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MONOGRAPHS ON BIOCHEMISTRY

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R. H. A. PLIMMER, D.Sc.

AND

SIR F. G. HOPKINS, M.A., M.B., D.Sc., F.R.S.

GENERAL PREFACE.

THE subject of Physiological Chemistry, or Biochemistry, is enlarging its borders to such an extent at the present time, that no single text-book upon the subject, without being cumbrous, can adequately deal with it as a whole, so as to give both a general and a detailed account of its present position. It is, moreover, difficult in the case of the larger text-books to keep abreast of so rapidly growing a science by means of new editions, and such volumes are therefore issued when much of their contents has become obsolete.

For this reason an attempt is being made to place this branch of science in a more accessible position by issuing a series of monographs upon the various chapters of the subject, each independent of and yet dependent upon the others, so that from time to time, as new material and the demand therefor necessitate, a new edition of each monograph can be issued without re-issuing the whole series. In this way, both the expenses of publication and the expense to the purchaser will be diminished, and by a moderate outlay it will be possible to obtain a full account of any particular subject as nearly current as possible.

The editors of these monographs have kept two objects in view: firstly, that each author should be himself working at the subject with which he deals; and, secondly, that a *Bibliography*, as complete as possible, should be included, in order to avoid cross references, which are apt to be wrongly cited, and in order that each monograph may yield full and independent information of the work which has been done upon the subject.

It has been decided as a general scheme that the volumes first issued shall deal with the pure chemistry of physiological products and with certain general aspects of the subject. Subsequent monographs will be devoted to such questions as the chemistry of special tissues and particular aspects of metabolism. So the series, if continued, will proceed from Physiological Chemistry to what may be now more properly termed Chemical Physiology. This will depend upon the success which the first series achieves, and upon the divisions of the subject which may be of interest at the time.

R. H. A. P.
F. G. H.

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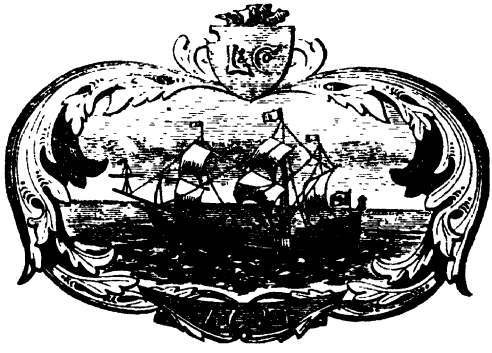
J. B. LEATHES, M.A., M.B., F.R.S.

PROFESSOR OF PHYSIOLOGY IN THE UNIVERSITY OF SHEFFIELD

AND

H. S. RAPER, C.B.E., M.B., CH.B., D.SC.

BRACKENBURY PROFESSOR OF PHYSIOLOGY IN THE UNIVERSITY OF MANCHESTER



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PREFACE TO SECOND EDITION.

Fifteen years have passed since the first edition of this monograph appeared and nearly ten since it has been out of print.

The issue of the monograph in this series on lecithine and allied substances by Professor H. Maclean has made any attempt at the systematic treatment of the phospholipines unnecessary. We have however attempted to treat of the physiology of fats in a less cursory way than was done in the first edition, so that what occupied in it barely a score of pages is now dealt with in seven chapters, forming the major part of the monograph, and the book is very considerably lengthened in consequence. We hope that its usefulness to those interested in the fats, whether they approach the subject from the chemical or from the physiological side, may have increased in proportion. We should again like to commend the study of the fats both to the chemist and the physiologist. The subject presents many problems which can only be attacked profitably by their co-operation, each contributing his special technique and outlook. It is only in this way that the part played by the fats in the animal or plant will be made manifest.

J. B. L.
H. S. R.

PREFACE TO FIRST EDITION.

THERE are many who study Physiology without appreciating how far the chemistry of the fats has been advanced by technical and scientific chemists. At the same time, there may be chemists, versed in the chemistry of fats, who do not appreciate all that this subject may mean to the biologist. This small book, which is aimed to reach both these classes of readers, and so runs the risk of missing both, must be considered to have attained its object if it proves useful to either. For the field of biochemical work to which it refers needs workers, who may be either physiologists who have trained themselves chemically, or chemists who are alive to the legitimate aspirations of biology.

J. B. L.

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INTRODUCTION.

THERE is a large group of substances widely distributed through the animal and vegetable kingdoms which have one property in common, that they contain in their chemical constitution and are mainly composed of the radicals of fatty acids, principally and generally only the higher ones. These compounds with but few well-attested exceptions form a group by themselves that does not overlap the borders of the other groups of substances, the proteins and carbohydrates, which together with them constitute the material in which the phenomena of life are manifested. They are at least as sharply defined a group as either of these, and they hardly can be said to have less physiological significance. And yet there is no one comprehensive name to include them all. The term fat has a precisely defined chemical meaning which restricts its application to a certain number only of the substances of this group, and yet many of these substances that are in the strict chemical sense fats have on account of their physical properties to be spoken of as oils, while some are generally known as waxes: of the compounds, on the other hand, that are not in the strict sense fats, some have had assigned to them the term "wax," a term for which also a chemical definition has been devised that does not correspond to the ordinary meaning of the word, while for a large class, the physiological importance of which is daily coming more into evidence, no better general name has been proposed than "lipoids," which is at once a cloak for ignorance and an indefinable limbo into which any one can thrust anything of which he knows little or nothing, including often what is not a compound of any fatty acid at all.

Of these compounds of fatty acids occurring in nature the commonest are esters of the triatomic alcohol glycerol. It is these esters that are by definition fats when they are solid at the ordinary temperature and oils when they are liquid. Such esters may contain one, two or three fatty acid radicals, the mono-, di- or triglycerides as they are respectively called; for instance the esters formed by butyric acid with glycerol are:—

1. the α and β monobutyrins—

$$\alpha\left\{\begin{array}{l}CH_2 . OOC . C_3H_7\\ |\\ CH . OH\\ |\\ CH_2 . OH\end{array}\right. \qquad \beta\left\{\begin{array}{l}CH_2 . OH\\ |\\ CH . OOC . C_3H_7\\ |\\ CH_2 . OH.\end{array}\right.$$

Of these the α form must clearly be capable of occurring in two stereoisomeric optically active modifications :—

2. the $\alpha\beta$ and the $\alpha\alpha$ dibutyrin—

$$\alpha\beta\left\{\begin{array}{l}CH_2 . OOC . C_3H_7\\ |\\ CH . OOC . C_3H_7\\ |\\ CH_2 . OH\end{array}\right. \qquad \text{and } \alpha\alpha\left\{\begin{array}{l}CH_2 . OOC . C_3H_7\\ |\\ CH . OH\\ |\\ CH_2 . OOC . C_3H_7\end{array}\right.$$

the $\alpha\beta$ form being also capable of stereoisomerism :—

3. tributyrin—

$$\begin{array}{l}CH_2 . OOC . C_3H_7\\ CH . OOC . C_3H_7\\ CH_2 . OOC . C_3H_7.\end{array}$$

But in one and the same diglyceride or triglyceride molecule the fatty acid radicals may be not all the same as in the above instances ; there may be two different radicals in a diglyceride and two or even three different ones in a triglyceride ; and then other stereoisomeric modifications become possible in the case of the $\alpha\alpha$ diglycerides and also of the triglycerides.

When it is remembered that glycerides of a large number of fatty acids occur in nature, it is clear that the number of arrangements of these in glycerol esters must be large indeed.

Again, when a glyceride contains less than three fatty acid radicals the place of the third or possibly of the second and third may be taken by an acid of a different kind. In lecithine, for instance, there are two fatty acid radicals, and the third hydroxyl group of the glycerol is coupled by an ester union with phosphoric acid. Since phosphoric acid is a tribasic acid, the acid hydroxyl groups which are not engaged by the glycerol in such a compound are free to enter into combination with other groups, alcohols or bases ; as, for instance, in lecithine, with the alcoholic base choline, and in cephaline with amino-ethanol.

Then, besides glycerol, there are several other alcohols that are found associated with fatty acids, and some of these are very widely distributed, if only in relatively small amounts.

Lastly, it must be remembered that fats and oils as they occur in nature are never single chemical substances, seldom indeed entirely composed of glycerides. In any fat or oil there is a mixture of several

glycerides with varying quantities of cholesterol or substances related to it and often free fatty acids or soaps as well.

These considerations show that the number of compounds of fatty acids which have to be dealt with in biological chemistry, and for which we have no single comprehensive name, is very large; and also that the precise constitution of any one such compound may, even when it is not complicated by the presence of phosphoric acid, be difficult to determine, whereas, where this complication does exist, the difficulty is bound to be very greatly increased. In fact at the present time these more complicated compounds of the fatty acids are of all compounds with which animal or vegetable chemistry has to deal among the most difficult to investigate, if not the most obscure.

NOTE ON TERMINOLOGY.

The following terms will be used in these pages:—

Lipines, to denote substances of a fat-like nature yielding on hydrolysis fatty acids or derivatives of fatty acids and containing in their molecule either nitrogen or nitrogen and phosphorus.

Phospholipines, to denote compounds of fatty acids that contain phosphorus and nitrogen.

Galactolipines, to denote compounds of fatty acids that contain nitrogen and galactose.

The phospholipines correspond to the phosphatides of Thudichum, and include lecithine, cephaline, sphingomyeline, and perhaps other less completely defined substances.

The galactolipines correspond to the cerebrins, cerebrosides or cerebrogalactosides of various authors.

The lipines include too the amido lipotides and cerebrin acids of Thudichum, among them sphingosine and certain other substances described by others.

CHAPTER I.

THE SUBSTANCES THAT ENTER INTO THE COMPOSITION OF FATS.

A. THE FATTY ACIDS.
B. GLYCEROL AND GLYCERIDES.
C. OTHER ALCOHOLS AND THEIR FATTY ACID ESTERS.

A. THE FATTY ACIDS.

THE acids with which we are concerned belong to more than one series; members of the following series are known to occur in fats and waxes:—

I. The saturated fatty acids with the general formula $C_nH_{2n}O_2$, the series taking its name from the acid in which n = 2, acetic acid.

II. The unsaturated fatty acids among which members of the following series are known to occur:—

(*a*) Acids with the general formula $C_nH_{2n-2}O_2$, the series taking its name either from acrylic acid (n = 3) or oleic acid (n = 18).

(*b*) Acids with the general formula $C_nH_{2n-4}O_2$, the series taking its name from linoleic acid (n = 18).

(*c*) Acids with the general formula $C_nH_{2n-6}O_2$, the series taking its name from linolenic acid (n = 18).

(*d*) Acids with the general formula $C_nH_{2n-8}O_2$.

(*e*) Acids with the general formula $C_nH_{2n-10}O_2$.

III. Saturated hydroxy-acids $C_nH_{2n}O_3$ rarely occur.

IV. Unsaturated hydroxy-acids: the only acids known have the formula $C_nH_{2n-2}O_3$ (n = 18), and are named after the one occurring in castor oil, ricinoleic acid, from which the others are artificially prepared.

V. Saturated dihydroxy-acids: the only acid known to occur in fats, has the formula $C_nH_{2n}O_4$ (n = 18), it is found as a glyceride forming 1 per cent. of castor oil.

VI. Saturated dibasic acids: the only acid known to occur in fats, has the formula $C_nH_{2n-2}O_4$ (n = 22), and occurs as a glyceride in the vegetable product known as Japan wax.

VII. Certain cyclic acids are described as occurring in the vegetable chaulmoogra oil.

I. THE SATURATED FATTY ACIDS: $C_nH_{2n}O_2$.

The acids of this series, up to and including the acid containing ten carbon atoms, capric acid, are fluids at the ordinary temperature; those members of the series that contain more than ten carbon atoms are

solids, the melting point of which rises with the molecular weight. But the melting point of acids with an uneven number of carbon atoms is lower than that of either of its neighbours in the series, while the difference between the melting points of neighbours diminishes as the molecular weight increases.

The lowest members containing four or less than four atoms of carbon are miscible with water in all proportions; as the molecular weight increases beyond this, the solubility of the acids rapidly diminishes to zero. Caproic acid is slightly soluble in cold water, caprylic acid requires 400 parts of boiling water to dissolve it, whereas capric and lauric acids dissolve only in traces even in boiling water, and the acids higher in the series still are practically insoluble in water.

The physical property, however, which is most useful in the grouping of these acids is one common to all the lower members of the series, from capric acid down, that of being easily distilled with steam; these acids are the volatile fatty acids. The acids next above capric acid in the series, lauric and myristic, pass over in a current of steam merely in traces, and the acids higher still barely even in traces. By reference therefore to this property the acids are fairly sharply divided into two groups, the volatile and the non-volatile acids.

With increasing molecular weight the boiling point of the acids also rises, at atmospheric pressure by increments of about 20° C. with each additional carbon atom, from formic acid (B.P. 100·8) to capric acid (B.P. 268). Beyond this temperature the acids that have a higher boiling point are decomposed, and their boiling points therefore can be determined only at lower pressures. In an absolute vacuum lauric acid boils at 89°, stearic acid at 128°; at 15 mm. pressure stearic acid boils at 232°.

The volatile acids up to and including butyric acid smell pungently acid; caproic, caprylic and capric acids have, as the names imply, offensive goatlike odours.

The solidifying point of melted acids is lower than the melting point, and generally can be more sharply determined.

The melting point of mixtures of the acids is lower than would be calculated from the melting points of the pure acids composing the mixture, lower than that of either component unless there is considerably more of that component which has the higher melting point. Thus a mixture of 1 part of stearic acid with 8 parts of palmitic acid melts at 59 to 60° C. and one composed of 47·5 per cent. stearic and 52·5 per cent. palmitic acid melts at 54·8° C. In this respect the fatty acids behave similarly to the metals in alloys. They form also eutectic

mixtures, tending to crystallise in certain fixed proportions with constant melting and solidifying point. Such a mixture of stearic and palmitic acids in molecular proportions has frequently been described as a chemical entity, margaric acid, which has been generally believed not to occur in nature.[1]

It is a remarkable fact, which has frequently been commented on, that the fatty acids that occur in nature, with the exception of the lowest members of this series, are almost without a well-certified exception acids that contain an even number of carbon atoms; and also with very few and rare exceptions they are the normal acids, with chains of carbon atoms that do not branch.

