* This is a complementary factor for the production of greenish cocoon color (Hasimoto, 1941a).

(vi) *Green c, Gc.* This green cocoon gene acts independently of *Ga* and *Gb;* it is linked with white-sided egg (*Se*) in chromosome XV (Hasimoto, 1941a; Kei, 1943).

(vii) *Flesh, F.* This produces a reddish-yellow or salmon color. It is very common in European races and is met with also in certain Chinese yellows. It is hypostatic to *C;* it exhibits flesh color only in presence of *Y* (Cleghorn, 1918; Ogura, 1931a).

(viii) *Sooty plain white, a.* Tanaka (1924, 1925) found that F_1 larvae from the cross sooty plain (extracted from Bagdad race) × white cocoon race are all yellow blooded and spin yellow cocoons. By further experiments, he proved that sooty plain lacks a certain fundamental

gene indispensable for production of yellow cocoon, namely, *a/a*, in which the blood and cocoon are both white in spite of the presence of other color factors. This was confirmed by Jucci (1935).

(ix) *Yellow cocoon, Yc.* This gene makes the cocoon yellow in the presence of $+^a$ and *Y*. Genotypes of various races may be summed up as follows: yellow blood, yellow cocoon, $+^a\ Y\ Yc$; yellow blood, white cocoon, $+^a\ Y\ +^{Yc}$; most white blood, white cocoon races, $+^a\ +^Y\ +^{Yc}$ or $+^a\ +^Y\ Yc$; sooty plain (white blood, white cocoon), $a\ Y\ Yc$. Symbols of cocoon color factors were somewhat confusing hithertofore. They have recently been standardized by the National Committee of Genetics and National Committee of Breeding, Japan Science Council.

(x) *Rusty, Rc.* The cocoon is yellowish brown externally; the color is lighter when the inner layer is white than it is when the inner layer is yellow. Heterozygotes are lighter than homozygotes. When the inner layer is white and the individual is heterozygous for *Rc*, the color approaches white (Kei, 1943).

b. *Cocoon Structures.* (i) *Flossy (cocon graine), Fl.* The cocoon layer is loosely composed, giving a cotton-like appearance. It is a simple dominant (Takasaki, 1947).

(ii) *Perforated a, b, c, ha, Hb, Hc.* One end of the cocoon is elongated and open at the tip in variable degrees. All three genes act cumulatively; *ha* is recessive, the others dominant, to the normal (Kobari, 1933).

4. *Pupal Characters*

a. *Pupal Shapes.* (i) *Wingless (flügellos), fl.* Both fore and hind wings are absent in the pupa and moth. The second and third thoracic legs are primitive; hence copulation is very difficult. It often dies from loss of blood in the area of wings during pupation and emergence of moth. This is a simple recessive. It was reported first by Shiba and Ishiwata, later by Katsuki (1935); recently it was found linked with white egg-2 (Harizuka, 1948).

(ii) *Crayfish, cf.* Fore and hind wings are swollen and protrude laterally from the body; emergence and copulation are difficult, but no abnormality appears in the genital organs or in fertility. The larval stage is normal. This is a spontaneous mutation found by Suzuki (1942) —a simple recessive linked with chocolate.

(iii) *Curled wing, Cw.* The apex of the fore wing is curled up; wings of moth shorter than normal. It is a simple dominant (Suzuki, 1948).

(iv) *Vestigial, Vg.* This is induced by X-rays. The larva is not abnormal; wings of the pupa and moth are more or less poorly developed,

the wing size highly variable, mostly club-shaped approaching apterous when extreme. The gene is located on a Z chromosome which is deficient for $+^{od}$ region. It is a recessive lethal. In the cross *od* × *Vg*, 50% of F_1 females die in the egg and 50% of F_1 males exhibit the recessive character *od* (Tazima, 1947).

b. *Pupal Colors.* (i) *Black pupa, bp.* Gamo (1923) described a black pupa which was inherited as a simple recessive. According to Harizuka (1940, 1942), there are two different strains of black pupae, one of which behaves as a simple Mendelian recessive, but the other does not. The former is linked with the knobbed and is dependent on the temperature in the pre-pupation period. The higher the temperature, the lighter is the pupal color, the lightest approaching the normal amber color. The other strain is more irregular in inheritance, but the frequency of appearance of black pupae seems to be heritable.

(ii) *White winged Pupa, Wp.* The wings of the pupa are very light in color, often nearly white. This is an incomplete dominant (Harizuka, 1947).

c. *Naked Pupa.* (i) *Naked a, Nda.* Middle and posterior regions of silk glands are degenerated. This is a complete dominant (Nakano, 1937).

(ii) *Naked b, Ndb.* About 50% of the larvae spin cocoons, the rest pupate without spinning (Nakano, 1937).

5. *Adult Characters*

a. *Body Colors.* As a rule, wing colors are directly related to body colors, so for convenience they may be described together. Beliajeff (1937) classified the wing colors into several types: (*a*) pure white, (*b*) white with dark designs, (*c*) plain dark, (*d*) dark with patterns, (*e*) almost black; and some intermediates. He assumed three to four or more autosomal polymeric genes Ni^1, Ni^2, $\cdots$, for dark color, and several autosomal polymeric genes St^1, St^2, $\cdots$, for designs, and their exaggerator *Ml.* The design genes (St^1, etc.) are hypostatic to *Ml.* *Ml* has a recessive semi-lethal effect, lowering the viability of females. Ito (1949) denoted the black imago by *Bi* and showed that it is dominant to the normal, but the color intensity is very variable in different races, especially in F_1 hybrids.

(i) *Wild melanism, Wm.* The moth of the wild silkworm (*Bombyx mandarina*) is black; the F_1 of the cross with the domestic normal is intermediate, and various wing colors segregate in the F_2. Tanaka (1919) attributed this to some multiple factors, one of which was represented as *Wm* by Hirobe (1947).

(ii) *Wild wing spot, Ws.* This is black spot on the apex of anterior wing, a characteristic of the wild silkworm; it is dominant to the normal (Hirobe, 1947).

(iii) *Degenerated radius, rv.* Distal portions of several wing veins are lost, and consequently the wing is highly liable to rupture. This abnormality occurs most frequently in the radius vein and less frequently in the media, cubitus, and anal veins (Hashida, 1934).

(iv) *Short wing, Swi.* This is induced by X-rays. The wings are short. It is dominant to the normal (Takasaki, 1947).

b. *Antennae.* (i) *Yellow antenna, ya.* Takasaki (1947) got a mutant moth, in an X-ray experiment, in which one side or a part of the antenna was yellowish white in contrast to the dark brown of the normal part. He considered this the result of a mutable gene. Astaurov (1935) obtained an antennal mutation by X-ray radiation, but the offspring could not be observed owing to the high mortality of the irradiated animals.

c. *Compound Eye Colors.* Eye colors are closely related to egg colors. Normal egg-color genes make eyes black, the red-egg gene gives dark-red eyes, and the white-egg genes give white eyes as a rule. So-called white-3 (w_3) is not really white but lightly colored, and the moth is black-eyed. Though some egg colors are maternal in inheritance, eye colors of the moth are all inherited in the usual way (Toyama, 1910). A similar case was observed in *Ephestia,* in which the eye colors of F_1 young larvae always coincide with those of the mothers (i.e., maternal inheritance), but dominant gene actions are manifested in later instars and moths (i.e., usual inheritance).

(i) *Lustrous, lu.* Compound eyes have a luster caused by the reflection of light on the ommatidia surface. This is recessive to the normal (Uda, 1930).

d. *Abnormal Reproductive Organs.* (i) *Deformed gonad, Gd.* Sakata (1938, 1940) obtained this mutation by a chemical treatment accompanied by a physical stimulus. Mutant moths have five ovarioles on each side in the female and five testicular follicles in each testis in the male, in contrast to four in the normal. They have extra crescents in the first abdominal segment. The wings of the pupa are short; hind wings of the moth are split. The character is recessive lethal, and homozygotes die within the eggs or shortly after hatching. Sakata afterwards (1939) obtained a strain free from the lethality as a result of crossing to a Chinese polyvoltine race. The non-lethal strain exhibits recessive supernumerary legs in the first and second abdominal segments, the third thoracic legs being defective. Most of these characteristics are

dominant, except the supernumerary legs and defective thoracic legs, which are recessive. This is a fine example of pleiotropy of a single gene.

(ii) *Male sterility, Stm.* This mutation is induced by treating the gonads of *Gd* heterozygotes with a chemical reagent. Larvae, pupae, and moths are apparently normal, but all males are sterile, *Stm* gene is transmitted only by the female (Sakata, 1947).

(iii) *Degenerated penis muscles, slp.* One or two pairs of three copulatory muscles are degenerate; the male moth usually is unable to fertilize eggs, but occasionally a male is fertile if only one pair of muscles is affected. Penetrance of the character is closely related to temperature; a low temperature in the larval stage combined with a high temperature in the stage from cocooning to emergence is most favorable to the manifestation (up to 91%). In the F_1 of the cross normal ♀ × *slp* ♂, about 1% of the males are sterile; sterile males are produced with far greater frequency in the reciprocal cross. In either cross, percentages of sterile males increase in later generations (Umeya, 1931, 1932). Umeya interpreted this result as a case of non-Mendelian inheritance, but Tanaka (1934) tried to explain it by assuming several modifiers which have cumulative effects and are transmitted by the female only. Although Umeya (1936) adopted this hypothesis once, he abandoned it later and insisted that, in this case, the egg protoplasm must play an important role.

(iv) *Oligospermy, slo.* Males scarcely produce eupyrene sperms and are nearly sterile; the females are fertile. In crossing the female of this strain with a normal male, F_1 males are all normal, and 20 to 30% abnormals appear in F_2. There is some tendency for the proportion of abnormal males to increase gradually in later generations. Their percentage varies greatly in different families, 20 to 30% and 50% being most frequent, but it may go up to 90% or more in extreme cases (Suzuki and Omura, 1944).

6. *Genes Responsible for Metamorphosis and Growth*

(i) *Retarded, Rt.* This is X-ray induced and dominant to the normal. Homozygotes are lethal, the lethal action operating chiefly in the pupal stage. The development of the *Rt*/+ larva is retarded by 2 or more days, but it passes through the pupal stage more quickly than the normal, and the emergence of the moth is almost simultaneous with the normal. The body is markedly smaller than the normal. This is probably a chromosome aberration, perhaps an inversion accompanied by a deficiency (Tanaka, 1934, 1935).

7. *Lethals*

Lethal factors which do not show any morphologic characteristics are listed here.

(i) *Lethal-10, l-10.* The embryo develops normally until the dark-spotted stage, 3 to 4 days before hatching; then all homozygotes die (Shimodaira, 1947).

(ii) *Lethal-72, l-72.* Homozygotes die within the eggs (Takasaki, 1947).

(iii) *Blastokinesis lethal, l-bk.* Embryos are killed in the blastokinesis stage (Umeya, 1927).

(iv) *Bluish egg lethal, l-bl.* Lethal action takes place after the embryonal body is completely formed on the day before hatching (Umeya, 1927).

(v) *Dark-spotted egg lethal, l-de.* The embryo is nearly completed, the head is black, as in the normal, but the body remains yellowish grey and the bristles colorless, when the embryo dies. The development up to that stage is retarded ½ to 1 day (Nishikawa, 1925).

(vi) *Dark-spotted egg lethal 2, l-ds.* The embryo dies in the same stage as by *l-de,* but the two are not allelic (Nishikawa, 1930).

(vii) *Elongate lethal,* e^l. The lethal action takes place from the end of embryonic life to the larval stage. This is allelic to the elongate (*e*). The dominance relation is as follows: $+^e > e > e^l$ (Kogure, 1935).

(viii) *Hibernating egg lethal, l-hb.* Lethal action occurs in the hibernating embryo up to the "longest" stage (Umeya, 1927).

(ix) *Lethal-k, l-k.* The embryo is killed a few days before hatching (Hasimoto, 1934).

(x) *Lethal-m, l-m.* This lethal is obtained in the gray-egg strain; it is recessive, acting in the embryonic stage, and is linked with *zebra* (Hasimoto, 1940).

(xi) *Sex-linked opaque lethal, l-os.* Homozygotes die in an early embryonic stage. This is allelic to the sex-linked translucent *os* (Tanaka, 1933).

(xii) *Four sex-linked lethals, l-sa, l-sb, l-sc, l-sd.* These are lethals reported by Nishikawa (1934), among which *l-sb* seems to be synonymous with *l-os.* They act as follows: *l-sa* and *l-sd* kill the embryos just before hatching; *l-sc* operates mostly in embryonal, and sometimes in early larval life; *l-sb* is allelic to *os.* They are located thus: *l-sa* not far from *od; l-sd,* very close to *od;* and *l-sc* very close to *os.*

(xiii) *Red-egg lethal, l-re.* The embryo dies at the "longest" stage (Nishikawa, 1925).

(xiv) *Red-egg lethal 2, l-rs.* Another lethal which kills the embryo nearly at the same stage as *l-re* (Nishikawa, 1930).

III. Linkage

1. *Characteristic Features of Linkage in the Silkworm*

Drosophila and *Bombyx* are the only organisms hitherto known in which crossing over occurs only in the homogametic sex. Darlington (1934) attributed the lack of crossing over in the male of *Drosophila pseudobscura* to an anomalous pairing of chromatids in the reduction division of the spermatocyte and the absence of chiasmata between them. Naville (1937) noticed in the silkworm that oogenesis passes from pachytene to diplotene and diakinesis, whereas spermatogenesis passes through pachytene, second contraction, resting stage, and diakinesis but not diplotene. The crossing over takes place, according to him, in the second contraction and resting stage, which are absent in the female. Maeda (1939) observed chiasmata formed in the spermatocytes of the silkworm as in other animals but no chiasma formation in the oöcytes in which univalent chromosomes are merely attached at one end. He calculated, after Belling's method, the terminal recombination value and got 49.5%, which is very close to the 49.6 unit distance between two genes located in extreme ends of the Z chromosome, *os* and *od*.

Crossing-over values may, of course, be calculated more easily from backcrosses. But we can obtain them from F_2 results by the following formula, where n designates the ratio of non-crossovers to crossovers formed in gametogenesis.

	Phenotypes				
	AB	Ab	aB	ab	*Formula for calculating n value*
Linkage phase					
Coupling	$3n+2$	1	1	n	$n = \frac{1}{2}\left(\frac{\text{AB}+\text{ab}}{\text{Ab}+\text{aB}} - \frac{\text{Ab}+\text{aB}}{2}\right)$
Repulsion	$2n+2$	$n+1$	$n+1$	0	

It is evident that F_2 results of the repulsion phase have nothing to do with the determination of n value, the F_2 ratio always being 2:1:1 without regarding the value of n.

Tanaka (1926) observed a phenomenon of interference in Z chromosome. His result is shown below:

Loci	Total Numbers	Crossovers	Recombination Values
os–e	15333	5583	36.41% (1)
e–od	8383	1107	13.21% (2)
os–od	1686	779	46.20% (3)

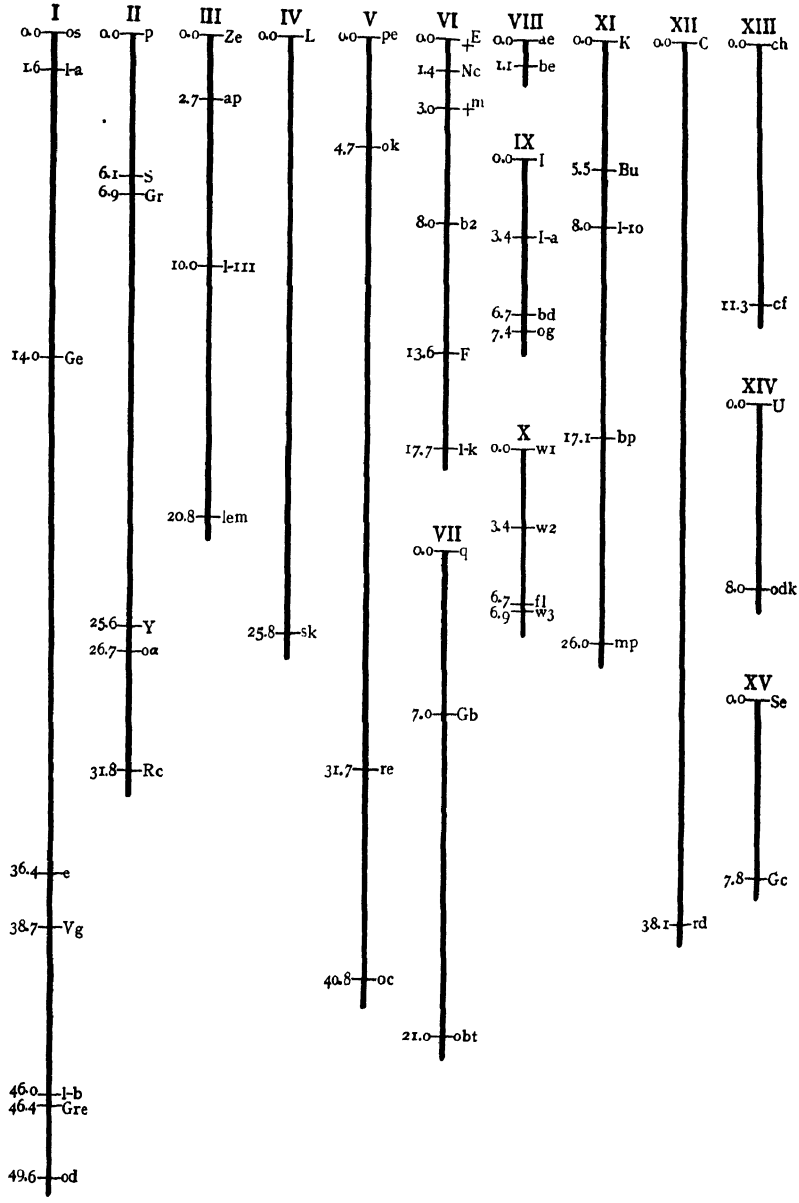


FIG. 13. Latest chromosome maps of the silkworm.

Thus [(1) + (2)] − (3) = 3.42%, which is smaller by 1.39 than 4.81%, the theoretical double crossing over which might occur between *os* and *od* if there were no interference. Hasimoto (1940) made a three-point test between genes located in chromosome V. The crosses were *pe re oc* ♀ × *pe* + *oc*/+ *re* + ♂ and *pe re oc* ♀ × + *re oc*/*pe* + + ♂. He got recombination values of 31.3% between *pe* and *re* and 8.8% between *re* and *oc*. Double crossing over between *pe* and *oc* was 0.4% instead of 2.8%. The deficiency in double crossing over should be considered as due to the interference. Takasaki (1940) pointed out that coincidence in the loci $+^{p}-Y-oal$ is nearly 0.

2. *Explanation of Chromosome Maps*

So far we have 15 linkage maps, representing 15 out of 28 chromosomes. No cytological map has been drawn for the silkworm. In the following list, $p^{S} = p^{M}$ means that p^{S} is equistatic to p^{M}, and $+^{p}>p$ designates that $+^{p}$ is epistatic to p.

a. *Chromosome I* (*Z Chromosome*).