Many of the most important properties of the acids are contained in the table of physical constants of the acids appended, and in the brief review of the several individual acids that here follows, note will be made only of a few additional points that may be most frequently of value in the study of fats :—

Formic acid occurs in sweat, urine and meat juice, and in the bodies of ants, especially the red ant (Stumper, 1922). Also in human blood (Stepp, 1920). It is formed by *B. Coli communis* from sugar (A. Harden, 1901) ; and from glycerine in the human body (E. Salkowski, 1919).

When heated with strong sulphuric acid it breaks up into carbon monoxide and water. It is readily oxidised by metallic oxides, *e.g.*, HgO, which is reduced to Hg_2O and the metal ; and by contact with rhodium it is converted into carbon dioxide and water.

All its salts are soluble in water with the exception of the mercurous salt, which spontaneously decomposes by reduction of the mercurous oxide to the metal and formation of carbon dioxide. The zinc salt is insoluble in alcohol, distinguishing it from the acetate and butyrate.

It is recognised by the fact that neutral solutions of its salts reduce silver nitrate, and when treated with neutral ferric chloride give a red colour and, on warming, a yellow precipitate of basic salt.

Acetic acid occurs in sweat, muscles, the liver, fæces, urine and sometimes in the stomach.

The silver salt may be precipitated on adding silver nitrate to a fairly concentrated solution of an acetate, and can be crystallised in fine needles from hot water ; the crystals contain 64·65 per cent. of silver.

It is recognised by the smell of the ethyl ester formed when ethyl alcohol is added to a solution of the acid or its salts, and the mixture treated with strong sulphuric acid, and also by the behaviour of neutral

[1] Klimont and Mayer (1914, 1915) claim to have found dipalmitomargarin in goose fat and trimargarin in horse fat.

solutions of its salts with ferric chloride in which it resembles formic acid.

Propionic acid occurs in sweat and in fæces.

It can be salted out of its aqueous solutions by means of calcium chloride like butyric acid.

All its salts are soluble, but the silver salt the least soluble.

Its basic lead salt is easily soluble in cold but not in hot water, in which respect it differs from the salts of formic, acetic and butyric acids.

Normal butyric acid occurs in sweat, fæces and urine, and in the form of glycerides, to the extent of about 6 per cent., in butter.

Freshly distilled it smells like acetic acid, diluted with water it has a rancid smell. Miscible with water in all proportions, it can be salted out like propionic acid by means of calcium or sodium chloride.

Its salts are easily soluble in water, with the exception of its silver salt, of which 1 part dissolves in about 200 of water at 14°; its mercurous and lead salts are also comparatively insoluble.

Calcium butyrate, crystallising with 1 molecule of water, is soluble in alcohol, and is more soluble in cold than in hot water; at 0° C. 100 parts of water dissolve 19·4 parts of this salt, of which 23 per cent. separates out on heating to 70° C., the temperature at which it is least soluble. The barium salt crystallises with 4 molecules of water, and is more soluble in water than the barium salts of lower members of the series. The smell of the ethyl ester, resembling that of pineapples, is characteristic. It is oxidised by chromic acid, by nitric acid and acid solutions of permanganates so as to give acetic and carbonic acids When boiled with strong nitric acid some succinic acid is formed. By the action of permanganate in alkaline solution oxalic acid can be obtained. Oxidation of the ammonium or alkali salts with hydrogen peroxide gives rise to the production of acetoacetic acid and acetone as well as some succinic acid.

Isobutyric acid occurs in fæces and among the products of bacterial action upon proteins. It is said also to be present in sesame oil in traces.

It is less soluble in water (1 in 5) than the normal acid.

Its calcium salt crystallises with 5 molecules of water, and unlike the normal butyrate, is more soluble in hot than in cold water.

Valerianic acid is said to be formed by certain bacteria from lactic acid. Its calcium salt resembles that of butyric acid in its solubility, and in crystallising with 1 molecule of water.

Isovalerianic acid (isopropylacetic acid) was stated by Chevreul (1817) to occur as a glyceride in the blubber of porpoises. It occurs among the products of protein decomposition, for instance in cheese.

It dissolves in 23·6 parts of water at 20° C., and is thrown out of solution on adding calcium chloride.

Its salts when thrown upon water show a rotary movement; those of the alkaline earths and of the alkalis are soluble. The calcium salt crystallises from cold water with 3 molecules, from water above 50° C., with 1 molecule of water.

Normal caproic acid, $C_6H_{12}O_2$, occurs in fæces and in the butyric fermentation of sugar, and in the form of glycerides, to the extent of about 1·2 per cent., in butter, and also in cocoa-nut and palm-nut oils.

It is slightly soluble in water.

Its calcium salt crystallises with 1 molecule of water, 4·4 parts of the anhydrous salt dissolve in 100 parts of water at 21 to 22° C.

The barium salt crystallises with 2 molecules of water, and is rather more soluble (11·1 parts in 100 of water at 10·5° C.).

Crystals of the zinc salt are precipitated when caproic acid is poured on to a solution of zinc acetate; by this it may be distinguished from butyric and valerianic acids.

Caprylic acid, $C_8H_{16}O_2$, occurs in sweat and in the form of glycerides in the butter of cow's and goat's milk, and also in cocoa-nut and palm-nut oils.

The calcium salt is less soluble than the barium salt; of the latter 0·62 grm. dissolves in 100 grms. of water at 20° C.

The lead salt may be crystallised from alcohol, m.p. 83·5 to 84·5°.

Capric Acid, $C_{10}H_{20}O_2$, occurs in the form of glycerides in the milk of cows and goats, and in cocoa-nut and palm-nut oils, and as potassium salt in wool washings.

The acid dissolves only in 1,000 times its weight of water and crystallises in needles melting at 31·3° C.

Its alkaline salts dissolve readily in water, its other salts do not; the barium salt is slightly soluble in boiling water, and separates on cooling in crystalline plates; it dissolves, however, readily in alcohol. Esters of capric and of caprylic acid occur in fusel oil and have been obtained for commercial purposes from this source.

Lauric acid, $C_{12}H_{24}O_2$, occurs as glyceride in milk forming from 2 to 3 per cent. of the fatty acids (Browne), more abundantly in spermaceti and in laurel oil, cocoanut oil, palm kernel oil (55 per cent.) and certain other vegetable oils.

Its alkaline salts are soluble, and differ from those of higher acids in being less easily salted out of their solutions; a solution of sodium laurate requires 17 per cent. of sodium chloride to be completely salted out, sodium stearate only 5 per cent., while a dilute solution of sodium

caproate is not precipitated by saturation with salt. Use is made of this property in the manufacture of so-called marine soaps.

The other salts are almost insoluble in water; in 1 litre of water at 100°, there dissolves 0·55 grm. of calcium salt, 0·70 of barium salt, 0·19 of zinc salt, and 0·01 of lead salt. These salts dissolve more readily in alcohol.

Myristic acid, $C_{14}H_{28}O_2$, occurs as a glyceride in milk, in which it forms about 10 per cent. of the fatty acids present (Browne, 1899), and in traces in lard, in wool-fat, in cod-liver oil, in large quantities in certain vegetable fats (nutmeg butter, palm kernel oil and others).

A crystalline potassium salt may be obtained, and also a barium salt that crystallises from alcohol, in which it is somewhat soluble when hot.

Palmitic acid, $C_{16}H_{32}O_2$, occurs combined with glycerol in most animal and vegetable fats, especially in palm oil and Japan "wax"; also as cetyl ester in spermaceti, as myricyl ester in beeswax, and as ceryl ester in opium wax. Myrtle "wax" contains palmitic and lauric acids only in the form of glycerides. In cow's milk it forms nearly 40 per cent. of the fatty acids present as glycerides, and is the most abundant of all these (Browne).

100 parts of absolute alcohol dissolve at 19·5° C. 9·3 parts of the acid, boiling alcohol dissolves it easily. It is not very readily dissolved by petroleum ether. It dissolves without change in sulphuric acid and can be recovered by diluting with water.

The alkaline salts dissolve readily in water, less readily in alcohol; 100 parts of 95 per cent. alcohol dissolve 1·14 grm. of potassium salt at 22° C. The silver salt is obtained in crystals by adding alcoholic silver nitrate solution to a solution of ammonium palmitate in alcohol. The calcium, barium and lead salts are very slightly soluble in alcohol; the lead salt is almost insoluble in ether (18 mgrs. in 100 c.c.).

The hydrolytic dissociation of the alkaline salts of fatty acids by water is especially important in connection with the soaps of the higher acids. Alkaline palmitates yield a clear solution in a small volume of hot water, which on cooling sets to a jelly. If the hot clear solution be largely diluted it becomes clouded, owing to the liberation of free fatty acid and of alkali by hydrolysis. The free fatty acid can be removed by shaking with toluene, and also the amount of alkali set free determined by salting out the unhydrolysed soap and free fatty acid together, dissolving the curd in alcohol, and titrating the solution with alkali. Sodium palmitate diluted with 900 parts of water and then treated in this way was found to contain 4·20 per cent. of sodium

instead of the 8·27 per cent. present in $C_{16}H_{31}O_2Na$. In the "acid salt" with the composition $C_{16}H_{31}O_2Na + C_{16}H_{32}O_2$ there would be 4·31 per cent. of sodium.

This phenomenon has sometimes been referred to as if the monobasic fatty acids formed acid salts. Acid soaps, it is true, are easily obtained, soaps, that is, containing more or less free fatty acid, but there is no evidence that there is any molecular proportion between the soap and the fatty acid. It is purely accidental that the figures just quoted appear to give colour to such a conception. Krafft and Stern boiled pure sodium palmitate with measured quantities of water, and estimated the percentage of sodium in the salt that separated out on cooling; when 200 c.c. of water was taken for 1 grm. of sodium palmitate, the soap obtained contained 7·01 per cent. Na, with 300 c.c. 6·84 per cent., with 400 c.c. 6·60 per cent., and with 500 c.c. 6·04 per cent. The dissociation increases with the dilution. If the partially dissociated soap solution is shaken repeatedly with toluene, very nearly the whole of the palmitic acid can finally be removed. (Krafft and Wiglow, and Lewkowitsch; see also McBain and others, 1922.)

The salts of stearic acid are still more liable to this hydrolytic dissociation than the palmitates. Oleates on the other hand are to a less degree hydrolysed on dilution. Sodium oleate can be dissolved in 10 parts of water, and remains clear on cooling; on diluting this solution twenty times a slight opalescence appears, but it is not perceptibly stronger at four times this dilution. So-called neutral soaps, which are in popular demand, are prepared from olive oil, or by the addition of excess of fatty acid, so as to reduce the hydrolytic liberation of alkali.

This hydrolytic dissociation is prevented by the addition of alcohols, ethyl alcohol, 40 per cent., methyl alcohol, amyl alcohol 15 per cent. or glycerol. Hence the rule that in titrating fatty acids with aqueous alkalis, alcohol should be always present at the end of the titration in a concentration of about 50 per cent.

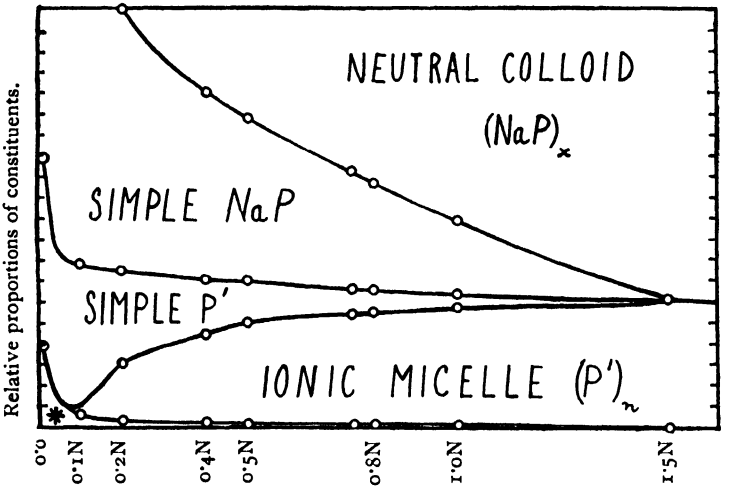
Hydrolytic dissociation is also impeded by excess of alkali, and it is the alkali set free in hydrolytic dissociation that puts a limit to the dissociation and prevents it being complete as might otherwise be expected, from the fact that the liberated fatty acid is insoluble and therefore removed from the sphere of action. With a volatile base like ammonia this limit tends to fail, and ammonia soaps are not stable; they decompose on heating or even on standing.

Soaps, when dissolved in water, produce solutions which exhibit some of the properties, both of crystalloids and colloids. These solu-

tions are, therefore, of great interest. By varying the acid or basic constituent of the soap, and examining the change in properties of the solution brought about thereby, much light has been thrown on the nature of the substances present. The most extensive investigation of this subject has been carried out by McBain and his colleagues, and a brief summary may be given of the results obtained up to the present, and the conclusions to which they have led.