Sex-Linked Genes

Symbol	*Locus*	*References*	*Remarks*
os	0.0	Tanaka, 1926	.....
l-os	0.0	Tanaka, 1933	*l-os–os*, 0
l-sa	?	Nishikawa, 1930	Closely linked with *os*
l-sb	0.0	Nishikawa, 1932	May be synonym of *l-os*
l-sc	0.0±	Nishikawa, 1934	.....
l-a	1.6	Tanaka, 1943	.....
Ge	14.0	Aruga, 1943	*Ge–os*, 14.11; *Ge–e*, 23.07; *Ge–od*, 36.17
e	36.4	Tanaka, 1924	*e–os*, 36.41; *e–od*, 13.21
l-e	36.4	Kogure, 1935	*l-e–os*, 34.0; *l-e–od*, 12.0; *l-e–e*, 0.0
Vg	38.7	Tazima, 1944	Right side of this locus deficient
l-b	46.0	Tanaka, 1943	.....
Gre	46.4	Aruga, 1943	*Gre–Ge*, 32.47; *Gre–od*, 3.24
od	49.6	Tanaka, 1926	*od–os*, 46.20
mod	49.6	Hatamura	+ *od* > low degree *mod* > high degree *mod* > *od*

Besides these, late-developing gene (Nagatomo, 1926-1943), silk-weight gene (Shen, 1926, 1928) and multiple alleles influencing voltinism *Ev*, ev_1, ev_2 (Muroga, 1943, 1947, 1948) are said to be sex-linked, but their linkage relationships have not yet been worked out.

b. *Chromosome II.* This is the first linkage group discovered in

the silkworm (Tanaka, 1913b), and it is remarkable in that it contains the largest number of genes studied, especially 16 alleles in the 0.0 locus.

Genes in *p*-Linkage Group

Symbol	*Locus*	*References*	*Remarks*
p	0.0	Tanaka, 1913b, 1916	$p^s = p^M > +^p > p$
p^s	0.0	Tanaka, 1913b, 1916	p^s–*Y*, 27.22
p^M	0.0	Tanaka, 1913b, 1916	p^M–*Y*, 23.66
$+^p$	0.0	Tanaka, 1913b, 1916	$+^p$–*Y*, 25.54
			Weighted mean, 25.59
p^B	0.0	Kawaguchi, 1933	p^B–*Y*, 23.74; $p^B > p^s$ allelic
p^L	0.0	Chikushi, 1938	p^L–*Y*, 20.64; $+^p > p^L > p$, p^L–S^w, 7.63
p^{Sa}	0.0	Tazima, 1938a	p^{Sa}–*Y*, 1.8; $p^s > p^{Sa} > p$, $p^M = p^{Sa} = +^p$
p'	0.0	Kawaguchi, 1942	$+^p > p' > p$
$+^{p1}$	0.0	Tanaka, 1942	$+^{p1} > +^{p3} > +^{p2} > +^{p4}$ allelic
$+^{p2}$	0.0	Tanaka, 1942	....
$+^{p3}$	0.0	Tanaka, 1942	Standard type
$+^{p4}$	0.0	Tanaka, 1942	Accompanies black pupa
p^{Sa3}	0.0	Tazima, 1943	p^{Sa3}–*Y*, 23.0; $p^{Sa3} = p^s$, allelic
p^{St}	0.0	Takasaki, 1947	p^{St}–*Y*, 21.8; $p^s > p^{St} = +^p > p$
p^D	0.0	Shimodaira	p^D–*Y*, 22.3; $p^D > +^p$
p^{Ma}	0.0	Takasaki and Mitarai, 1944	p^{Ma}–*Y*, 25.8; $p^{Ma} = p^s = p^M > +^p$
Ae	4.9	Kei, 1943	*Ae*–$+^p$, 4.89; *Ae*–*Y*, 19.6; probably allelic to *Gr*
S	6.1	Tanaka, 1929, 1933	*S*–$+^p$, 6.1; inhibits *Gr-16*
S^d	6.1	Tanaka, 1929, 1933	$S > S^d$, allelic
S^{1a}	6.1	Tanaka, 1929, 1933	Allelic to *S* and S^d
S^w	6.1	Chikushi, 1938	p–S^w–*Y*, 2.87 and 17.14; S^w–*Y*, 20.76
S^s	6.1	Takasaki, 1947	p^s–S^s–*Y*, 1.5 and 21.4; p^M–S^s–*Y*, 1.8 and 20.7; allelic to *S*
Gr	6.9	Tanaka, 1942, 1943	Not inhibited by *S*
Gr-16	6.9	Takasaki, 1947	$+^p$–*Gr-16*–*Y*, 4.1 and 16.4; allelic to *Gr*
Y	25.6	Tanaka, 1913b, 1916 1925	.....
oal	26.7	Takasaki, 1940	$+^p$ (p^s)–*Y*–*oal*, 26.0 and 1.1
Rc	31.8	Kei, 1943	*Rc*–$+^p$, 31.82; *Rc*–*Ae*, 26.43; *Rc*–*Y*, 4.44

Takasaki (1947) found a light gray egg (*Lg*) which always accompanies p^{Sa}. This type shows the normal egg color whenever the p^{S} region of chromosome II is deficient, but it exhibits the gray color if the deficiency is compensated by a normal chromosome II or by a fragment of the same chromosome.

c. *Chromosome III.* A considerable degree of confusion existed in this group. Ogura (1922) was the first to find a linkage between zebra (*Ze*) and *w* (*lem*). Tanaka (1927) used the symbol *l* (lemon) for *w*, but this was misprinted as *I* in his chromosome map. Kosminsky (1932) questioned the linkage between *Ze* and *I* (inhibitor of the yellow blood *Y*) and represented the lemon by *v*, describing the *Ze–v* linkage value as 22.97. Delmas (1935) reported the linkage value between *Ze* and *j* (jaune, i.e., lemon) as 19.1% and considered that Tanaka's map to be correct. Meanwhile, some Japanese authors had drawn a map putting *v*, without understanding what *v* means, on the opposite side of *lem*, so that chromosome III was made twice the actual length. Moreover, *v* was again mistaken for a lethal gene. This confusion was straightened out by Hirobe (1944), and the present map has been drawn by him.

Genes in *Ze*-Linkage Group

Symbol	*Locus*	*References*	*Remarks*
Ze	0.0	Ogura, 1922	*Ze–lem*, 20.83
		Kosminsky, 1932	*Ze–lem*, 22.97
		Delmas, 1935	*Ze–lem*, 18.4–19.1
ap	2.7±	Hirobe, 1944	*ap–Ze*, 2.7
l-III	10.0±	Hasimoto, 1940	*l-III–Ze*, 10.0±
let	20.4±	Kosminsky, 1932	*let–Ze*, 20.37; data insufficient
lem	20.8	Ogura, 1922 (*w*)	Kosminsky's *v*, Delmas' *j*

The locus of the lethal described by Bobrow and Friesen is not yet determined.

d. *Chromosome IV.*

Genes in *L*-Linkage Group

Symbol	*Locus*	*References*	*Remarks*
L	0.0	Tanaka and Matsuno, 1927	*L–sk*, 24.47–27.07
sk	25.8	Tanaka and Matsuno, 1927	.....

2. *Aberrations in Chromosome II*

a. *Monosomics and Trisomics.* Takasaki and Tazima (1944, and in press) obtained a few monosomics and trisomics of chromosome II in X-ray experiments. In the case of trisomics, each chromosome II was marked differently. The experiments led to the conclusion that three II chromosomes were complete and had synapsed freely with each other. Under the microscope, the trisomic strain sometimes showed an extra small univalent chromosome. In the cases in which the small chromosome was not present, a trivalent chromosome existed, as was proved experimentally, though it could not be detected microscopically. In the monosomic strain, 1 univalent and 27 bivalent chromosomes can be seen.

It is interesting to note that chromosome II seems to be one of the smallest cytologically, as inferred from the examination of trisomics, translocations, and other aberrations, but in spite of this, its chromosome map is the longest (Kawaguchi, 1936; Kawaguchi and Suzuki, 1946, 1947).

b. *Fragmentation.* (i) *Dark rusty marking.* This new marking was obtained from centrifuged eggs by Kawaguchi (1936); a small chromosome fragment is always present. This fragment perhaps originated as a chromatid of chromosome II and remains as a monad without splitting in maturation divisions, so that one-fourth of the gametes receive the fragment and in three-fourths it is absent, thus resulting in a ratio of 1 dark rusty to 3 normal in the backcross, and 7 to 9 in the F_2.

(ii) *X-ray mottling.* Besides natural mottlings, a number of mottlings produced by X-ray irradiation are now known. They are *S*-mottled (Tanaka, Kogure, Aruga, Takasaki), *od*-mottled (Hasimoto, Aruga, Tazima), *os*-mottled (Morohoshi, Tazima), and *M*- and *Ze*-mottled (Aruga). These are all due to a chromosome fragment or fragments bearing corresponding dominant genes which are occasionally eliminated in somatic divisions. These fragments are always situated in the periphery of the spindle in cell division and were assumed to lack the spindle fibers (Morohoshi, 1938; Takasaki, 1938). Later Takasaki (in press) proved the existence of the mutual translocation between the fragments and some unknown chromosome, thus showing that they possess spindle fibers. They are eliminated together with the unknown chromosome. The elimination of chromosomes with the spindle fiber has been observed by Takasaki and Tazima.

c. *Translocation.* Kawaguchi (1936) obtained a mutation by centrifuging; the left portion of the second chromosome was translocated to one of the non-homologous autosomes of a moderate size. This trans-

location is fairly stable, and crossing over occurs between it and the normal chromosome II with a frequency of about 3.6%.

Aruga (1940, 1949) reported a case of a single translocation (M^4) which he obtained in an X-rayed strain. This is a translocation of the p^M region to another autosome, resulting in a complete loss of linkage between Y and p^M. Tazima (1943) observed that attached p^{Sa} chromosomes became tetrasomic by non-disjunction and that they segregated quite independently from free second chromosomes. Microscopic study showed the presence of only 28 chromosomes. This indicates that the attached chromosomes are translocated to some autosome. For a description of the W chromosome translocation, the readers are referred to Section IV, Induced Mutations. Aruga's (1940) finding of tripartite chromosomes suggest a complex segmental interchange between unknown chromosomes.

d. *Duplication.* Several examples of supposed duplication have been reported by Tazima, Aruga, and Takasaki. One of the characteristic effects of duplication is the change of intensity of markings. Takasaki (1947) listed the phenotypic effects of the duplicated fragments of the left end of the chromosome II containing p^S gene as follows:

A List of Phenotypic Effects of the Duplicated p^s Locus (Takasaki, 1947)

Darker than p^s/p^s $(2n)$	Equal to p^s/p^s $(2n)$	Equal to p^s/p $(2n)$	Lighter than p^s/p $(2n)$
$p^s/p^s/p^s$ $(2n+1)$	$p^s/p^s/p$ $(2n+1)$	$p^s/p/p$ $(2n+1)$	
$p^s/p^s/p^s/p$ $(2n+2)$	$p^s/p^s/p/p$ $(2n+2)$	$p^s/p/p/p$ $(2n+2)$	
	$p^s/p^s/p^s$ $(3n)$	$p^s/p^s/p$ $(3n)$	$p^s/p/p$ $(3n)$

According to Takasaki, the duplication does not exert a lethal effect.

e. *Compensating Action of Deficient Chromosomes.* As long as the chromosome fragments of a single chromosome are retained in the same cell, they compensate each other physiologically although the linkage is lost between them (Kogure, 1936). Takasaki (1940) determined growth rates of various combinations involving different deficiencies of chromosome II, as follows: $+^p--/-Y+^{oal}/+++ \geqq -Y+^{oal}/+++ > +^p--> +^p--/-Y+^{oal} > 0/+++$ (where $+^p--$ represents a chromosome II deficient for the right half and $-Y+^{oal}$ denotes a chromosome II deficient for the left half). He also proved the compensating action of Sm fragment with a chromosome II deficient for the left portion.

f. *Size of Duplications and Deficiencies.* The length of a deficiency of chromosome II ($-Y+^{oal}$) may be measured by the crossing-over value

in the *p-Y* region, while the size of the Sm duplication may be estimated by the crossing over with the normal chromosome II. The size of the Sm fragment can also be inferred from the frequency of its elimination because it may be assumed that the smaller the size, the higher the frequency of elimination. A third method of measurement is microscopic

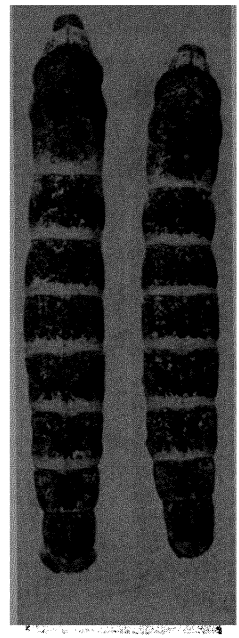


FIG. 20. S-mottled, larva.

examination. Takasaki (1944, and in press) believes that none of these methods gives an exact result. He put forth a so-called compensation method. Suppose the chromosome-II constitutions of *x* and *y* strains to be $(Sm)^x/(-Y)^x/(-Y)^x$ and $(Sm)^y/(-Y)^y/(-Y)^y$, respectively. Cross these strains with each other and produce $(Sm)^y/(-Y)^x/(-Y)^x$ and $(Sm)^x/(-Y)^y/(-Y)^y$ by recombination, and observe which of them survives. If the former combination survives and the latter does not, that would show that the $(Sm)^y$ fragment is larger than the $(Sm)^x$. The merit of this method is that any comparative difference, however small, between fragments in given strains can be detected, though the actual

length is not exactly known. When the Sm fragment is comparatively small in *x* strain, the *Y*-deficient chromosome must be comparatively large in the same strain. In this way, Takasaki tested several different strains and listed fragments and deficient chromosomes according to their sizes.

3. *Non-Disjunction*

Tanaka (1939) described a "low sex ratio" strain in which males are usually less numerous than females and sex ratios are often irregular, i.e., sometimes equal to or even higher than 100. By crossing this strain to a sex-linked recessive elongate (*e*), a certain number of exceptions appeared which could be explained by non-disjunction of the Z

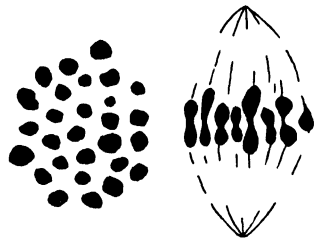


FIG. 21. Chromosomes in the first maturation division of the ZO male (from Tanaka, 1939). A monosomic chromosome (presumably Z) is clearly seen in the side view to the right).

chromosome in oogenesis. If the non-disjunction occurs at the maturation division of an egg, ZW and 0 eggs will be produced, and hence ZZW and Z0 zygotes will be formed after fertilization by Z sperm. All exceptions could be accounted for by the assumption that Z0 is male, but the fate of ZZW is not yet known. The frequency of non-disjunction was counted as about 8%. This assumption found cytological support when Tanaka observed a monad (presumably Z chromosome) in the lateral view of the reduction division of the first spermatocyte in an exceptional male. The Z0 males are retarded in growth and much smaller than the normal (a little over one-third of normal in body weight); they are apt to die or to be lost during culture, especially under unfavorable conditions, before identification of the sex becomes possible in the last instar. The irregularity of sex ratios is perhaps due to the fluctuating viability of Z0 males. From the size of the monad, Tanaka estimated that Z is one of the medium-sized chromosomes in the silkworm.

Tazima (1938, 1943) found many examples of non-disjunction of

chromosome II in his p^{Sa} strain. The non-disjunction occurs in both sexes, though it is less frequent in the female, the frequency being 0.23% on the average. He later ascertained that two chromosomes II which go to the same pole by non-disjunction are always attached at the left end, and the attachment is usually stable, but occasionally dissociation occurs and the chromosome II with the p^{Sa} gene and that bearing the $+^{p}$ gene are detached. Tazima (1943, 1944) also observed an additional non-disjunction which occurred in the individual with $\overset{\frown}{\text{II II}}$ II $(2n + 1)$ chromosomes to give rise to $\overset{\frown}{\text{II II}}$ II and 0 gametes; hence the former produced, after fertilization by the normal gamete, $\overset{\frown}{\text{II II}}$ II II $(2n + 2)$ zygote.

Takasaki (1940) observed a frequent non-disjunction in the deficient chromosome II. The frequency was much higher in the male and in extreme cases reached 50%.

In addition to non-disjunction occurring in the germ tract, many somatic non-disjunctions were observed. Takasaki (1942) found in the integument of larvae heterozygous for Sm fragment, Sm/*p*/*p*, adjoining

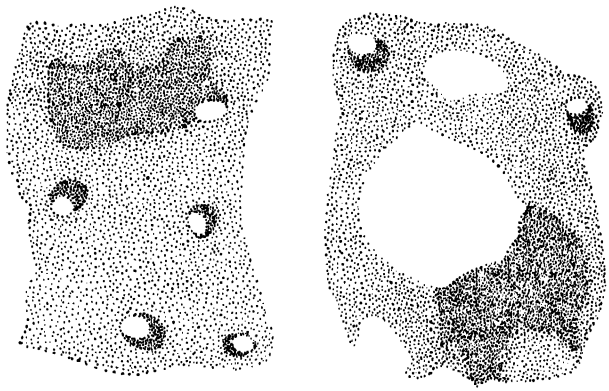


FIG. 22. Twin patches in the skin due to non-disjunction in the somatic cells of larvae with Sm fragment (from Takasaki, 1942). Darker patches are Sm/Sm/*p*/*p*, lighter areas are Sm/*p*/*p*, and white patches are *p*/*p*.

twin patches, one of which was dark colored and the other white. The dark-colored patch was Sm/Sm/*p*/*p*, and the white portion *p*/*p* was derived from Sm/*p*/*p* by a somatic non-disjunction. The portion consisting of Sm/*p*/*p* is lightly dark-colored. The area of Sm/Sm/*p*/*p* and

p/p patches on the skin of Sm/*p/p* animals was about 12.5%, and that of Sm/*p/p* portion about 87.5%.

4. *Elimination*

Sm is a fragment of the left end of chromosome II which was found in an X-ray experiment. This fragment is frequently eliminated in somatic as well as germinal cells. There are known several mottled translucent strains which are produced by repeated somatic elimination of the chromosome bearing the dominant opaque gene allelic to the recessive translucent. In both Sm strain and mottled translucent, the germinal elimination is considerably more frequent in males than in females, while no sexual difference is noticeable in the somatic elimination. There seems to be some parallelism between elimination frequencies in somatic and in germinal cells.

In some strains, the mosaicism often appears more or less symmetrically. Takasaki (1948, 1949) tested the offspring of many individuals of such symmetrical type and came to the conclusion that those which are provided with more white patches on the ventral side give more white offspring lacking Sm, while the phenotype of the dorsal side has no relation to the segregation in the offspring. According to him, the observed sexual difference in the elimination frequencies may be due to a larger number of cell divisions in the spermatogenesis than in the oogenesis, the elimination taking place in each cell division.