A soap solution may exist in three forms. These are: the sol, the gel and the curd. The sol is fluid and transparent; the gel is rigid, elastic and transparent, and the curd is white and opaque Each form is capable of transformation into the other two by varying the temperature. The sol is the stable form at higher temperatures and the curd at lower. The gel is relatively unstable. The methods by which these three forms have been investigated have included, among others, the measurement of electrical conductivity, osmotic pressure, H-ion concentration, Na-ion concentration, vapour pressure and the use of ultrafiltration. From the data obtained, McBain suggests that, in the sol form, and in moderately dilute solution (about N/10), the principal constituents are undissociated soap molecules, sodium ions and fatty acid ions. The amount of hydrolysis is small, unless the solution is very dilute. In normal solution only about 0·1 per cent of the soap is hydrolysed, so that the hydroxyl ion concentration is very low, and is quite insufficient to account for the properties of the sol. When the solutions are more concentrated, then the principal constituents are (1) aggregates of molecules of the undissociated soap, which are hydrated, and (2) aggregates of fatty acid ions, which form an ionic micelle. This micelle is highly charged, conducts well and accounts for the relatively high electrical conductivity of strong soap solutions, which is of the same order as that of salts of the simple fatty acids. The ionic micelle, because of its high charge which is due to its constituent ions, attaches to itself both water and undissociated soap molecules. Perhaps the most important evidence for the presence of the ionic micelle is the fact that the osmotic activity of the sol is about half that of a salt, whereas the electrical conductivity is equal to that of a salt, so that half the conductivity is due to some constituent that displays little or no osmotic activity. The ionic micelle conducts better than the ions which compose it; hence the conductivity curves fall at first, and then rise asymptotically as dilution increases. This corresponds with the change from the micellar into the ordinary ionic state.

The relative proportions of the various constituents of solutions of pure sodium palmitate at 90°. The asterisk marks the field showing the proportion of acid soap, 2NaP, HP, present.



Total concentration of solution.
(From "Transactions of Chemical Society," 1922, p. 629.)

The gel form of a soap solution is identical with the sol in composition, but it is rigid and elastic. The osmotic activity and electrical conductivity of the sol and gel are the same. This indicates the presence in the gel form of a filamentous structure, formed by a grouping together of micelles in such a way that the micelles, which are the units of such a structure, do not lose their individual properties.

The curd form contains soap which is not in solution but has separated out in a manner analogous to crystallisation. It is made up of fine fibres, which are visible with the ultramicroscope. A felt like network is formed, which includes a dilute soap solution within its meshes. On warming, the fibres dissolve, and, on cooling, they reappear. These deductions on the structure of the curd are supported by osmotic pressure and conductivity measurements, and by the examination of the enmeshed liquid, which may be squeezed out of the curd by applying pressure to it.

Margaric acid (Daturic acid), $C_{17}H_{34}O_2$, is said to exist in Datura oil, the acid isolated from this oil being indistinguishable in all its properties from synthetic heptadecylic acid (Bömer and Limprich). It is slightly more soluble in alcohol than palmitic acid.

Stearic acid, $C_{18}H_{36}O_2$, occurs as a glyceride in most solid fats, most abundantly in the vegetable fats, shea butter and cacao butter.

In milk it forms less than 2 per cent. of the fatty acids present as

glycerides, according to Browne, but as much as 10 to 15 per cent., according to Smedley.

In 100 parts of absolute alcohol at 20° C. 2·5 parts of stearic acid dissolve.

The salts undergo hydrolytic dissociation in water, like the palmitates; even the insoluble barium and calcium salts give up their bases on washing with water.

The calcium and barium salts are almost insoluble in alcohol; the magnesium salt dissolves in hot alcohol, and crystallises out on cooling almost entirely.

Lead stearate is almost insoluble in ether, 15 mgrs. dissolving in 100 c.c.

Sodium and potassium stearates dissolve in hot alcohol; on dilution of their solutions with much water "acid salts" crystallise out.

The copper, lead and silver salts are amorphous.

Arachidic acid, $C_{20}H_{40}O_2$, has been found in traces as glyceride in cow's milk, and in the fat of dermoid cysts, more abundantly in arachis or pea-nut oil (5 per cent.), and certain other vegetable fats.

The acid dissolves easily in boiling alcohol, very little in cold, much less than stearic acid. It dissolves readily in ether or chloroform, and is also soluble in benzene and petroleum ether.

Its salts are very similar to the stearates; but the copper and silver salts crystallise from alcohol.

Behenic acid, $C_{22}H_{44}O_2$, occurs in oil of ben, used in the East for cosmetics.

It crystallises in needles, which are more soluble in ether (1 in 500 at 16° C.) than in alcohol (1 in 1000 at 17° C.).

Lignoceric acid, $C_{24}H_{48}O_2$, occurs in lignite tar (Meyer, Brod and Soyka, 1913); in beechwood tar (Brigl and Fuchs, 1922), and as glyceride in arachis oil. It is very slightly soluble in cold alcohol, readily soluble in ether, benzene and carbon bisulphide. The acid of this elementary composition obtained from lignite has been shown by Meyer, Brod and Soyka not to be the normal straight chain acid; this is true, too, of the acid which occurs in combination with sphingosine and galactose in the galactolipine, kerasine (Levene and West, 1914; Levene and Taylor, 1922), and also in combination with sphingosine, phosphoric acid and choline in the phospholipine sphingomyeline. In the kidney of the ox and the horse, compounds are found, probably kerasine and sphingomyeline, from which this same acid can be prepared (Rosenheim and Maclean, 1915).

From beechwood tar Brigl and Fuchs separated two acids of this

composition, one apparently the normal C_{24} acid, and another melting much lower, 74° C. An acid, too, of the composition $C_{24}H_{48}O_2$, having a lower melting point, is said to occur combined with higher alcohols in carnaubic wax and in wool-fat. There is some doubt as to its existence.

Cerotic acid, $C_{26}H_{52}O_2$, occurs free in beeswax and in carnauba wax; in Chinese wax, opium wax and in wool-fat, it occurs combined with ceryl alcohol.

It is extracted from beeswax by boiling alcohol, and crystallises out completely on cooling, in curved needles.

The acid does not dissolve in dilute boiling aqueous soda, but dissolves in boiling alcoholic potash, separating out on cooling. The sodium and potassium salts dissolve in boiling water. The molecular weight has been determined by titration.

Melissic acid, $C_{30}H_{60}O_2$, occurs free in beeswax.

It dissolves readily in hot acohol, chloroform, carbon bisulphide, or petroleum ether, less readily in ether and methyl alcohol.

The lead salt, insoluble in alcohol or ether, dissolves in boiling chloroform, and crystallises from toluene in needles, melting at 118° to 119° C.

Recently the acids in beeswax have been described as four in number, and different from the two previously recognised. According to Gascard and Damoy (1923), these acids are neocerotic $C_{25}H_{50}O_2$, m.p. 77·8°; cerotic $C_{27}H_{54}O_2$, m.p. 82·5°, identical with the acid in Chinese wax; montanic $C_{29}H_{58}O_2$, m.p. 86·8°, and melissic $C_{31}H_{62}O_2$, m.p. 90°; the new formulæ all giving an uneven number of carbon atoms.

II. The Unsaturated Acids.

These acids contain at some point in the chain one or more pairs of carbon atoms united by a double union. This enables them to combine with halogens and so become saturated, a property which is made use of for determining quantitatively the amount of such unsaturated acids or their glycerides present in a mixture, or the number of double bonds in the molecule of an isolated acid or its ester.

The double union may clearly occur, in the case of the higher members of the series, in a number of different possible situations, and the properties of the acids with a normal chain of a given number of carbon atoms are found to differ with different positions of the double union. For instance the three acids,

$$CH_2 : CH . CH_2 . CH_2\,COOH$$
$$CH_3 . CH : CH . CH_2\,COOH$$
$$CH_3 . CH_2 . CH : CH\,COOH$$

are all known, and differ from each other in their properties. The position of the double union in the case of some of the higher members of the series, with only one unsaturated linkage in the chain, has been conclusively determined, by making use of the fact that an unsaturated linkage is liable to become saturated by the entry of hydroxyl groups, and when that is the case further oxidation leads to the chain being broken at this point; the identification of the products of this cleavage indicates the position of the double linkage in the original chain. Instances of the successful application of this procedure will be found in the account given below of the determination of the constitution of oleic and erucic acids.

Some of the cases of isomerism in these acids have in this way been shown to be due to differences in the position of the double union. [1]

Other cases depend on a different fact, namely, that the two unsaturated carbon atoms may have their valencies satisfied by two different arrangements of the same groups, differing from one another in that, in the one the two hydrogen atoms are on the same side, in the other they are on opposite sides, thus—

$$\begin{array}{l} R{-}C{-}H \\ \quad\| \\ R_1{-}C{-}H \end{array} \quad \text{and} \quad \begin{array}{l} R{-}C{-}H \\ \quad\| \\ H{-}C{-}R_1. \end{array}$$

Instances of this are found in the case of oleic acid and its isomer elaidic acid, and of hypogæic and gadic, erucic and brassidic acids (Fittig). That the isomerism in these cases is satisfactorily explained in this way, is borne out by the fact that such isomeric acids, when oxidised and split into two halves in the manner referred to immediately above, yield identical cleavage products. It is remarkable, however, that the measures which bring about the conversion of oleic acid into elaidic acid are without effect in the case of isomers of oleic acid, in which the double union is in a different position from that which it has in oleic, and that in these cases cis-transisomerism is not known to occur though theoretically equally possible.

The acids more unsaturated than those of the oleic series occur characteristically in the vegetable drying and semi-drying oils, and also in animal tissues, for instance, in the liver of the cod and many other fish, and also in the liver, heart and kidney of mammalian animals. In the latter they are in part at any rate present in combination with glycerophosphoric acid as phospholipines.

[1] Some of the unsaturated acids when fused with potash or even when boiled with an alkali, undergo a change consisting in a transposition of the unsaturated link, *e.g.*, hydrosorbic acid,

$CH_3CH_2CH = CH\,CH_2\,COOH$ becomes
$CH_3CH_2CH_2\,CH = CH\,COOH.$

TABLE OF THE SATURATED FATTY ACIDS.

	Formula.	Mol. Wt.	Solidifying Point.	Melting Point.	Boiling Point.	B.P. of Ethyl Ester.	B.P. of Methyl Ester.	M.P. of Amide.	Sp. Gr.
Formic . .	$HCOOH$	46	—	+ 8·6	100·8(760)	55	—	—	1·2415 at 0°
Acetic . .	CH_3COOH	60	—	16·5	118	77·5	—	82-83	1·0701 at 0°
Propionic .	C_2H_5COOH	74	—	− 22	140·9	98·8	—	79	1·0133 at 0°
n. Butyric .	C_3H_7COOH	88	− 19	− 6·5	163·5(760)	119·9(760)	102·3(760)	115	0·9781 at 0°
Isobutyric .	$CH_3\diagdown\!\diagup CHCOOH$, CH_3	88	—	− 47	155·5(760)	110·1	—	128	0·9651 at 0°
n. Valeric .	C_4H_9COOH	102	—	− 58	186(760)	144·6	127·3	—	0·958 at 0°
Isopropyl acetic	$CH_3\diagdown\!\diagup CHCH_2COOH$, CH_3	102	− 57	− 36	173·7(760)	134·3	116·7	127	0·9647 at 0°
Ethyl methyl acetic . .	$CH_3\diagdown\!\diagup CH\,.\,COOH$, C_2H_5	102	—	—	175	—	—	—	0·941 at 21°
Trimethyl acetic	$CH_3\diagdown C\,.\,COOH$, CH_3	102	—	35	164	—	—	—	0·905 at 50°
n. Caproic .	$C_5H_{11}COOH$	116	—	− 20	204·5-205 (760)	165-166	149·6	100	0·945 at 0°

Isobutyl acetic .	CH_3 $>$ $CHCH_2CH_2COOH$ CH_3	116	below − 18	− 9·5	202-203 (770)	—	—	—	0·925 at 20°
Caprylic . .	$C_7H_{15}COOH$	144	12	16	236-237(761)	207-208	192-194	97-98	0·927 at 0°
Capric . .	$C_9H_{19}COOH$	172	—	31·3	270(760)	243-245	223-224	108	0·930 at 37°
Lauric . .	$C_{11}H_{23}COOH$	200	—	[2] 48·0	176(15) [1] 89(0)	[Solid − 10] 269	141(15°)	110	0·875 at 43·5°
Myristic . .	$C_{13}H_{27}COOH$	228	—	[2] 58·0	200·5(15) [1] 98(0)	[Solid 10-11] 102(25)	[M.P. 19] 291(751)	102	0·862 at 53·6°
Palmitic . .	$C_{15}H_{31}COOH$	256	62·6	[2] 63-64	340-356(760) 215(15) [1] 114(0)	[M.P. 24] 185(10)	[M.P. 28]	106-107	0·853 at 62·6°
Margaric (Daturic) . .	$C_{16}H_{33}COOH$	270	58·8	59·3	227(100)	—	—	—	—
Stearic . .	$C_{17}H_{35}COOH$	284	69·3	[3] 70·5-71·5	[ca. 360(760)] 232(15) [1] 128(0)	[M.P. 32·5-33·5] 200(10) [3] 152(0·18)	[M.P. 38·5-39·5]	108·5-109	0·845 at 69·3°
Arachidic .	$C_{19}H_{39}COOH$	312	—	76·77	—	[M.P. 41·5-42·5] 284(100)	[M.P. 46-47]	108	—
Behenic . .	$C_{21}H_{43}COOH$	340	77-79	81-82	306(60)	[M.P. 48·5-49·5]	[M.P. 53-54]	111	—
n. Tetracosanic [3]	$C_{23}H_{47}COOH$	368	—	84·5-85·5	—	M.P. 55·5-56·5 B.P. 198·9 at 0·24	M.P. 59-60	—	—
Lignoceric .	$C_{23}H_{47}COOH$	368	—	(1)74(2)80·5	—	—	—	—	—
Cerotic . .	$C_{25}H_{51}COOH$	396	—	78	—	—	[M.P. 60]	109	—
Melissic . .	$C_{29}H_{59}COOH$	452	—	91	—	[M.P. 73]	[M.P. 74·5]	116	—

[1] Caldwell and Hurtley, *Jour. Chem. Soc.*, 1909, 853.
[2] Levene and West, *Jour. Biol. Chem.*, 1914, 18, 463.
[3] Levene and Taylor, *Jour. Biol. Chem.*, 1924, 59, 905, where data for other synthetic normal higher fatty acids are given.

The drying oils are of great commercial and technical importance because of the changes which they undergo when exposed to the air, by which they increase in weight, become saturated by combination with oxygen and are converted into varnishes. Chemical changes of the same kind are undergone by the equally unsaturated fatty acids of animal tissues, though the physical properties of the resulting products are different; cod-liver oil does not "dry" like linseed oil, although it becomes sticky.

The larger the number of unsaturated unions that occur in a fatty acid or its glyceride the more unstable it is, and the more readily it takes up oxygen; the fact that in those tissues of the animal body in which fats are burnt and not merely stored in reserve, the fatty acids found are more unsaturated than those of the glycerides which are found in the adipose tissue, or contained in the food, points to the former having been made ready for oxidation, while the reserve is kept in the inactive form; the body stores its powder wet for safety, and dries it only when required for use.

The chemistry of the acids more unsaturated than the oleic series has as yet been but little developed; the constitution of none of these acids has yet perhaps been finally determined, and some of the most highly unsaturated acids are known only by their halogen derivatives.

(*a*) *The Oleic Series*, $C_nH_{2n-2}O_2$.

Tiglic acid, $C_5H_8O_2$ occurs as glyceride in croton oil, and is stereo-isomeric with angelic acid, the dibromo-derivative of which is converted by sodium amalgam into tiglic acid. By its constitution, as the expanded formula shows, it is an α-methyl crotonic acid.

$$CH_3 - CH = \underset{\displaystyle CH_3}{\overset{\displaystyle |}{C}} - COOH$$

It is soluble in water.

The calcium salt, unlike that of angelic acid, is more soluble in hot than cold water. The barium and silver salts crystallise, the former with 4 molecules of water, which are given off on standing over sulphuric acid.

On fusion with caustic potash it yields acetic and propionic acids (Fittig).

Hypogæic Acid and its Isomers, $C_{16}H_{30}O_2$.

Hypogæic acid occurs in pea-nut or arachis oil as glyceride, and also in maize oil.

It is easily soluble in cold alcohol, and crystallises in needles. On distillation it decomposes and yields sebacic acid, $C_8H_{16}(COOH)_2$.

Nitrous acid fumes bring about its conversion into *gaïdic acid* by cis-trans isomeric transformation (Schroder).

Physetoleic acid occurs as the cetyl ester in sperm oil and as the glyceride in Caspian seal oil.

It differs from hypogæic acid in that no stereoisomeric transformation is brought about in it by nitrous acid, and on distillation no sebacic acid is formed.

The barium salt crystallises out of boiling alcohol.

The lead salt is soluble in ether (Ljubarsky).

Palmitoleic acid.—This name has been proposed by Lewkowitsch for an unnamed acid found by Bull in cod-liver oil, and also in herring oil, differing from the isomeric acids as shown in the table of constants. It forms about 6 per cent. of the total acids of cod-liver oil.

Lycopodic acid occurs as glyceride in lycopodium spores. It is fluid at ordinary temperatures. On fusion with potash it yields isobutyric and lauric acids, and when oxidised with potassium permanganate yields a dihydroxypalmitic, isocaproic and hydroxycapric acid (Langer and Rathje).

Δ^{α} *hypogæic acid* has been synthetically prepared from α-iodopalmitic acid by the action of alcoholic potash, which gives α-hydroxypalmitic acid and the corresponding unsaturated acid—

$$CH_3(CH_2)_{12}.CH = CH.COOH.$$

The calcium salt ($+ 3H_2O$) is insoluble in water (Ponzio).

Oleic Acid and its Isomers, $C_{18}H_{34}O_2$.

Oleic acid occurs as glyceride in most fats and oils, and as a general rule in larger quantities than any other fatty acid. In cow's milk it forms about one-third of the fatty acids present as glycerides.

Oleic acid is a colourless, odourless fluid, setting to an ice-like solid at 11·5° to 12° and melting at 13·5° or a few tenths of a degree higher. It is dimorphous, and the second modification forms opaque masses of slender needles melting above 18°. It cannot be distilled at atmospheric pressure but may be distilled in quantities of a few grams at a time under a pressure of 2 mm. without much change (Lapworth).

It is said to distil unchanged in a current of steam at 250°. Insoluble in water, it is far more soluble in alcohol than the corresponding saturated acid, dissolving even in cold diluted alcohol.

The *salts* of oleic acid can mostly be melted without change and are soluble in alcohol, some of them even in ether. The alkaline salts are

more soluble than the salts of the higher saturated acids, and are much less liable to hydrolytic dissociation than these.

Sodium oleate, which can be crystallised from absolute alcohol, dissolves in 10 parts of water at 12° C., in 20·6 parts of alcohol (specific gravity 0·821) at 13°, and in 100 parts of boiling ether. Potassium oleate, which is a jelly, dissolves in 4 parts of cold water, 2·17 parts of alcohol (specific gravity 0·821) at 10°, and 29·1 parts of boiling ether (Chevreul).

Calcium oleate, a powdery solid, dissolves in alcohol and ether. Barium oleate, a crystalline powder, insoluble in water and only slightly in alcohol or benzene, dissolves in hot benzene if 5 per cent. or less of 95 per cent. ethyl alcohol is present, and then on cooling separates out in crystals almost quantitatively (Farnsteiner); amyl alcohol has much the same effect as ethyl alcohol, but here again small quantities of water must be present (Lapworth).

The lead, iron and copper salts dissolve easily in ether. The lead salt melts at 80° C.

The *preparation*[1] of oleic acid from olive oil or lard depends on the solubility of the lead salt in organic solvents such as ether, petroleum or toluene. Starting from olive oil, the latter is saponified, usually by alcoholic potash, the acids set free with a mineral acid, and dissolved in warm, dilute ammonia. Lead acetate solution is now added in excess, the precipitated lead salts being separated from the aqueous liquor by decantation, washing and draining, dried and extracted with ether, benzene or toluene. As the salts of the saturated acids are relatively sparingly soluble, the bulk of these, but not all, are thus removed, but some 4 to 6 per cent. of lead palmitate remain.

The acids may be recovered from the dissolved lead salts by shaking the cold solution with diluted hydrochloric acid, filtering from lead chloride, and evaporating off the organic solvent; the residue is now fractionally distilled using an efficient column, at a pressure of not more than 2 mm. The middle fractions contain the smallest proportion of saturated fatty acids, and may be converted into barium salts by dissolving them in benzene or toluene, adding an equal bulk of amyl alcohol and heating with a slight excess of powdered barium hydroxide. The barium oleate formed remains in solution while the latter is hot, but separates in crystals on cooling; by recrystallisation of the barium oleate from a mixture of toluene and amyl alcohol, it may ultimately be obtained free from salts of acids more highly unsaturated than oleic

[1] The authors are indebted to Professor Lapworth for details of the preparation and the physical constants of oleic acid. See also Lapworth, Pearson and Mottram (1925), *Biochem. Jl.*, **19**, 7.

acid and with but a very small percentage of barium palmitate. The oleic acid may be obtained from the purified salt by decomposing the latter with warm, diluted hydrochloric acid, and if thought necessary distilled in a vacuum not exceeding 2 mm.

Reactions and Constitution.—Oleic acid cannot be distilled at atmospheric pressure as it decomposes on heating into a number of substances which have thrown no light on its constitution, but among which have been detected acetic, caprylic, capric and sebacic acids, all of which have an even number of carbon atoms.

It is doubtful whether pure oleic acid is appreciably acted on by air at the ordinary temperature. But a stream of air at 120° or a higher temperature converts it into acids insoluble in petroleum ether, and of a higher specific gravity, presumably hydroxy-acids.

On oxidation with nitric acid all the volatile acids from formic to capric are formed and also several dicarboxylic acids, glutaric, adipic, pimelic and suberic. Permanganate of potassium oxidises oleic acid in alkaline solution, giving at low temperatures mainly a dihydroxystearic acid, M.P. 134° (Edmed), at a raised temperature pelargonic and azelaic acids, the mono- and dicarboxylic acids respectively containing nine carbon atoms. The stages in these changes are represented thus:

$$(1)\ CH_3 . (CH_2)_7 . \underset{OH}{\overset{H}{C}} . \underset{OH}{\overset{H}{C}} . (CH_2)_7 . COOH$$

dihydroxystearic acid;

$$(2)\ CH_3 . (CH_2)_7 . CO . \underset{OH}{\overset{H}{C}} (CH_2)_7 . COOH;$$

this intermediate product has been isolated (Holde and Markusson), and can be converted by chromic acid into

$$(3)\ CH_3 . (CH_2)_7 . CO . CO . (CH_2)_7 . COOH,$$

which is presumably the next product; this then gives rise to

$$(4)\ CH_3 . (CH_2)_7 . COOH$$

pelargonic acid and $COOH . (CH_2)_7 . COOH$, azelaic acid.

This makes it probable that the double bond in oleic acid is in the middle of the chain; for allyl acetic acid, the constitution of which is known in other ways, when thus oxidised, gives formic and succinic acids by breaking up at the point where the double bond occurs:—

$$CH_2 : CH . CH_2 . CH_2\ COOH \rightarrow HCOOH + COOH . CH_2 . CH_2 . COOH.$$

There is no change in the position of the double linkage previous to oxidation, and the cleavage products would reveal the true constitution of the original acid were it not previously known.

This probability is increased by the behaviour of oleic acid with ozone. The three atoms of oxygen enter at the unsaturated point in the molecule as in the case of other unsaturated compounds with the formation of an ozonide (Molinari and Soncini, Harries and Thieme).

A third reaction pointing to the same constitution for oleic acid is that described by Baruch. If oleic acid be saturated with bromine and the resulting dibromostearic acid treated with alcoholic potash, stearolic acid is formed, which contains a triple bond, in the place of the double one of oleic acid,

$$C_8H_{17} . C \equiv C . (CH_2)_7 . COOH.$$

This substance is converted by concentrated sulphuric acid into keto-stearic acid,

$$C_8H_{17} . CO . CH_2 . (CH_2)_7 . COOH.$$

This compound reacts with hydroxylamine giving a mixture of the two oximes:

$$(1)\ C_8H_{17} . \underset{\underset{N . OH}{\|}}{C} . (CH_2)_8\ COOH \text{ and } (2)\ C_8H_{17} . \underset{\underset{HO . N}{\|}}{C} . (CH_2)_8\ COOH,$$

which, with concentrated sulphuric acid, undergo the Beckmann transformation and give rise to

$$(1)\ C_8H_{17} . NH . CO . (CH_2)_8 . COOH \text{ and } (2)\ C_8H_{17} . CO . NH . (CH_2)_8 . COOH.$$

And these substances are broken up by strong hydrochloric acid into

$$\overset{(1)}{\begin{cases} C_8H_{17}NH_2, \text{ octylamine} \\ \text{and} \\ COOH . (CH_2)_8 . COOH, \text{ sebacic acid.} \end{cases}} \qquad \overset{(2)}{\begin{cases} C_8H_{17}\ COOH, \text{ pelargonic acid} \\ \text{and} \\ NH_2\ (CH_2)_8 . COOH, \text{ amino-nonylic acid.} \end{cases}}$$

These three changes, pointing to the same position for the double bond in oleic acid, are satisfactory proof that it is in the position which they indicate, namely, exactly in the middle of the chain.

It is true that on fusion with caustic potash oleic acid is broken down into palmitic acid, containing an uninterruptedly saturated chain of sixteen carbon atoms, and acetic and oxalic acids, which shows that under these conditions the weak spot, that is to say the double linkage, is between the α and β carbon atoms. But the migration of a double link in such circumstances is not without parallel (Meyer and Jacobson: see above, p. 15, footnote). Klimont suggests that the spatial arrangement of the spiral chain of carbon atoms may make a migration to a position six places down the chain intelligible. Noordwyn finds that, besides palmitic and acetic acids, other homologues lower than the former and higher that the latter are formed at the same time.

The evidence that there is as to the course of the biochemical oxidation of oleic acid will be referred to in a later section. There is at

any rate no evidence of the formation in animals of either pelargonic or azelaic acid.

Oleic acid dissolves in strong sulphuric acid, being converted into the ester of a hydroxystearic acid, which hydroxy-acid, set free by saponification with boiling water, is believed to have the hydroxyl group attached to the ninth or tenth carbon atom (*vide infra* iso-oleic acid).

Like other unsaturated compounds, oleic acid decolorises bromine water and absorbs 2 atoms of this or other halogens.

The reduction of oleic acid to stearic acid can be readily effected in the laboratory by heating with hydriodic acid and red phosphorus, but is now generally done by hydrogen in the presence of a catalyst. For the commercial hydrogenation of unsaturated fatty acids or fats the catalyst used is nickel. In the laboratory the use of platinum or palladium is more convenient. The following method for the preparation of an active platinum catalyst is recommended by Feulgen. Five grms. of platinum chloride are dissolved in 5 c.c. of water, 7 c.c. of 40 per cent. formaldehyde are added and then 5 grms. of NaOH in 10 c.c. of water are introduced gradually, the liquids being well cooled. Allow to stand half an hour and complete the reduction of the platinum by immersing the flask for 15 minutes in a water bath at 55° C. Pour the fluid into a half-litre flask half filled with water and shake very vigorously for a few minutes. The platinum is thus obtained as a rapidly settling, flocculent precipitate and the supernatant liquid is almost colourless. Pour off the latter, add water strongly acidified with acetic acid and again shake vigorously till the precipitate is flocculent. The washing may be repeated until chlorides are removed when the precipitate is filtered off by suction and dried *in vacuo* over sulphuric acid. In admitting air into the dessicator care must be used as the platinum may become so hot that it glows. Feulgen recommends that the metal at this stage be ground up and rewashed. The catalyst is very active. One grm. of oleic acid in 10 c.c. of glacial acetic acid is completely reduced in 5 minutes by shaking it with hydrogen in the presence of 0·1 grm. of the catalyst.