5. *Polyploidy*

Tanaka (1928) found a few exceptional females in a mosaic strain and attributed them to polyploidy. Goldschmidt and Katsuki (1928) observed the formation of triploid egg nuclei in a mosaic strain and their union with haploid (normal) sperm nuclei. Since then numerous triploid, tetraploid, and hexaploid silkworms have been obtained by Tanaka and Kawaguchi, Hasimoto, Sato, Kawaguchi, Hirobe, and Tsujita by centrifugation, high and low temperatures, artificial parthenogenesis, and colchicine. The triploid is completely sterile because of abnormal chromosome distribution in maturation divisions. The tetraploid is otherwise normal, but some times tetravalent chromosomes are formed, which seem to affect fertility. The frequencies of the tetravalent formation in males and females are 3.7% and 1.0%, and the fertility percentages in males and females are 18.8% and 79.6%, respectively (Kawaguchi, 1938).

Tazima (1938, 1943) described a mixoploidy in his strain with attached chromosomes II—one of the first spermatocytes showed 56 bivalent chromosomes. In one of the strains with aberrant chromosomes,

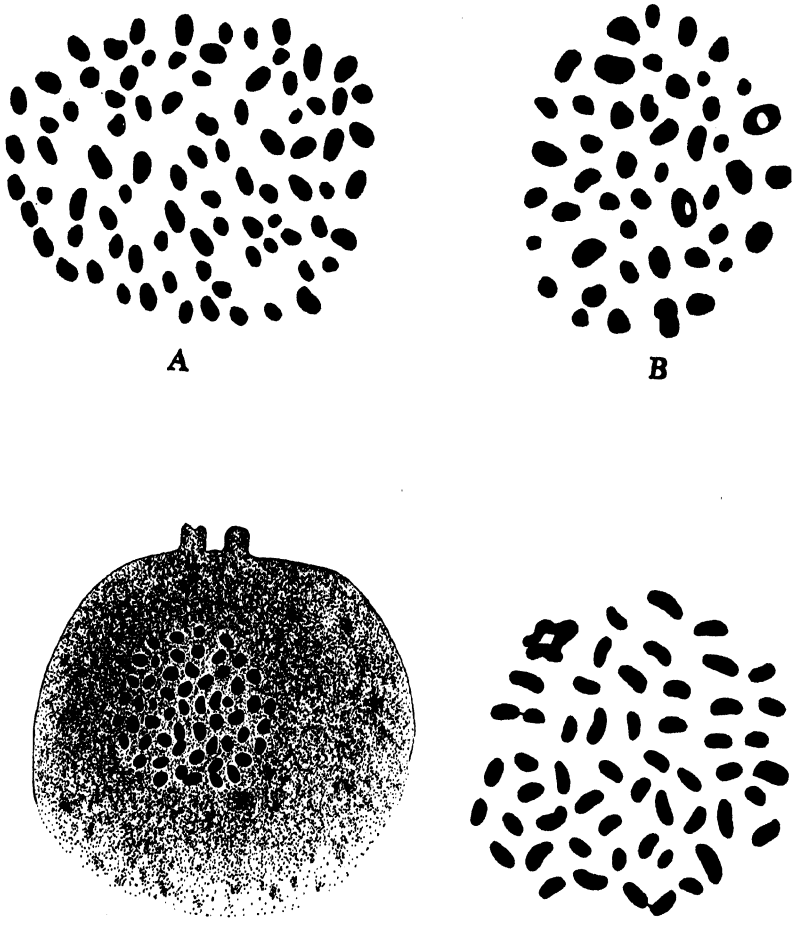


FIG. 23. Polyploidy (from Kawaguchi, 1938). *A*, Triploid spermatogonial division (84 chromosomes). *B*, equational plate of the first maturation division of triploid spermatocyte. *C*, first maturation division of tetraploid spermatocyte (56 chromosomes), drawn from microphotograph. *D*, first maturation division of tetraploid oöcyte.

dark-striped 1, Aruga (1939) counted 115 chromosomes in a first spermatocyte mixed among cells with the normal number. The same author (1940) reported a spermatocyte with diploid chromosomes, 6 of which formed two tripartite sets.

6. *Aneuploidy*

Monosomics of the Z chromosome and those of chromosome II are all retarded in growth, small in size, and liable to die or to be lost in the larval stage.

Tazima's strain with the attached chromosomes II is usually accompanied by one free chromosome II to form $\overset{\frown}{\text{II II}}$ II. The attached chromosomes sometimes dissociate and give rise to three independent chromosomes II II II, a typical trisomic $2n + 1$. In the metaphase of the first maturation division of $2n + 1$ individuals, there are usually seen 28 diads and 1 monad, the latter going to one or the other pole without dividing. In certain cases, however, a trivalent chromosome is formed, as has been observed by Tsujita (1949) cytologically and by Takasaki (1944, and in press) experimentally. The extra chromosome, as a rule, goes to the pole simultaneously with other chromosomes, but sometimes its retardation or precosity is observed. When retarded, it seems to be lost, as was proved by Takasaki experimentally. It has been ascertained that the crossing-over takes place with an equal chance between three homologous chromosomes II II II, each of which is marked with different genes.

VI. Sex Determination

1. *Sex-Linked Inheritance*

The first case of sex linkage was found by Tanaka (1917) in the inheritance of *os* translucent in an Italian race, Giallo puro indigeno. He (1923, 1926) discovered a second (*e*) and third (*od*) sex-linked genes; this was followed by Aruga's giant egg (*Ge*) and Tazima's vestigial wing (V_g). All of them are inherited according to the *Abraxas* type, and the sex-chromosome composition is ZZ (male) and ZW (female), the diploid number of chromosomes being 56 in both sexes.

2. *Sex-Linked Lethals*

Tanaka described two sex-linked lethals which do not show any morphological effect, *l-a* and *l-b*. Nishikawa reported four sex-linked lethals. Tanaka is now working to identify these lethals. Tazima's V_g is a dominant sex-linked character with a recessive lethal action.

3. *Sex-Limited Inheritance*

Several strains in which an autosome was translocated to the W chromosome have been obtained by Tazima and Hasimoto. If the W-translocated autosome bears a dominant gene, and that female is crossed

to a recessive male, the dominant character will be transmitted from the mother to daughters only.

4. *Cytological Identification of Sex Chromosomes*

In the gametogenesis of the silkworm, we do not find any morphologically distinct chromosomes which may be the sex chromosome. Kawaguchi (1928, 1933) found one or two chromosomes partly embedded in the nucleolus of the ovarian cell before differentiating into the egg or nutritive cell. Similar figures have been observed by him in many other

FIG. 24. Supposed sex chromosomes connected with the nucleolus (from Kawaguchi, 1938). Nuclei in the oöcytes before differentiation into egg and nutritive cells.

Lepidopterous insects. He also showed that in triploids three chromosomes are connected with one nucleolus, and he came to the conclusion that the chromosomes in question are Z and W. They become detached from the nucleolus later, condense, and behave like other chromosomes in nuclear divisions.

5. *Sex-Determining Capacity of W Chromosome*

There is a marked contrast between the Y chromosome in *Drosophila* and the W chromosome in *Bombyx*. In *Drosophila* Y has no positive male-determining effect, and X0 individual is male only because of the presence of one X, whereas in *Bombyx* W has a positive female-determining power, and Z0 is male because of the absence of W. The proof for this conclusion is as follows.

(i) Tanaka's case (mentioned above) in which the Z0 individual was male experimentally and cytologically.

(ii) Tazima (in press) X-rayed $+^{od}\cdot\widehat{W\cdot+^{p}\cdot p^{sa}}$ females which possess translocated chromosomes marked with distinct genes then mated them to *od/od, p/p* males with ordinary diploid chromosomes. As a result of irradiation, the repeated somatic elimination of one or more translocated chromosomes occurred in the same individuals. These animals proved to be complex gynandromorphs, and showed that the cells

from which W chromosome (together with translocated chromosomes $+^{od}$, $+^{p}$, or p^{sa}) is lost, are transformed into the male cells even if the individual started its development as a female.

(iii) ZZW trisomic individuals produced by non-disjunction were all females (Tazima, 1941).

(iv) The sex-chromosome composition of polyploids is described by Hasimoto and Kawaguchi. Tazima, experimenting with his W-translocated strain, came to the following conclusion:

Triploid		*Tetraploid*	
3A + ZZ	♂	4A + ZZZW	♀
3A + ZZZ	♂	4A + ZZWW	♀
3A + ZZW	♀		
3A + ZWW	dies		
3A + ZZZW	♀		

6. *Racial Difference*

Are there any racial differences in sexual potency in different races of the silkworm comparable to those in *Lymantria*? The answer to this question is: No! A large number of intercrossings between different races (sometimes remote) was carried out, but no case of intersex has yet been found. The case which Tanaka (1929) described as an "intersex" now seems to be better explained as a gynandromorph. To test this, Tazima repeatedly crossed males of several Japanese and Chinese races to females of a W-translocation strain for more than ten generations without finding a single intersex.

7. *Location of the Male-Determining Gene in Z*

Tazima (1944, and in press) succeeded in producing Z chromosomes with different deficiencies, Z^{rd} (lacking the right end), Z^{md} (the middle portion deleted), Z^{ld} (the left end lost), Z^{r} (the right end fragment), and Z^{l} (the left end fragment). Sexes observed in various combinations of these deficient Z chromosomes are as shown. No abnormality

2A + Z^{+} Z^{ld}	♂	2A + Z^{+} $\overset{\frown}{Z^{r}W}$	♀
2A + Z^{+} Z^{md}	♂	2A + Z^{+} $ZZ^{l}W$	♀
2A + Z^{+} Z^{rd}	♂	2A + Z^{+} $ZZ^{r}W$	♀

in sexual characters was seen in these individuals. All females with one deficient Z (hypoploid) die irrespective of deleted portions, whereas in hyperploid types which have an extra fragment both sexes survive and do not show any abnormal sexual characteristics.