For the preparation of a nickel catalyst many methods have been proposed (see Maxted, 1919). The method recommended by Rupe, Akermann and Takagi (1918), is as follows:—

100 grms. of finely powdered porous pot is mixed with 250 grms. nickel sulphate and 400 c.c. water and to this with good mechanical stirring the calculated amount of dilute caustic soda is added to precipitate the nickel as hydroxide. 2 to 3 litres of water are added, the whole boiled up and then poured into a large volume of cold water,

Decant the supernatant liquid and centrifuge or filter off the precipitate by suction. Repeat the boiling up and washing until the mass has no alkaline reaction. Finally dry at 140° C. and powder finely in a mortar or mill. The oxide is reduced to nickel by heating in a tube at 370° to 380° in a current of hydrogen. It is allowed to cool in an atmosphere of CO_2 and may be preserved in sealed tubes in an atmosphere of this gas for many months without losing its activity.

A method in which colloidal palladium is used as the catalyst is described by Paal and Oehme.

Elaidic acid, $C_{18}H_{34}O_2$. This isomer of oleic acid is formed when nitrous acid fumes are allowed to act on oleic acid for a few minutes or when it is heated with sulphurous acid or sodium sulphite to 180° to 200° C.: also when heated with phosphoric or phosphorous acid (Fokin, 1911).

On cooling the fluid sets to a white solid crystalline mass.

The acid melts at 44·5°, and distils *in vacuo* unchanged.

It is easily soluble in warm alcohol.

The solubility of its salts resembles that of the salts of stearic acid rather than oleic acid; the lead salt, for instance, is almost insoluble in ether.

The relation of elaidic acid to oleic acid is shown to be that of a cis-trans stereoisomer by the following considerations:—

On reduction with hydriodic acid and phosphorus it gives stearic acid.

On oxidation with permanganate in alkaline solution, a different dihydroxystearic acid is obtained, as shown by the lower melting point (99° instead of 134°), the greater solubility in alcohol and the greater readiness with which it is oxidised. But on the other hand, when further oxidised, it yields the same cleavage products, pelargonic and azelaic acids.

With ozone an ozonide is formed which, on heating with water, splits in exactly the same way and yields the same products as the ozonide of oleic acid.

With halogens it yields different addition products from those obtained from oleic acid. But on addition of hydriodic acid to its molecule and then treating the product with alcoholic potash, oleic acid is obtained as well as another acid, iso-oleic acid.

It is remarkable that oxidation with permanganate in acid solution gives with elaidic acid the dihydroxystearic acid which alkaline permanganate gives with oleic acid, and *vice versa*, and that the similar observation has been made in the case of erucic and brassidic acids (Albitzky).

On fusion with potash it gives palmitic, acetic and oxalic acids; on treatment with sulphuric acid and then with alcoholic potash it gives the hydroxystearic acid which is obtained in this way from oleic acid too.

The constitution of elaidic and oleic acids is therefore represented by the formulæ:—

$$CH_3.(CH_2)_7.CH \qquad \text{and} \qquad H.C.(CH_2)_7.CH_3.$$
$$\quad\quad\quad

stearic acid has been prepared, the melting point of which was 77° to 78° C.

Fokin has obtained other isomeric modifications of oleic acid. By treating ricinoleic acid with hydrogen in the presence of platinous hydroxide λ-hydroxystearic acid was obtained; this was converted into λ-bromostearic acid, which on boiling with alcoholic potash gave two oleic acids; a solid acid melting at 34° to 36° C., shown to be $\Delta\lambda$ ($\Delta^{11\cdot12}$), and a liquid acid solidifying at about 6° to 8° C., shown to be $\Delta\kappa$ ($\Delta^{12\cdot13}$). There is reason for thinking that linkages in the even odd position (according to the notation here given in brackets) give rise to solid acids, those in the odd even positions to liquids. It is possible that the solid acids may be elaidic modifications.

Rapic acid, $C_{18}H_{34}O_2$, occurs as glyceride in rape or colza oil, which is expressed from the seeds of the cabbage and other related crucifers.

It differs from oleic acid in not solidifying when acted on by nitrous acid, in not solidifying on cooling, and in the properties of its zinc salts. Its constitution is not known (Reimer and Will), though since its di-iodo derivative has been converted into stearic acid it must have a normal chain.

The following evidence of another oleic acid with the unsaturated link between the sixth and seventh carbon atoms was obtained by Hartley. On oxidising the fatty acids from the liver of the pig with permanganate in alkaline solution at a low temperature, a dihydroxystearic acid was obtained with the melting point 129·5°, which was constant after recrystallisation from various solvents. This melting point is different from the melting point of dihydroxystearic acid prepared from olive oil by Edmed, and of the $\Delta^{\alpha\beta}$ acid synthesised by Le Sueur. On oxidising his dihydroxystearic acid with hot permanganate of potassium Hartley obtained no trace of pelargonic or of azelaic acid, but he found and identified caproic acid, and obtained some indirect evidence for the formation of decamethylene dicarboxylic acid, though this acid was not isolated. Oxalic acid was found at the same time, so probably the dicarboxylic moiety was attacked in the reaction. In the liver of the pig, therefore, an oleic acid occurs, differing from the common one, which is found, as Hartley showed, in the connective tissue of the pig, in having its unsaturated linkage between the sixth and seventh carbon atoms, reckoning from the unoxidised end of the molecule. Reference will be made to the probable significance of this observation later.

Higher Members of the Oleic Series.

Gadoleic acid, $C_{20}H_{38}O_2$, occurs as glyceride in cod-liver oil, in herring oil and in sperm oil (Bull).

On oxidation with permanganate in the cold it yields a dihydroxy acid, crystallising out of alcohol with the melting point, 127·5° to 128°.

Erucic acid, $C_{22}H_{42}O_2$, $[CH_3(CH_2)_7 . CH : CH . (CH_2)_{11} . COOH]$, occurs as glyceride in colza oil, mustard-seed oil and other vegetable oils. The same or an isomeric acid has been found to be present in cod-liver oil by Bull.

In its chemical behaviour it closely resembles oleic acid, but its lead salt is not so soluble in cold ether.

Its barium salt crystallises from alcohol.

It is converted by nitrous acid into the solid brassidic acid. From its dibromide a behenolic acid similar to stearolic acid has been obtained, and from this a keto acid and its oximes, which, similarly to those obtained from oleic acid (*vide supra*, p. 22, *sub* oleic acid), broke up in two different ways, as follows :—

I. $\begin{cases} C_8H_{17}COOH \text{ pelargonic acid} \\ \text{and} \\ NH_2(CH_2)_{12}COOH_{13} \text{ amino-brassylic acid,} \end{cases}$ and II. $\begin{cases} C_8H_{17}NH_2 \text{ octylamine} \\ \text{and} \\ COOH(CH_2)_{12}COOH, \end{cases}$

showing its constitution to be that given above (Baruch).

On fusion with caustic potash it yields arachidic and acetic acids; on reduction with hydriodic acid and phosphorus, behenic acid.

Brassidic acid, $C_{22}H_{42}O_2$. This is a cis-trans isomeride of erucic acid formed from it in the same way as elaidic acid is from oleic acid by nitrous acid fumes. Erucic acid is warmed with dilute nitric acid to 60° to 70° till gas is given off, and then at once cooled.

Iso-erucic acid, $C_{22}H_{42}O_2$. This acid has been prepared by methods similar to those which yield iso-oleic acid (Alexandroff and Saytzeff, Ponzio).

(*b*) *The Linoleic Series*, $C_nH_{2n-4}O_2$.

The acids that have been described in this series have the formula $C_{18}H_{32}O_2$. The acid obtained from linseed oil is the one that has been most studied, but the constitution of even this acid has not yet been determined. An acid, or possibly two acids, of this formula occur in the fat of the pig's liver, and the same is true of cotton-seed oil. And in many vegetable oils a similar acid has been shown to exist.

Two methods have been made use of for preparing the pure acid : (1) The tetrabromide was reduced by Hazura (1887) by means of zinc and alcoholic hydrochloric acid (*cf.*, Rollett, 1909); (2) the ethyl ester was prepared by Reformatzky and distilled at 180 mm. at 270°.

Two methods are used for identifying it when present in a mixture of fatty acids :—

(1) The *tetrabromide* is obtained by dissolving the acids in glacial acetic acid and adding bromine slowly : on evaporation the tetrabromide crystallises out. It can be separated from the bromides of other unsaturated acids inasmuch as it is nearly insoluble in petroleum ether, in which the bromide obtained from oleic acid dissolves easily, and because it is easily soluble in alcohol, ether, benzene, chloroform and acetic acid, whereas the hexabromide of linolenic acid dissolves easily only in benzene.

It can be crystallised from alcohol or glacial acetic acid in plates melting at 114°.

(2) The acids are oxidised with permanganate in alkaline solution at a low temperature : linoleic acid is thus converted into tetrahydroxystearic, or sativic acid, 1 grm. of which dissolves in 2 litres of boiling water, and crystallises out on cooling in long needles or prisms which melt at 173°. The acid is insoluble in ether, chloroform, and benzene, but dissolves easily in hot alcohol or glacial acetic acid. The dihydroxystearic acid formed at the same time from oleic acid is more soluble in ether, the hexahydroxystearic acid formed from linolenic acid more soluble in water. By heating linoleic acid with hydriodic acid and phosphorus to 200°, it may be converted into stearic acid.

The calcium, barium, zinc, copper, and lead salts dissolve in ether, the barium salt in benzene and petroleum ether. The lead, zinc, and manganese salts dissolve in linseed oil, and then accelerate its oxidation, and are therefore used as "driers." The constitution of the acid has not been determined; but azelaic acid has been found among the products of oxidation by permanganate in alkaline solution, and perhaps formic acid (Reformatzky); and by boiling the diozonide with water, Takahashi (1921), obtained, in addition to azelaic, butyric and glutaric acids.

Isomers of linoleic acid have been described from other sources : they are identified by the formation of a tetrabromostearic acid, and distinguished from linoleic acid by the melting point of the product so obtained, or of the acid itself from which it is formed.

Linoleic acid or an isomer is assumed to occur in certain animal fats since the iodine value of the mixed fatty acids yielded by them is higher than that of pure oleic acid.

Hartley has prepared and isolated from the mixed fatty acids, obtained by acting on pigs' livers with strong alkali and alcohol, a tetrabromide and also a tetrahydroxystearic acid with the melting point 175°. There were indications of the presence of a second acid mixed

with this one before it was purified, and it is possible that two acids of the formula $C_{18}H_{32}O_2$ occur in this organ.

The other acids with the formula $C_{18}H_{32}O_2$ which have been described occur each of them in some vegetable oil of local occurrence, and have not been studied as much as linoleic acid. One of these, tairiric acid, is said on oxidation to yield lauric acid $C_{12}H_{24}O_2$ and adipic acid $C_6H_{10}O_4$.

Elaeostearic acid yields valeric and azelaic acids on oxidation with ozone, and consequently contains double bonds probably between the fifth and sixth, and ninth and tenth carbon atoms. It is found in Japanese wood oil, which is described as the best drying oil known (Majima).

(*c*) *Acids of the Series*, $C_nH_{2n-6}O_2$.

The only acids that have been described in this series, as in the last, contain 18 carbon atoms. It is the glycerides of these acids still more than those of the acids of the linoleic series that confer upon the drying oils their peculiar properties.

Linolenic acid was first described by Hazura, then by Hehner and Mitchell, and more recently has been studied by Erdmann, Bedford, and Raspe, and by Coffey (1921). On brominating the acids obtained from linseed oil, a product containing six atoms of bromine is obtained which is soluble in benzene, but in no other ordinary solvent. It melts at 179°. By boiling 50 grms. of this substance with 300 c.c. of 95 per cent. alcohol, and 100 grms. of zinc filings, Erdmann and Bedford obtained a mixture of zinc salt and ethyl ester free from bromine, and after removing the zinc and saponifying, an acid substance which distilled *in vacuo* at 157° to 158° C., and gave on analysis the figures required by the formula $C_{18}H_{30}O_2$. Two acids were probably present, denoted α and β linolenic acids, one derived from the other by, it is supposed, steric change. For from the mixture only 23 per cent. of the original amount of hexabromide could be reformed, the rest absorbing no longer six, but only four, atoms of bromine, and giving a liquid bromide: (Rollett (1909), however, and Coffey (1921), maintain that from the hexabromide, the acid reformed is one and the same linolenic acid). An attempt to throw light on the constitution of these acids was made by converting the mixture into the ozonides, and decomposing these with water. Azelaic acid was obtained in sufficient quantity to account for half the molecule of both modifications, and in addition to this among the oxidation products malonic acid and propionic aldehyde were identified. The final elucidation of the constitution of these acids must, therefore, be left for the future.

Hazura and Hehner and Mitchell also recovered the acid from the hexabromide by reduction with zinc and alcoholic hydrochloric acid, and found that it absorbed not far short of the theoretical amount of iodine (iodine value, 241·8 and 245).

Hazura prepared another derivative by oxidising the acids from linseed oil with permanganate in the cold. Two hexahydroxystearic acids were obtained, linusic and isolinusic acids. These acids are more soluble in water than sativic or tetrahydroxystearic acid, less soluble in alcohol, and insoluble in ether. Linusic acid crystallises in rhombic plates or in needles, with the melting point 203° to 205°. Isolinusic acid crystallises in prisms, melting at 173° to 175° C. It has the same elementary composition as linusic acid, but is more soluble in water. But the subject is one that requires further study before these data can be regarded as final.

Linolenic acid can be reduced quantitatively to stearic acid when heated with pumice coated with reduced metallic nickel in a stream of hydrogen at from 170° to 200° C. The amount of hydrogen absorbed corresponds closely with the amount required by theory (Erdmann and Bedford).

(*d*) *Acids of the Series*, $C_nH_{2n-8}O_2$.

Isanic acid is the name given to an acid found in a vegetable oil from the French Congo, to which the formula $C_{14}H_{20}O_2$ is ascribed on account of the analysis of the acid and some of its salts, and the molecular weight of the acid. But the acid absorbed only two atoms of bromine and reduction with hydriodic acid did not give results pointing to its being an aliphatic acid.

Therapic acid is the name given to an acid corresponding to a bromination product obtained by Heyerdahl from the fatty acids of cod-liver oil which, on analysis, appeared to have the composition $C_{17}H_{26}Br_8O_2$.

Arachidonic acid, $C_{20}H_{32}O_2$. From the liver of pigs an octabromide has been prepared by Hartley (1909), the analysis of several preparations of which pointed to an acid $C_{20}H_{32}O_2$. The evidence for the occurrence of this acid was made conclusive by the isolation of an acid from the products of permanganate oxidation which, on analysis, proved to be the octahydroxy derivative of this same acid.

Levene (1922) obtained it from lecithine, whether prepared from egg yolk or from the liver, and identified it by saturating it with hydrogen and analysis of the arachidic acid so formed, and also by analysis of the octabromide.

(*e*) *Acids of the Series*, $C_nH_{2n-10}O_2$.

Clupanodonic acid, $C_{22}H_{34}O_2$, was found by Tsujimoto in Japanese sardine oil, in cod and sunfish liver oils, in herring and in certain species of whale oils. The acid was at first given the formula $C_{18}H_{28}O_2$, which would place it in the previous series. It is separated by the solubility of its lithium salt in acetone containing a little water (6 c.c. in 100 of the mixture). Probably associated with this acid in sardine oil are other highly unsaturated acids, *e.g.*, $C_{20}H_{30}O_2$ (Tsujimoto), or $C_{20}H_{32}O_2$ (Maijima and Okada). Clupanodonic acid would have the iodine value 384. The acid with this iodine value has been obtained from cod liver oil by Southgate using the lithium salt method, and distilling *in vacuo* the methyl esters.

III. Saturated Hydroxy Acids, $C_nH_{2n}O_3$.

Juniperic acid, $C_{12}H_{24}O_3$, occurs with sabinic acid in certain coniferæ as a natural etholide or product of the condensation of a hydroxy acid with another acid—the alcoholic hydroxyl making such a compound possible. It has been identified as hydroxy-lauric acid, as it can be reduced to lauric acid, and oxidised to decamethylene dicarboxylic acid.

Sabinic acid, $C_{16}H_{32}O_3$, which occurs with juniperic acid, has been similarly shown to be Ω-hydroxy-palmitic acid (Bougault).

Lanopalmic acid, $C_{16}H_{32}O_3$, was obtained from wool fat; it forms a potash salt readily soluble in cold alcohol. The acid itself is insoluble in water, but dissolves in it on warming if a little alcohol is added. It crystallises out on cooling. M.p., 87° to 88° C.; solidifying point, 85° to 83° C. (Darmstadter and Lifschütz).

Cocceric acid, $C_{31}H_{62}O_3$, occurs in cochineal wax combined with cocceryl alcohol. The acid is slightly soluble in cold alcohol, ether, benzene, petroleum ether and acetic acid, and crystallises from alcohol. M.p., 92° to 93° C. (Liebermann).

Hydroxystearic acid is said by Erben to form one-fifteenth of the fatty acids in the fat of human chyle.

Cerebronic acid (phrenosinic acid, Rosenheim, 1914; neurostearic acid, Thudichum), $C_{25}H_{50}O_3$, optically active α-hydroxypentacosoic acid, occurs only in combination with sphingosine and galactose in the galactolipine phrenosine.

II. UNSATURATED ACIDS: OLEIC SERIES, $C_nH_{2n-2}O_2$.

		Mol. W.	Solidifying Point.	Melting Point.	Boiling Point.	B.P. of Ethyl Ester.	B.P. of Methyl Ester.	Sp. Gr.	M.P. of Dihydroxy Acid.
Tiglic . .	$C_5H_8O_2$ $CH_3CH : C . COOH$ CH_3	110	—	64-65	198·5(760)	—	—	0·964(76)	—
Hypogæic .	$C_{16}H_{30}O_2$	254	—	33	230(10)	—	—	—	—
Gaidic . .	$C_{16}H_{30}O_2$	254	—	39	—	—	—	—	—
Δα-Hypogæic	$C_{16}H_{30}O_2$	254	45	49	—	—	—	—	—
Physetoleic .	$C_{16}H_{30}O_2$	254	—	30	—	—	—	—	115
Palmitoleic .	$C_{16}H_{30}O_2$	254	− 1·5	—	—	—	185-186(10)	—	125
Lycopodic .	$C_{16}H_{30}O_2$	254	—	Fluid	—	—	—	—	—
Oleic . .	$C_{18}H_{34}O_2$	282	11·5-12	13·5 or 18	223 (10) 166(0·25)	—	*ca.* 206 (10)	0·898(15°)	131·5-132 Le Sueur
Elaidic .	$C_{18}H_{34}O_2$	282	—	44-45	130(0)[1]	370 with decomposition	—	—	99-100
Δα-Oleic .	$C_{18}H_{34}O_2$ $C_{15}H_{31}CH : CHCOOH$	282	56-55	58-59	—	M.p. 25-26	—	—	126 Solid 124-3
Isooleic .	$C_{18}H_{34}O_2$ $CH_3(CH_2)_8$ or $_6CH : CH$ $(CH_2)_6$ or $_8COOH$	282	—	44-45	—	—	—	—	77-78 Solid 66-64
Rapic . .	$C_{18}H_{34}O_2$	282	—	Fluid	—	—	—	—	—
Gadoleic .	$C_{20}H_{38}O_2$	310	—	24-25	—	—	*ca.* 224 (10)	—	127·5-128
Erucic . .	$C_{22}H_{42}O_2$	338	—	33-34	256 (10) 179 (0)	above 360 unchanged	*ca.* 240 (10)	—	132-134
Brassidic .	$C_{22}H_{42}O_2$	338	56	65	180 (0)	—	—	—	98-99
Isoerucic .	$C_{22}H_{42}O_2$	338	—	54-56	—	—	—	—	86-88

[1] Caldwell and Hurtley, *Jour. Chem. Soc.*, 1909, 853.

IV. Unsaturated Hydroxy Acids, $C_nH_{2n-2}O_3$.

Ricinoleic acid.—The glyceride of this acid is the principal constituent of castor oil. It absorbs two atoms of bromine and gives a monoacetyl derivative with acetic anhydride, and is consequently a hydroxy oleic acid. On reduction with hydriodic acid it gives stearic acid, and has therefore a normal chain of carbon atoms.

The constitution of the acid has not been definitively settled, but is probably represented by the formula of Goldsobel, which is

$$CH_3(CH_2)_5\,CHOH\,CH_2CH = CH(CH_2)_7\,COOH.$$

This is based on evidence of the same kind as that of Baruch with regard to the constitution of oleic and erucic acids, namely, the formation of a ricinostearolic acid, a keto-hydroxystearic acid, and its oximes; amongst the cleavage products of the latter, after undergoing Beckmann's transformation, was found a γ-dekalactone and azelaic acid. Haller found also, after the action of ozone, on hydrolysis of the ozonide formed, β-hydroxy-pelargonic and azelaic acids; and further evidence for this formula has been given by Thoms and Deckert, by asansky, by Chonowsky and by Noorduyn. But a different formula with which the facts do not so well agree was proposed by Krafft.

On distillation with dilute nitric acid it yields heptoic, oxalic and azelaic acids, which products the formula of Goldsobel might lead one to expect. On fusion with potash it yields sebacic acid and secondary octyl alcohol.

The pure acid is obtained by repeated crystallisation of its barium salt from alcohol. It melts at 4° to 5°, cannot be distilled unchanged, but at 50 mm. and at 250° C. it is converted partly into an acid of the composition $C_{18}H_{32}O_2$ which distils over and solidifies on cooling below zero. Some polymerisation also takes place when it is heated (Rassow).

It is soluble in all proportions in alcohol and in ether, and insoluble in petroleum ether.

It is optically active, α_D when the acid itself is examined in the polarimeter being + 6·67°.

The calcium salt crystallises in scales from alcohol, and melts at 80°; the barium salt crystallises in plates.

The crystalline lead salt is soluble in ether, and melts at 100°.

The methyl ester boils at 245° at 10 mm. ; α_D + 3·8.

The ethyl ester boils at 258° at 13 mm. : α_D + 4·07.

Ricinelaidic acid, stereoisomeric with ricinoleic, is formed when the latter is heated with nitric acid till red fumes form and then quickly

cooled. The solid product, pressed free from unchanged substance, is crystallised from alcohol or petroleum ether.

The crystalline needles melt at 52° to 53°.

Ricinic acid, an isomeride of ricinoleic acid, has been obtained by heating the barium salt of the latter (Krafft; *cf.* Walden).

Isoricinoleic acid, another isomeride, is said to be obtained by the action of sulphuric acid. It reacts with phenyl hydrazine and hydroxylamine, and is therefore a keto acid. It dissolves readily in petroleum ether in which medium ricinoleic acid is insoluble.

V. Saturated Dihydroxy Acids, $C_nH_{2n-1}.(OH)_2.COOH$.

A *dihydroxystearic acid* occurs in nature only in castor oil so far as is at present known.

This solid acid can be obtained by cooling the acids from castor oil to below 12°, and expressing the crystals that form. Stearic acid is removed by dissolving in toluene, and the insoluble dihydroxy acid crystallised from alcohol.

The melting point, 141° to 143° C., is different from that of the dihydroxy acids obtained from oleic, elaidic, Δ_a-oleic or iso-oleic acids (Juillard).

Lanoceric acid, $C_{30}H_{60}O_4$, is obtained as a potassium salt soluble in dilute alcohol on saponifying wool fat with alcoholic potash.

The acid melts at 104° to 105°, but loses a molecule of water, from the two hydroxyl groups, and then melts subsequently at 102°. On boiling with dilute hydrochloric acid, it forms a lactone melting at 86° C. (Darmstädter and Lifschütz).

VI. Saturated Dibasic Acids, $C_nH_{2n}(COOH)_2$.

Japanic acid, $C_{22}H_{42}O_4$, is the only dibasic acid occurring in natural fats, small quantities of what is probably the mixed glyceride of this and palmitic acid being present in Japan wax.

It crystallises from alcohol or chloroform in plates which are heavier than water.

On heating to 100° carbon dioxide is given off, and a ketone is formed for which the constitution $C_{10}H_{20}.CO.C_{10}H_{20}$ is given (Geitel and V. der Want).

VII. Cyclic Acids.

Chaulmoogric acid, $C_{18}H_{32}O_2$.—This acid, obtained by Power and Gornall from the vegetable Chaulmoogra oil, has the same composition as linoleic acid; it absorbs, however, only two atoms of bromine, and when reduced by hydriodic acid and phosphorus it yields

an acid of the formula $C_{18}H_{34}O_2$. The second unsaturated linkage is supposed therefore to occur in a cyclic formation.

On oxidation with permanganate it yields a dihydroxy acid, and with excess of the oxidising agent decomposes into lower dibasic acids.

Hydnocarpic acid, $C_{18}H_{32}O_2$, occurring in the same and also in other vegetable oils, is for similar reasons also regarded as a cyclic acid (Power and Barrowcliff).

B. GLYCEROL AND THE GLYCERIDES OF FATTY ACIDS.

1. *Glycerol.*

The triatomic alcohol glycerol occurs in all natural fats and oils, and is formed in the fermentation of sugar by yeast. The ordinary commercial source of glycerol has been natural fats and oils, from which it is obtained as a by-product in the manufacture of soap. The shortage of fats and oils during the Great War led to the discovery that the preparation of glycerol from sugar by the action of yeast could be carried out in the presence of large amounts of sulphite on a large scale. Enormous quantities of glycerol were obtained in this way (Connstein and Lüdecke, Neuberg and Reinfürth).

At ordinary temperatures it is a viscid fluid, and solidifies to a mass of rhombic crystals when cooled much below zero, or at zero if sown with crystals previously obtained. These crystals melt at 20° C. It boils at atmospheric pressure at 290° C. (Mendelejeff), but unless free from salts and impurities decomposes before this temperature is reached. At 0·25 mm. pressure it boils at 143° C. (Fischer and Harries). Its vapour pressure at 118° C. is less than 0·25 mm., at 161° C. 6·5 mm., at 220° C. 101 mm., and at 260° C. 385 mm.

It is miscible with water in all proportions, and highly hygroscopic. When mixed with water, contraction and a rise of temperature occurs. It cannot be completely freed from water *in vacuo* over sulphuric acid (Struwe). It is readily volatilised with water vapour, so that aqueous solutions heated on a water-bath lose glycerol; in this way it is impossible to obtain solutions of a concentration higher than 70 per cent.

The specific gravity at 15° C. of glycerol and its aqueous solutions is (Gerlach):—

100 per cent. glycerol,	1·265	50 per cent. glycerol,	1·129
90 „ „	1·240	40 „ „	1·102
80 „ „	1·213	30 „ „	1·075
70 „ „	1·185	20 „ „	1·049
60 „ „	1·157	10 „ „	1·024

Glycerol is miscible with alcohol, only slightly soluble in ether (1 in 500), but readily dissolves in a mixture of alcohol and ether. It is insoluble in chloroform, petroleum ether, and carbon bisulphide.

It is a good solvent for many salts (Klewer), 100 parts dissolving, *e.g.*, 50 of zinc chloride, 40 of potassium iodide, 30 of copper sulphate, 20 of ammonium chloride or carbonate, 10 of barium chloride or copper acetate, 8 of sodium carbonate, 7·5 of mercuric chloride, and 1 of calcium oleate.