8. *Location of the Female-Determining Gene in W*

Tazima assumes that the translocation $\widehat{Z^{+} \cdot W \cdot +^{p} \cdot p^{Sa}}$ is probably a product of crossing over between Z and W in a $\widehat{W \cdot +^{p} \cdot p^{Sa}}/Z^{+}$ female. If this supposition is correct, a W chromosome which has lost a part by crossing over still retains its female-determining power. He also obtained a mosaic from a $\widehat{Z^{+} \cdot W \cdot +^{p} \cdot p^{Sa}}$ female as a dissociation induced by X-raying. That mosaic had a $+^{p} \cdot p^{Sa}$ constitution and simultaneously was a gynandromorph. According to him, the individual was $\widehat{W \cdot +^{p} \cdot p^{Sa}}$ to start with, but the mosaicism and gynandromorphism, were brought about by the elimination of the $\widehat{W \cdot +^{p} \cdot p^{Sa}}$-chromosome in somatic cells. On basis of these observations, Tazima believes that the female gene or genes might be concentrated in a certain portion of W chromosome, though the exact location is as yet impossible to determine.

In summing up all reported data on sex determination in the silkworm, we must admit that the female-determining capacity of the W chromosome is unusually strong, and everywhere that W is present femaleness shows up, whereas if W is absent maleness is brought about, irrespective of the number of Z chromosomes. It is evident, therefore, that *Bombyx* is different from *Lymantria* so far as the mode of sex determination is concerned.

VII. Embryological Genetics

1. *Parthenogenesis*

The development of unfertilized eggs takes place quite frequently in the silkworm, as was reported by several authors during the past sixty years. This problem was studied in detail by Sato and Kawaguchi. Kawaguchi (1934) obtained 0.0003 to 0.003% larvae from unfertilized eggs through parthenogenesis. Sato (1925) observed that parthenogenesis could be accelerated by immersing the eggs in a warm solution of hydrochloric acid. Similar results were obtained by centrifugation (Kawaguchi) and by immersing eggs in hot water (Hasimoto).

Parthenogenetic larvae are generally weak and less viable and often show various abnormalities in the male gonad, whereas a vigorous male can sometimes fertilize several females. Parthenogenetic females lay as many eggs as normal females.

In the first division of the haploid nucleus of a parthenogenetic egg,

two daughter nuclei unite, and the diploidy is re-established. If that does not happen, embryonal development cannot proceed beyond the blastoderm stage. Triploid and tetraploid cells are often found in parthenogenetic silkworms.

Both sexes are produced by parthenogenesis, though males are more numerous than females. The formation of males can easily be accounted for by the doubling of haploid chromosomes (27A + Z). But how are females (54A + ZW) produced? Kawaguchi tried to explain it by fusion of a polar body with the egg nucleus. Sato, however, failed to find the "polar body fertilization" cytologically and came to the conclusion that females are products of the non-disjunction of Z and W chromosomes. As to autosomal characters, segregation which actually occurs can take place as follows. Suppose that the female moth was heterozygous *Nn*. After the maturation divisions, some eggs will retain *N* and some others *n*. By doubling of chromosomes *NN* and *nn*, embryos will result, hence segregation of dominant and recessive characters occurs in nearly equal numbers.

2. *Merogony*

Hasimoto (1929, 1934) was the first to find merogony in the silkworm. He obtained a number of exceptional males by exposing the eggs from the cross +/+, +/+ ♀ × *od/od*, E^{Kp}/+ ♂ to a high temperature within one hour after deposition. The exceptional males were all homozygous for the sex-linked recessive gene *od* translucent. In regard to the autosomal dominant gene E^{Kp}, three phenotypes appeared among exceptional males—namely, E^{Kp}/E^{Kp}, E^{Kp}/+, and +/+, in the ratio 1:2:1. This result is explicable by assuming that the egg nuclei were inactivated by the high temperature, and the exceptional males developed from the union of two sperm nuclei. This conclusion is supported by cytologically observed polyspermy (Kawaguchi, 1926). Since then, Astaurov (1937), Tazima (1939), and Ichikawa (1947) observed numerous exceptions evidently derived by merogony by temperature shocks. Sato (1942) observed the union of two sperm nuclei cytologically. The exceptional males had diploid chromosomes and were quite normal in fertility.

3. *Mosaicism*

Mosaicism occurs frequently in the silkworm. It is brought about by different causes.

a. *Mosaicism Due to a Gene Mutation.* When a mutation takes place in one of the segmentation nuclei, a mosaic will result. If the mutation occurs at the two-cell stage, a bilateral mosaic will be produced. For in-

stance, Tanaka's bilateral p^s-S mosaic and Tazima's (1942) egg-color mosaics are considered to be due to spontaneous mutations. If, on the contrary, the somatic mutation occurs at a later stage, the mutated area will become smaller. The mottled characters due to mutable genes fall into the same category.

b. *Mosaicism Due to Chromosome Aberrations.* (i) *Mosaics due to elimination of a chromosome fragment.* Examples of this are *S*-mottled, *os*-mottled.

(ii) *Mosaics due to polyploidy.* During embryonal development, divisions of certain cells get disturbed and polyploid cells are produced, hence a mixoploidy or a mosaic of the ploidy; for example, Hirobe's colchicine product.

c. *Mosaicism Due to Unusual Modes of Fertilization.* (i) *Binucleated egg.* When a mature egg retains the second polar nucleus, it will have two nuclei, each of which is fertilized by a sperm and constructs half the future body; for example, the hereditary mosaicism of Goldschmidt and Katsuki. Many mosaics produced by artificial stimuli might belong to this category.

(ii) *Polar-body fertilization.* One side of the embryo originates from the ordinarily fertilized egg nucleus, the other side from the union of one of two nuclei produced by the division of the first polar body with the second polar-body nucleus. Such a phenomenon is observed, though rarely, when eggs are exposed to high temperatures just after laying.

(iii) *Dispermic merogony.* A part of an embryo develops from the fertilized egg nucleus and the other part from the union of two sperm nuclei, hence a gynandromorph or a mosaic.

Why are the two embryos not formed within the same egg in these cases? Tazima attributes this to the more important role played by the egg protoplasm than by the nucleus in the differentiation of the embryonal body (predetermination), especially in the early development of an insect egg.

4. *Hereditary Monstrosities*

It is not rare for a considerable number of monstrosities to appear among the silkworm larvae hatched by an artificial stimulus, i.e., immersing into hydrochloric acid solution. The majority of these monstrosities are, however, non-hereditary. Tanaka has kept several strains of hereditary malformations for over twenty years. The penetrance in these strains is rarely 100%, but it is often very low. The type of abnormality in the same strain is usually not fixed but is variable. Apparently normal individuals give the same results in succeeding

generations as the malformed ones, even after a continued selection. Selection for a specific type of malformation is not effective. Abnormalities in these strains are concerned with the external organs (integument, segments, thoracic legs, abdominal legs, ocelli, spiracles, and caudal horn) as well as the internal organs (muscles, ganglions, dorsal vessels, trachea, silk glands, Malpighian vessels). The internal abnormalities sometimes coincide with certain external ones but sometimes do not. Several reported examples of hereditary monstrosities are given below.

a. *Hind-Body Abnormality.* The abnormality occurs chiefly in 5th abdominal segment and its vicinity; penetrance is 30 to 90%, mostly 50 to 60%. The F_1 of the cross with the normal strain is normal. Segregation occurs in the F_2 and later generations, but the percentage of monstrosity is comparatively low (Omura, 1949).

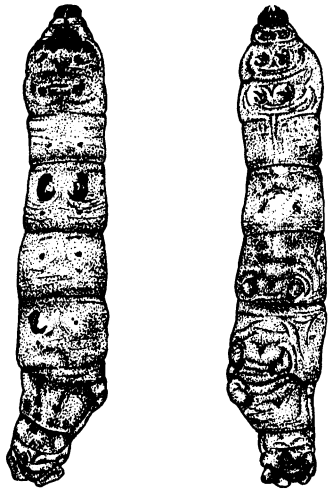


FIG. 25. Hereditary monster (from Omura, 1949). Dorsal (*left*) and ventral (*right*) sides of the same individual.

b. *Abnormal Corselet, Ac.* This is a fissure along the dorsal median line of the second thoracic segment. It is a recessive lethal, X-ray induced (Aruga, 1939).

c. *Abnormal Segment, As.* The anterior four abdominal segments are constricted on the level of the intersegmental membrane. This is an incomplete dominant to the normal, a recessive lethal. It suppresses the knobbed gene. It is X-ray induced. Possibly it is a chromosome aberration (Aruga, 1943).

d. *Vomitor, vo(ex)*. This is normal when young. In the fifth instar the larva vomits gastric fluid during or after feeding. Owing to the poor development of the mandibular muscles, mulberrry leaves are swallowed in larger pieces than by normal larvae, and the large pieces interrupt the closure of cardiac valves, hence causing vomiting. This is a recessive spontaneous mutation (Nakano, 1948).

e. *Apodal, ap.* All thoracic legs are rudimentary, and consequently feeding is somewhat difficult. Mortality is high, especially during ecdysis and pupation. The moth is unable to walk. Male moths can be assisted to copulate and are fertile, but females are completely sterile (Hirobe, 1944).

f. *Hereditary Mosaic, mo.* Owing to habitual retrogression of the polar body, double-nucleated eggs are formed, hence mosaics and gynandromorphs. Reproductive organs are malformed as a rule in gynandromorphs.