Dehydrating agents are apt to remove 2 molecules of water and convert it into acrolein, the aldehyde of acrylic acid, $CH_2 = CH . CHO$. This occurs especially on heating with acid potassium sulphate.

It is readily oxidised by most oxidising agents; ozone yields carbonic, formic, and propionic acids (Gorup); hydrogen peroxide, with a trace of ferrous sulphate, yields glyceric aldehyde. Nitric acid gives oxalic, glyceric, and other acids (Heintz). With sulphuric acid and manganese dioxide, formic acid and carbonic acid are formed. With dry potassium permanganate it burns explosively (Dvorak); in the presence of excess of alkali this reagent oxidises it quantitatively to oxalic acid and carbonic acid, 4 molecules of permanganate giving up 3 of oxygen (Fox and Wanklyn). It reduces many metallic oxides or salts. If heated with silver nitrate on the water-bath till discoloration occurs, on then adding ammonia a mirror is formed. Gold, platinum, and mercury salts are reduced in the presence of alkalis, mercury salts simply under the influence of sunlight. Fehling's solution is not reduced by dilute solutions, but strong ones act slowly upon it.

Halogens partly oxidise, partly give rise to the corresponding hydrines. The lead compound of glycerine is converted by bromine vapour into the mixture of glyceric aldehyde and dihydroxyacetone, in which the latter predominates, and which is known as glycerose (Fischer and Tafel).

Electrolysis gives rise to formic, acetic, oxalic and glyceric acids and trioxymethylene.

Micro-organisms produce many changes in glycerol. Yeast is said to convert it into propionic acid; the organisms in putrid meat or cheese in the presence of chalk give rise to acetic and propionic, and at the same time to butyric, valeric, and caproic acids, and ethyl and higher alcohols.

Bacillus butyricus forms lactic and butyric acid and butyl alcohol (Fitz, Emmerling).

Glycerol forms alcoholates with bases; for instance, the mono- and disodium glycerylates, intensely hygroscopic crystalline compounds

readily decomposed by water; and the lead glycerylate obtained by heating 500 grms. of lead hydroxide with 100 grms. of boiling 85 per cent. glycerine (Fischer and Tafel).

Esters of glycerol may be formed containing one, two, or three acid radicals. Those containing one such radical may have it attached to the central carbon atom of the chain, or to one of the terminal ones; and those containing two may have the central or one terminal hydroxyl left unaltered. If there are different acid radicals attached to the same glycerol molecule isomers will arise according to the positions taken up by those acid radicals. The esters formed by the action of the halogen acids, *e.g.*, mono- and dichlorhydrines, and the trichlorhydrine formed from the latter by phosphorus pentachloride, are fluids differing from glycerol in solubility and other properties. Of the esters of nitric acid trinitro-glycerol is familiar for its explosive and pharmacological properties. The ester of phosphoric acid, glycero-phosphoric acid, $CH_2OH \cdot CHOH \cdot CH_2 \cdot OPO_3H_2$, which is found in traces in the urine and blood, is contained in lecithine and cephaline, widely distributed through the animal and vegetable kingdoms. It is obtained by the saponification of these compounds with baryta as a soluble barium salt; and it can be synthesised by the action of phosphorus pentoxide or of metaphosphoric acid upon glycerol. Its calcium salt is more soluble in cold than in hot water, and can be obtained in glistening scales by heating a strong solution. The free acid is unstable.

2. *The Glycerides of Fatty Acids.*

The glycerides of fatty acids occur in animals stored in the connective tissue cells of adipose tissue which is found principally in the subcutaneous tissue, the bone marrow, beneath certain parts of the peritoneal, pericardial and pleural serous membranes, and in the interstitial tissue of the voluntary muscles. In this adipose tissue fat the glycerides are as a rule to a small extent esters of stearic, to a larger extent of palmitic, and most of all of oleic acid. In certain animals the glycerides of other fatty acids occur. In lard the liquid fatty acids have properties (iodine value, *vide infra*) which indicate that 10 per cent. of the acids entering into the composition of this fat are of the linoleic series (Twitchell). In the fat of the horse and the hare there is similar evidence for the presence of glycerides of other acids. It is further remarkable that in certain species the fat of animals kept in captivity differs from that of those living wild. The evidence that points to the presence of linoleic acid esters in the fat of the domesticated pig points to there being more of such esters in the fat of the wild boar.

And the same difference is particularly well marked in the rabbit (iodine value of fatty acids from tame rabbit 64, from wild rabbit 101), and the duck (iodine value of fat of tame duck 58, of wild duck 85) (Amthor and Zinck).

On the other hand, this difference does not appear to hold for the cat, and the character of the fat of the chamois and deer living in freedom (iodine value 25 to 30) does not justify generalisations from the facts mentioned.

In the fat of cow's milk the esters of butyric and caproic acids occur in fair amounts (6 to 7 per cent. and 1 to 3 per cent. respectively), and those of the intermediate acids, caprylic, capric, and lauric in traces, as well as that of arachidic acid. The glycerides of myristic acid, according to Browne, occur in larger amount, nearly 10 per cent.

In plants the glycerides of many other acids besides stearic, palmitic and oleic acid occur; for instance, linoleic and linolenic in linseed oil, erucic in colza and rape oil, lauric in laurel oil, myristic in oil of nutmeg, arachidic acid in pea-nut oil, ricinoleic in castor oil, etc.

The fatty acid esters of glycerol that occur in nature contain in practically all cases three fatty acid radicals and are triglycerides; an ester containing two erucic acid groups, a dierucin has been separated from a sample of rape oil (Reimer and Will). These triglycerides have frequently been supposed to be each a compound of glycerol with three molecules of one and the same acid, *i.e.*, to be simple triglycerides. But several mixed triglycerides, or compounds of different acids with the same molecule of glycerol, have been separated from natural products. From mutton and beef fat, by means of fractional crystallisation from solutions in acetone or alcohol-ether, a distearopalmitin, a dipalmito-stearin and a dipalmito-olein have been separated, and by cooling down ethereal solutions to low temperatures similar mixed glycerides have been obtained from olive oil (Hansen, Kreis and Hafner, Bomer and Limprich, Holde and Stange, Schicht). From butter the mixed triglyceride, oleopalmitobutyrin, has been obtained by Blyth and Robertson, and from cocoa butter mixed triglycerides of oleic and palmitic with stearic or myristic acids respectively by Klimont and by Hansen. The fact that no tributyrin is found in butter shows that all the butyric acid at any rate is present in the form of mixed glycerides. On the other hand, trilaurin has been isolated by distillation of laurel oil in the vacuum of kathode light (Krafft). Other evidence that glycerides in natural products are not mixed has not been obtained and the probability is that as a rule they are mixed.

The glycerides of the fatty acids up to caprylic acid are liquids

heavier than water. Tricaprin melts at 31° C. and the glycerides of higher saturated fatty acids are solids lighter than water.

The melting points of simple glycerides of fatty acids are higher than those of the corresponding acids; those of the mixed glycerides are considerably lower than that of the fatty acid entering into their composition which has the highest melting point (Guth). For the solidifying point of mixtures of simple glycerides data are given by Kremann and Schantz.

Pure triglycerides melt if they have been solidified by rapid and brief cooling at a temperature which is lower than the temperature at which they melt if they have been cooled long enough to assume a stable crystalline form. The phenomenon described as the double melting point of triglycerides is probably to be explained by the existence of two crystalline modifications. If melted tristearin is rapidly cooled in a capillary tube a metastable crystalline modification appears which, when heated, melts at 54° to 55° C. If kept, however, at a temperature a few degrees higher than this (about 63° C.), the stable form is slowly deposited, which melts at 70·8° C. The determination of the melting point of a glyceride should be undertaken, therefore, only after the specimen has been kept in the solid cooled condition for some hours (Guth, Kreis and Hafner, Smits and Bokhorst, Grün).

The boiling points of glycerides are high, even in the case of those of the lower acids: triacetin 258° to 259° C., tributyrin 287·8° C. The highest triglyceride that has been boiled without decomposition in a vacuum is trimyristin; tripalmitin decomposes even in the kathode tube before its boiling point is reached (Krafft).

The synthesis of the individual glycerides has been effected in three ways:—

1. By heating glycerol and fatty acids, as was done by Berthelot, in sealed tubes to 200° to 240° C. The monoglyceride so obtained is then heated similarly with more of the acid, and the diglyceride is formed, and by repeating the process with this the triglyceride has been obtained. If a slow stream of dry air, or better, of CO_2, is led through a large excess of the acid heated with glycerol, the water formed in the reaction is carried off and complete esterification with the formation of triglyceride may be attained in one operation (Schey).

2. By heating the calculated amounts of hydrines with the alkaline salts of the fatty acids. It may be possible in this way to obtain the two different mono- or di-glycerides of a particular acid, starting, *e.g.*, with either α- or β-mono- or αβ- or αα-dichloro-hydrin. Several α-monoglycerides and αβ-diglycerides have been prepared in this way

(Guth). But Thieme showed that when the monochlorohydrin is heated with an alkali salt of a fatty acid the diglyceride and triglyceride are formed as well as the monoglyceride, because glycerol heated to 100° C. with an alkaline soap gives rise to the formation of some glyceride. He describes a method by which pure mono- and di-glycerides can be obtained from dichlorohydrin and α-chlorosulphonic acid. Grün and Schreyer used the α-monochlorohydrin in a different way, to react with myristyl chloride and subsequently with stearyl chloride and so obtained α-myristo- β-stearo-chlorohydrin. From this, by substituting hydroxyl for chlorine, α-myristo-β-stearin, a diglyceride with an asymmetrical carbon atom, was obtained. Abderhalden and Eichwald have shown how optically active glycerides may be prepared from optically active epibromohydrin or epihydrin alcohol, $CH_2(OH)\overset{\diagup O \diagdown}{CH} . CH_2$. The rotatory power is low and the methods for obtaining the active fats are at present troublesome and circuitous.

3. By heating the disulphuric acid ester of glycerol with fatty acids dissolved in sulphuric acid (Grün).

The glycerides may be hydrolysed by water alone, in the course of ages at atmospheric temperatures, as is the case with bog butter, in a few hours with superheated steam, and more rapidly in the presence of hydrochloric acid acting as a catalyst. Sulphuric acid acts as a hydrolysing agent more effectively than hydrochloric acid because it helps mechanically to bring the oil into a state of fine division or emulsification; but it acts on the unsaturated oleic acid, giving first an addition product which then is converted into monohydroxy stearic acid, an acid with a high melting point, 81° C. Twitchell's reagent is an aromatic derivative of sulphuric acid combined with fatty acid obtained by dissolving oleic acid in benzene, or naphthalene in oleic acid, and adding strong sulphuric acid. The aromatic sulphonic acid is the catalyst, and it acts similarly to but more rapidly than sulphuric acid, because it is soluble in fat, fatty acids and water alike. The addition of small quantities, 1 to 3 per cent., of lime or magnesia accelerates the action of steam in hydrolysing fats, and if similarly small quantities of the alkalis that give soluble soaps be added the acceleration is greater still.

At the temperature of boiling water, however, even the addition of sufficient alkali to combine with all the fatty acids obtainable from a fat, or even of excess of alkali, does not bring about hydrolysis sufficiently rapidly for laboratory purposes. In the laboratory hydrolysis is carried out usually by means of excess of alcoholic alkali solutions. In this case the greater solubility of fats in alcohol makes the alcoholysis, the

formation of ethyl esters of the fatty acids, occur more rapidly than hydrolysis, the formation of the acids themselves, which would occur in water alone. The ethyl esters in the presence of water, however, are themselves hydrolysed, and as the fatty acids are at once converted into soaps by the alkali no equilibrium point is reached in either the alcoholysis of the fat or the hydrolysis of the ethyl esters; the glycerides are consequently completely and rapidly converted into glycerol and soap. Caustic potash is preferable to soda as the potash soaps are more soluble in alcohol. The intermediate formation of ethyl esters in the hydrolysis of fats with alcoholic alkalis was demonstrated by Allen, by Kossel and Krüger, and also by Henriques. Esters of fatty acids are held by Euler to be ionised so as to give a positive acyl ion, $R.CO^{+}$, and a negative alkoxyl ion, $R.O^{-}$.

The removal of glycerol from its union with fatty acids in glycerides may be effected by alcohols containing as catalyst an acid instead of a base. Methyl or ethyl alcoholic solutions of hydrochloric acid (1 to 2 per cent.) give with fats methyl or ethyl esters of the fatty acids (Haller). In the reaction—

$$\begin{array}{l} CH_2OOCR \\ CH\ OOCR \\ CH_2OOCR \end{array} + 3CH_3OH \leftrightarrows 3CH_3OOCR + \begin{array}{l} CH_2OH \\ CH\ OH \\ CH_2OH \end{array}$$

the great preponderance of the mass action of the methyl alcohol in which the fat is dissolved over the glycerol originally in combination sets the equilibrium point very much towards the right side of the above equation, and the presence of hydrochloric acid causes this equilibrium point to be rapidly approached.

Enzymes that hydrolyse fats occur in the seeds in which vegetable oils are found, and during germination become active in preparing the food for the growing embryo. The enzyme in the seeds of the castor oil plant has been specially studied. It was usually said that it acted best in an acid medium; but according to Tanaka, whose work has been confirmed by Armstrong and Gosney, the acid is necessary for the liberation of the enzyme but should then be removed. The enzyme, insoluble in water, is specially adapted for action on insoluble glycerides of very weak acids. It acts best under neutral conditions and in the absence of water in more than traces. The ricinus enzyme has been used in the technical hydrolysis of fats and oils.