The influence of external conditions on the occurrence of malformations, hereditary and non-hereditary, is striking. In the hereditary naked pupa strain, which does not spin a cocoon owing to the degen erated silk glands, Takahashi (1949) found the following percentages of naked pupae in four lots: all instars reared in a high temperature, 68.4%; 1 to 3 instars reared in a high temperature, 4 to 5 instars, low temperature, 82.4%; all instars reared in a low temperature, 8.2%; 1 to 3 instars reared in a low temperature, 4 to 5 instars, high temperature, 0%.

In his monstrosity strain, Umeya (1949) noticed that the monstrosity percentage went up to 21.1% when the eggs were treated with hydrochloric acid 20 hours after deposition, whereas the percentage decreased if the acid was applied earlier or later. F_1 eggs from the cross monster ♀ × normal ♂ give rise to numerous monstrosities after the acid treatment, but F_1 eggs from the reciprocal cross do not. F_2 of either cross produces numerous abnormals under proper conditions. According to Umeya, this characteristic is a maternally inherited dominant.

5. *E Allelic Group*

E is one of the two largest allelic groups in the silkworm. The characteristics of each allele belonging to this group are described in Section II. Their common characteristics may be enumerated as follows: (*a*) These alleles act pleiotropically in various stages of the life cycle, and many of them manifest morphologic as well as physiological disorders in the insect. (*b*) Each allele seems to have its center of action; it affects only a definite segment but not the other although it may

be histologically similar. (*c*) The morphologic characters are all dominant, though sometimes the penetrance may be low, whereas the physiological character, the lethal action, is recessive. (*d*) Homozygotes are sometimes different from heterozygotes in certain characteristics. For example, E^{Kp}/E^{Kp} exhibits a pair of extra crescents on the third abdominal segment, but they are absent in $E^{Kp}/+$. In $E^{N}/+$ larvae, the extra crescents are present on the third abdominal segment and the star-

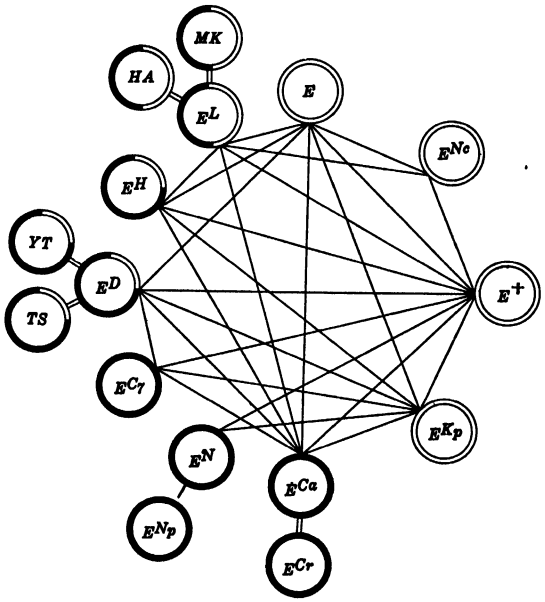


FIG. 26. Interrelations between *E* multiple-allelic genes (from Takasaki, in press). Genes connected by a single line have proved to be allelic; genes connected by two parallel lines are likely identical. Double circles mean absence of lethality. Thick circles represent complete lethality when homozygous, and thick arcs show partial lethals; the shorter the arc, the weaker the lethality.

spots are absent; the former characteristic is variable in penetrance, the latter more constant. On the contrary, the E^{N}/E^{N} embryo, which dies before the blastokinesis owing to the lethal action, has a pair of thoracic legs on each segment from the first to the eighth abdominal segments, 2 caudal horns, only 3 pairs of rudimentary spiracles, and a poorly developed tracheal system.

Takasaki (in press) proposed a hypothesis regarding the direction of genic action based on studies of the pleiotropic expression of charac-

teristics of genes belonging to the *E*-allelic group. He assumes the existence of polarity and direction of action for each gene. For example, E^{Ca} acts backwards, starting from the end of the first abdominal segment to the anal segment; thus the crescents on the second segment are repeated on the next segment to produce extra crescents, and the character of the fourth segment is moved to the next segment; hence star-spots on the fifth abdominal segment are erased. The star-spots often appear on the sixth and seventh segments.

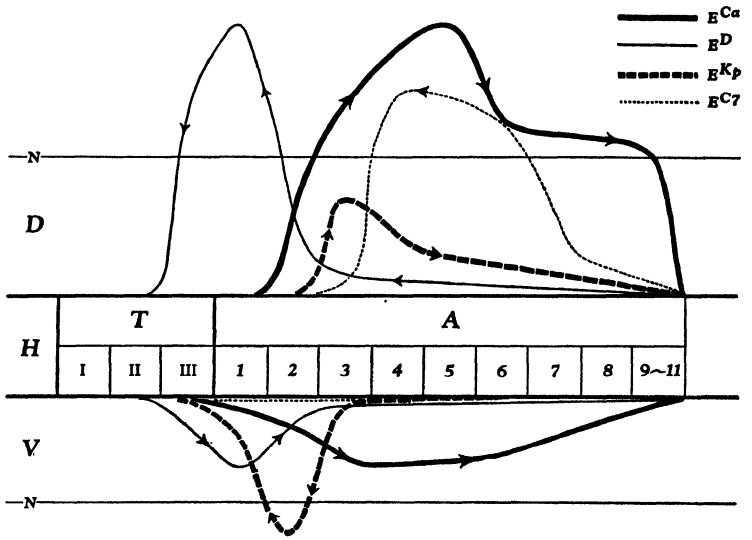


FIG. 27. Direction of the action of *E*-allelic genes (from Takasaki, in press). The directions and power of action not only of E^{Ca}, E^{D}, E^{Kp}, and E^{D^s} differ from each other, but they also differ on the dorsal (*D*) and ventral (*V*) sides; the heights of the curves designate the power of action in the thorax (*T*) and the abdomen (*A*). *H*, head; *N*, normal or standard line; I-III, thoracic segments; 1-11, abdominal segments of embryo.

The direction of action is definite for each gene. Certain genes act from anterior to posterior, others in a reverse direction. Still others act from anterior to posterior on the dorsal side of the embryo, but from posterior to anterior on the ventral side. The capacity for action is different at different levels of the embryonal body. The capacity of E^{Ca} to move in a backward direction is strongest in the fifth abdominal segment and is weaker in the second segment, so the loss of the star-spots on the fifth segment is a constant feature of the phenotype, whereas the extra crescents on the third segment have variable penetrance.

VIII. Inheritance of Certain Practically Important Characters

1. *Voltinism*

There are known univoltine, bivoltine, quadrivoltine, and multivoltine races in the silkworm. "Quadrivoltine" and "multivoltine" are usually called "tetravoltine" and "polyvoltine," respectively. It seems to me preferable to designate them with prefixes of Latin origin and I think the terms "univoltine" and "bivoltine" should be retained. In the early days of silkworm genetics, voltinism was often conceived to be transmitted after some non-Mendelian fashion. Toyama (1913) described voltinism and some egg colors as inherited maternally. Since then, the gene analysis of voltinism and the effect of environment on it have been studied by various authors, especially Watanabe (1918, 1919), Kogure (1933), Nagatomo (1942), Muroga (1935–1948), and Morohoshi (1950). Recently Nagatomo (in press) established three sex-linked alleles, $+^{Hs}$, Hs, and Hs^2, and three autosomal genes, H_1, H_2, and H_3. The static relation of the sex-linked genes is $Hs > Hs^2 > +^{Hs}$, and the epistasis is complete, while the autosomal genes have a cumulative effect which is proportional to the number of dominant genes. The hibernating power of each gene and the genotype of various races have been assumed to be as follows:

$Hs = 6$ $\quad Hs^2 = 3$ $\quad +^{Hs} = 0$

$H_1 = 1$ $\quad H_2 = 1$ $\quad H_3 = 1$

Univoltine $Hs\ H_1\ H_1\ H_2\ H_2\ H_3\ H_3$ (Hibernating value, (H.V.) = 12)

Bivoltine $Hs^2\ H_1\ H_1\ +^{Hs}\ +^{Hs}\ +^{H_3}\ +^{H_3}$ (H.V. = 5)

Pure multivoltine $+^{Hs}\ +^{H_1}\ +^{H_1}\ +^{H_2}\ +^{H_2}\ +^{H_3}\ +^{H_3}$ (H.V. = 0)

Univoltine females always lay hibernating eggs; bivoltine females lay hibernating eggs if they have been incubated at a high temperature (25°C.) and non-hibernating eggs if they have been incubated at a low temperature (15°C.). Multivoltine females always lay non-hibernating eggs. Several examples of crossing are given below; the hibernating values are in parentheses.

a. Cross between univoltine (U) and bivoltine (B)

U(12) ♀ × B(5) ♂ → ♀ (7) ♂ (10)

B(5) ♀ × U(12) ♂ → ♀ (10) ♂ (10)

b. Cross between univoltine (U) and multivoltine (M)

U(12) ♀ × M(0) ♂ → ♀ (3) ♂ (9)

M(0) ♀ × U(12) ♂ → ♀ (9) ♂ (9)

c. Cross between bivoltine (B) and multivoltine (M)

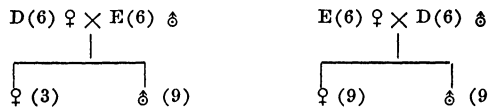
Nagatomo obtained the following three strains with different hibernating values by a recombination of voltinism genes.

Strain A: $Hs^s\ H_1\ H_1\ H_2\ H_2\ H_3\ +^{H_3}\ +^{H_3}$ (7)
H.V. stronger than the ordinary bivoltine.

Strain B: $+^{Hs}\ H_1\ H_1\ H_2\ H_2\ +^{H_3}\ +^{H_3}$ (4)
H.V. stronger than the ordinary quadrivoltine.

Strain C: $+^{Hs}\ H_1\ H_1\ +^{H_2}\ +^{H_2}\ +^{H_3}\ +^{H_3}$ (2)
It is difficult to make all eggs hibernate even at a low incubating temperature.

In addition, two bivolting strains which are different in genotypes but equal in H.V. have been developed. They are: strain D, $Hs\ +^{H_1}\ +^{H_1}\ +^{H_2}\ +^{H_2}\ +^{H_3}\ +^{H_3}$ (6); and strain E, $+^{Hs}\ H_1\ H_1\ H_2\ H_2\ H_3\ H_3$ (6). The F_1 generations of these strains are as follows:



This result is identical with that obtained by crossing univoltine and multivoltine. Various strains, including univoltine and multivoltine, with all possible H.V.'s, could be bred from the offspring of these crosses. Nagatomo grouped hibernating values into thirteen classes.

H.V.	*Voltinism*	*Characteristics*
0–1	Multivoltine	Always lay non-hibernating eggs
2–4	Intermediate between multivoltine and bivoltine	Lay some non-hibernating eggs at high incubating temperatures
5–7	Bivoltine	Lay non-hibernating eggs at low incubating temperatures, hibernating eggs at high incubating temperatures
8–10	Intermediate between bivoltine and univoltine	Lays both hibernating and non-hibernating eggs even at low incubating temperature
11–12	Univoltine	Always lay hibernating eggs

Morohoshi (1950) assumes that the hibernating value of the embryo is determined by the antagonistic action of two hormones secreted by the prothoracic glands and the corpora allata. The sex-linked hibernating genes control the secretion of prothoracic glands, whereas autosomal genes, which are located in chromosome VI, control the activity

of the corpora allata. Prothoracic gland hormone affects development or metamorphosis, and corpora allata hormone influences the growth of the animal. Voltinism as well as moltinism are both dependent upon the balance of these two kinds of hormones. Morohoshi visualizes their relation as follows:

	Prothoracic gland hormone (sex-linked genes)	*Corpora allata hormone* (autosomal genes)
Univoltine	Weak	Strong
Bivoltine	Intermediate	Intermediate
Multivoltine	Strong	Weak

2. *Moltinism*

Several genes for moltinism are known.

a. *Tetramolting,* $+^{M}$. This is the standard type; the larva spins a cocoon and pupates after the fourth molt.

b. *Trimolting,* M^{3}. The larva passes through three molts before spinning. This type is most common in primitive races and in the wild silkworm and it is a simple dominant to tetramolting (Toyama, 1912).

c. *Pentamolting,* M^{5}. The larva spins a cocoon after passing through five molts. This has been studied by Nakamura (1939, 1942), Ogura (1931), and others.

d. *Recessive Trimolting, mr.* This is a simple recessive to M^{3} and $+^{M}$. In crosses with M^{5} it gives only tetramolters in F_1; trimolters, tetramolters, and pentamolters are segregated in F_2. This condition is not yet fully analyzed (Asano, 1948). Chikushi (1949) also discovered a recessive trimolting strain.

Ogura (1931) found linkage between moltinism and a cocoon color (F) and the allelic relation between tri-, tetra-, and pentamolting. Nakamura (1942) observed, on the contrary, the segregation in a digenic ratio (12 trimolting : 3 tetramolting : 1 pentamolting) in the F_2 of the cross trimolting $\times$ pentamolting. Morohoshi (1939) supported Nakamura's findings. The discordance between Ogura and Nakamura is difficult to harmonize at present, but a possible explanation might be that genetically different trimolting and pentamolting strains exist. Morohoshi thinks that, in addition to autosomal genes, sex-linked genes take part in the determination of moltinism. Autosomal genes may be located not only in chromosome VI, but also in some other chromosomes. In addition, moltinism is easily modified by external conditions such as temperature, humidity, and light. Morohoshi (1950) thinks that quantitative differences are caused by two kinds of hormones as follows:

	Prothoracic gland hormone (sex-linked genes)	*Corpora allata hormone* (autosomal genes)
Trimolting	Strong	Strong
Tetramolting	Intermediate	Intermediate
Pentamolting	Weak	Weak

According to Morohoshi, if the races are identical in growth genes (corpora allata genes), a sex-linked inheritance of moltinism will be observed, whereas if they are identical in metamorphosis genes (prothoracic gland genes), the inheritance will not be sex linked.

Takasaki (1949) found that *E*-allelic genes differentially influence the change from tri- to tetramolting, the order of changing power being $E^{D} > E^{Oa} > +^{E}$.

3. *Cocoon and Silk Characters*

In crossing a univoltine or bivoltine race with a tetravoltine or multivoltine race, Nagatomo (1926, 1949), Shen (1926, 1928), Suzuki (1947), and Morohoshi (1949) observed sex linkage of various quantitative characters such as the length of larval life, the pupal weight, and the weight of cocoon layer. Nagatomo attributed this phenomenon to some sex-linked dominant *Lms* (sex-linked, late-developing) and its allele $+^{Lms}$ (early-developing). *Lms* retards development and is responsible for univoltine, tetramolting (*versus* trimolting), longer larval life, heavier pupal weight, heavier cocoon layer weight. According to Morohoshi, there are also autosomal genes responsible for those quantitative characters. The reason why sex linkage is not evident in crosses of related races is that they are identical in sex-linked genes affecting development.

The amount of floss silk which encloses the cocoon is at least partially influenced by a gene on the Z chromosome (Suzuki, 1947). Kobari (1940) selected proportion of the floss-silk weight to the total cocoon layer weight in + and − directions, and observed that the weight of total cocoon layer and the weight of reeled cocoon fiber are inversely proportional to the increase of floss silk, whereas cocoon-fiber thickness is directly proportional to it.

Superior neatness and lousiness-free characters of raw silk are incomplete dominants to inferior neatness and lousiness-rich characters.

4. *Inheritance of Defective Cocoons*

This title is, of course, an abridged form of the full title "inheritance of habits spinning defective cocoons."

a. *Open Cocoon.* The cocoon is often perforated on one or both

poles when the larva spins in cold and humid condition. But there are certain strains which hereditarily spin perforated cocoons even under favorable conditions (Takahashi, 1950; Kobari, 1938; and Suzuki, 1950). Suzuki carried out a selection experiment over twelve generations in which the open-cocoon percentage was increased to 90%. In crossing with a normal strain, F_1 cocoons varied from normal to the pointed form but were not open. It may be concluded, therefore, that the open cocoon is incompletely recessive to the normal. Kobari observed that open cocoons appear more frequently when the moth is exposed to a high temperature during the incubation period and young instars followed by a low temperature and high humidity during the spinning.

b. *Thin Equatorial Region.* The equatorial region of the cocoon is exceedingly thin so that the pupa is visible from outside through the net-like fibers. Kobari and Karasawa (1933) obtained a strain in which all cocoons became the thin-equatorial type after nine generations of selection. This character is inherited as an incomplete recessive; F_1 cocoons resemble normal more than thin-equatorial.

c. *Stained Cocoon.* Cocoons are often stained on the outside by various non-genetic factors. The stained cocoon in question is stained to a rusty or dark rusty color from inside, and this character is hereditary. (Kobari and Karasawa, 1937). The cause of staining is a weak acidic fluid excreted by the Malpighian vessels during the spinning.

5. *Heterosis*

The wide application of heterosis in the silkworm is matched only by that in corn. The practical value of heterosis was known to European silkworm raisers since the latter half of the nineteenth century. About forty years ago, the Sericultural Experiment Station of Japan started extensive experiments on this problem under the auspices of Toyama, and the results were published in 1917. This report shows that rearing period is shorter, mortality is lower, cocoon fiber is longer, reeled fiber weight is heavier, cocoon fiber is thicker, and double cocoon percentage is higher in F_1 generations compared with the averages of pure parental races. These results have been affirmed by later investigators (among others, Katsumata, 1948, and Morohoshi, 1948). Recently Osawa and Harada (1944) studied the value of heterosis statistically and established the following general rules. M = mean value of the parents, F = mean value of F_1, D = difference of values of male and female parents, δ = effect of heterosis $= F - M$.

a. F depends on M, but not on D.

b. If $D < 2\delta$, F is superior to either parent; if $D = 2\delta$, F is equal to the better one of the parents; if $D > 2\delta$, F is between the values of parents.

c. The greater is M, the smaller is δ.

The formula devised by them for calculation of F values from M are given below, in somewhat modified form.

Total cocoon weight	$F = 0.826M + 0.609$ g.
Duration of larval life	$F = 0.504M + 13.581$ hours
Mortality	$F = 0.424M + 3.007\%$
Double-cocoon rate	$F = 1.596M - 0.515$ %
Cocoon crop (good cocoons obtained from 10,000 hatched eggs)	$F = 0.697M + 8.010$ kg.
Cocoon-layer weight	$F = 0.845M + 11.404$cg.
Length of reeled cocoon fiber	$F = 0.737M + 318.18 \times 1.125$ m.
Weight of reeled cocoon fiber	$F = 0.875M + 9.519$ cg.
Thickness of reeled cocoon fiber	$F = 0.753M + 0.939$ denier

IX. References

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