Enzymes with a lipolytic action are also well known in animal physiology. The steapsin of pancreatic juice acts in an aqueous medium but is insoluble in water, glycerol, oil and fatty acids (Berczeller). It is easily destroyed by an acid reaction. The conditions which govern its action in the intestine will be discussed later. The other source of

animal lipase that has been most studied is the liver. Armstrong and Gosney comment on the inferiority of this enzyme as compared with that with which they worked and obtained from ricinus seeds; they are inclined to attribute it to the conditions under which the experiments with this animal enzyme have been conducted and the method of preparation. In the expressed juice of the liver, or other organ, it is present in suspension in water; whereas in the living cells it may be supposed that conditions prevail more like those which they found were required for the best results with ricinus enzyme. It should be noted, however, that the liver lipase is inhibited by the presence of small amounts of bile salts when ethyl butyrate is used as substrate. The liver enzyme acts not only upon the glycerides of higher fatty acids but on combinations of glycerol and other alcohols with other carboxylic acids. The reversibility of the action of the enzyme obtained from the pig's liver was detected by Kastle and Loewenhart by the development of the odour of ethyl butyrate when the enzyme was added to butyric acid and alcohol. The action on ethyl butyrate is inhibited by the presence of small amounts of bile salts. In the case of the ricinus enzyme the reversible action was beautifully shown in the experiments of Armstrong and Gosney: equilibrium in the absence of all but traces of water was attained with 38 per cent. of the oleic acid combined as glycerides and 62 per cent. as free acid; as the amount of water increased the amount of glyceride diminished, and the free acid increased, and the rate at which equilibrium was attained was at the same time reduced: the effect of increased amount of glycerol was to increase the proportion of glyceride, but as in the case of water, to retard the attainment of equilibrium.

Many bacteria when suspended in an emulsion with toluene can be shown to effect the hydrolysis of olive oil, ethyl butyrate and triacetin. *Staphylococcus pyogenes aureus*, *B. pyocyaneus*, *B coli* and *B. dysenteriæ* appear to be in this order more active than *B. tuberculosis* (Wells and Corper).

In the hydrolysis of glycerides by sulphuric acid, by water at high temperatures and by alkalis, it has been shown that the di- and monoglycerides are stages in the reaction, these intermediate products having been isolated when the reaction is arrested before completion (Thieme, Grün and Corelli; Lipp and Miller). Experimental observations have indeed been shown to be in fair agreement with what mathematical theory requires (Treub). There is good evidence that in the action of ricinus lipase the hydrolysis and also the synthesis of glycerides takes place similarly in stages (Armstrong and Gosney).

The changes undergone by fats and oils when they become rancid are probably initiated or favoured by enzymes that hydrolyse the glycerides. The free fatty acids are then oxidised by the oxygen of the air in the presence of traces of moisture. But the part played by enzymes and also by the action of light is a subject of some dispute. The changes consist in (1) the appearance of hydroxy acids and (2) of lower volatile fatty acids, their esters or aldehydes; (3) the hydrolysis of the fat; and (4) the disappearance of the liberated glycerol.

The more saturated acids the fat contains, the less liable is it to become rancid; cacao butter, for instance, is rarely found to undergo this change. The presence in a fat of other substances on which bacteria can grow increases the probability of rancidity; butter, for instance, is particularly prone to the change But a sterile fat may nevertheless become rancid; and, as Duclaux showed, the growth of bacteria in an impure fat, though it may promote a tendency to rancidity, is not the cause of the change. The taste of rancid fat is probably due to the formation of œnanthal and pelargonal from palmitoleic and oleic acids (Scala).

C. OTHER ALCOHOLS AND THEIR FATTY ACID ESTERS.

The term wax, which has from common use been applied to substances of very different chemical nature with certain obvious but ill-defined physical properties, has been given a place in chemical terminology which introduces the necessity for restricting its application in some directions and extending it in others. As a scientific term, a wax is defined as a fatty acid ester of some alcohol other than glycerol. Some substances therefore that have been always spoken of as waxes—for instance, myrtle wax or Japan wax—become technically fats; bees' wax itself is composed only in part of wax, and on the other hand wool fat is a mixture of waxes, and liquids such as sperm oil, which contains no glycerides, come to be "liquid waxes." The definition is an arbitrary one, and not very happy, as it would seem to include such substances as ethyl stearate and even ethyl acetate, which is probably more than is intended.

Alcohols that occur in Waxes.

These alcohols are insoluble in water, and are obtained by saponification of the waxes, conversion of the soaps into calcium salts, filtering, drying and extracting the mass with acetone or alcohol. With acetic anhydride the acetic esters are then formed, and distilled fractionally. For further identification the alcohols may be converted into the

corresponding acids by treating with soda lime. By means of sulphuric acid, phosphorus pentoxide or chloride of zinc they can be converted into the corresponding hydrocarbons.

The principal alcohols found in waxes are:—

Cetyl alcohol, $C_{16}H_{34}O$, m.p. 50°, found in spermaceti as ester of palmitic acid, is the principal component of this wax. It occurs too in the secretion of sebaceous glands of ducks and geese (De Jonge), in dermoid cysts (Ludwig), and in mutton bird oil, chiefly as oleate (Carter). It crystallises in scales from alcohol. The acetate is slightly soluble in cold alcohol, and crystallises in needles melting at 22° to 23° C.; it boils at 15 mm. at 200°.

Octadecyl alcohol, $C_{18}H_{38}O$, m.p. 59° C., also occurs as an ester in spermaceti and in the anal glands of geese (Röhmann). It is separated by distillation of the acetate, b.p. 222° to 223° C. at 15 mm. (Krafft).

Eicosyl alcohol, $C_{20}H_{42}O$, has been obtained from the fat of dermoid cysts by Ameseder, and identified by conversion into the corresponding acid, arachidic acid.

Carnaubyl alcohol, $C_{24}H_{50}O$, m.p. 68° to 69° C., is said to occur in wool fat (Lifschutz; *cf.* Röhmann).

Ceryl alcohol, $C_{26}H_{54}O$ (or $C_{27}H_{55}OH$ (Gascard)), m.p. 80° C., occurs as cerotate, m.p. 84° C., in Chinese wax and as palmitate in opium wax, respectively the principal components of these waxes. Also in wool fat, in carnauba wax, and in bees' wax.

An alcohol, $C_{27}H_{56}O$, m.p. 76° C., is found in carnauba wax (Heiduschka and Gareis).

Carnauba or melissyl alcohol, $C_{30}H_{62}O$, m.p. 88° C., is found free and as the ester of cerotic acid in carnauba wax. The same alcohol was supposed to occur in bees' wax, though it was not certain whether the formula was the one given above or that which Heiduschka and Gareis have found to be the correct one for myricyl or melissyl alcohol which as palmitate is the principal constituent of bees' wax, namely, $C_{31}H_{63}OH$: the alcohol which they find in carnauba wax has the formula with one carbon atom less. In bees' wax the other constituents are cerotic acid, and some free melissyl alcohol together with ceryl alcohol.

In addition to these, other alcohols of the aliphatic series have been described in various waxes, some of them being unsaturated substances. In sperm oil, for instance, unsaturated alcohols occur.

Furthermore, a glycol, $C_{25}H_{52}O_2$, occurs as an ester in carnauba wax, and another, $C_{30}H_{62}O_2$, cocceryl alcohol, as coccerate in cochineal.

The principal esters occurring in the best-known waxes are given in the following list:—

	M.P.	
Cetyl palmitate . .	53·5°	in spermaceti.
Ceryl palmitate . .	79°	in poppy wax.
Ceryl cerotate . .	82°	in Chinese wax.
Myricyl palmitate .	72°	in bees' wax.
Myricyl cerotate . .	—	in carnauba wax.

As a general rule, these waxes, with the exception of spermaceti, have to be boiled with alcoholic potash much longer than the glyceride in order to be saponified—bees' wax, for instance, for three hours. This is probably due to the low solubility of these solid waxes even in boiling alcohol.

Cholesterol, $C_{27}H_{46}O$, a cyclic compound and a secondary alcohol, was first found in gall-stones in 1775 by Conradi, and is found in all animal fats or oils in small quantities—0·1 to 0·5 per cent.—in bile, blood, milk, yolk of egg, and in various animal tissues, notably the medullated sheathes of nerve fibres, the liver, kidney, epidermis, hair and dermoid cysts. Cod-liver oil contains from 0·5 to 2·0 per cent. In the form of a silicate it occurs in birds' feathers (Drechsel).

It crystallises from chloroform in needles containing no water of crystallisation that melt at 148·4° to 150·8° (corr. Bömer). From alcohol, and sometimes from ether, crystals are formed with 1 molecule of water; they consist of rhombic plates frequently having one re-entering angle. The water of crystallisation is given up slowly over sulphuric acid and rapidly at 100° C. (Bömer).

Its specific gravity is given as between 1·03 and 1·07. It is lævorotatory, α_D at 15° C., calculated from a solution containing 2 grms. in 100 c.c. of ether, being − 31·1 (Hesse). It is quite insoluble in water: in 100 c.c. absolute alcohol, at 17·5° C., 1·08 grm. will dissolve. It is easily soluble in ether, chloroform, carbon bisulphide or benzene. In 100 c.c. of petroleum ether, boiling under 85° C., its solubility is 0·83 grm. at 19° C. (Bömer). In glacial acetic acid it is only slightly soluble in the cold and when heated dissolves with partial conversion into acetate. It dissolves in aceto-acetic ester, its esters do not (Liebreich).

It can be distilled, without decomposition at ordinary pressure if carefully heated, more readily under diminished pressure.

It absorbs two atoms of bromine, and the product dissolves much less readily in a mixture of equal parts of ether and acetic acid than the similar product obtained from sitosterol. If half the theoretical

amount of bromine be added to a solution in carbon bisulphide crystals having the composition of 1 molecule of cholesterol associated with 1 molecule of dibromide separate and these melt at 112° C. (Cloez).

On heating with soda lime, fatty acids are not formed as they are from aliphatic alcohols (Lewkowitsch).

On prolonged exposure to light and air its solubility diminishes, its m.p. is lowered and the colour reactions become indefinite.

The colour reactions for cholesterol are:—

1. *The Hager-Salkowski reaction:* if a few centigrams of cholesterol, dissolved in 2 c.c. of chloroform, are treated with the same amount of strong sulphuric acid, the chloroform assumes a crimson colour which gradually becomes more purple, while the sulphuric acid shows a green fluorescence. A drop of the purple chloroform solution in a basin turns blue, then green and finally yellow. If the purple solution be diluted with more chloroform it becomes colourless or acquires an intense blue colour, but on shaking again with the sulphuric acid the original colour is restored as the traces of moisture in the chloroform are removed (Salkowski).

2. *Liebermann's reaction.*—Cholesterol dissolved in acetic anhydride gives on adding strong sulphuric acid drop by drop a violet pink colour. The test is sharper, according to Burchard, if to 2 c.c. of a chloroform solution of cholesterol 20 drops of acetic anhydride and 1 drop of strong sulphuric acid are added. The colour changes to blue by which the reaction is distinguished from a similar reaction given by resin. Wool fat cholesterol reacts with a red colour rather than a violet-pink (Lewkowitsch).

3. Cholesterol with concentrated sulphuric acid and a trace of iodine assumes a violet colour that turns blue, green and red.

4. *Schultze's reaction.*—Cholesterol heated with a drop of nitric acid, till the acid is evaporated, leaves a yellow stain that with ammonia turns red.

5. Cholesterol, 0·1 mg. in 5 c.c. light petroleum, gives with excess of furfuraldehyde a faint purple colour appearing slowly, 0·5 mg., a strong colour instantly, on adding strong sulphuric acid (Harden and Robison).

6. Whitby describes as ten times more sensitive than Salkowski's reaction the following: to 2 c.c. of a solution of cholesterol in chloroform is added 2 c.c. of concentrated sulphuric acid containing 2 vols. per cent. of formalin; the fluids after shaking separate, the chloroform cherry-red in colour, the acid brownish-red with intense green phosphorescence. The upper layer pipetted off and treated with a drop or

two of acetic anhydride becomes bright blue, turning after some time green. Whitby describes also other colour reactions for sterols and discusses their meaning.

Cholesterol is obtained (1) from gall-stones by powdering and washing the powder with boiling water and then extracting it repeatedly with hot alcohol in which the lime salts of bile pigments do not dissolve. The alcohol extract is boiled with alcoholic potash and evaporated down and the cholesterol can then be separated from traces of soap with ether and crystallised from alcohol; (2) from the brain most readily, according to Rosenheim, by mixing the brain in a mortar with plaster of Paris and sand. The mass solidifies and can be easily reduced to a dry powder and then extracted with cold acetone. On evaporation of the acetone almost pure cholesterol separates out. The separation of cholesterol from fats will be dealt with later (p. 93).

The constitution of cholesterol, which has long baffled investigation, is still not finally cleared up.

That cholesterol is an alcohol is evident from the many esters that can be prepared and even exist in nature.

That it is a secondary alcohol follows from the formation of the corresponding ketone, cholestenone, $C_{27}H_{44}O$, on oxidation with copper oxide at 280° to 300° C. (Diels and Abderhalden), or on treating the dibromide dissolved in benzene with potassium permanganate and sulphuric acid and then removing the bromine with zinc and acetic acid after it has done its work in protecting the doubly-bound carbon atoms from oxidation. In this way Windaus obtained the ketone in a yield of 60 per cent. crystallised from methyl alcohol. Cholestenone melts at 81° to 82° C.

That it contains a doubly-bound carbon atom follows, from the fact that it adds on (1) the atoms of hydrochloric acid, when this gas is passed through a solution of cholesterol in alcohol and ether: the chlorocholestanol, of which 50 per cent is obtained, melts at 154° C. (Mauthner and Suida); (2) two atoms of bromine when dissolved in carbon bisulphide and treated with a solution of bromine in the same solvent, or when cholesterol in 10 per cent. solution in ether is treated with half its weight of bromine in 10 per cent. solution in glacial acetic acid (Windaus); the dibromide melts with decomposition at 102° to 103° C.; (3) and that with Hübl's solution it gives the theoretical iodine value (*vide infra*, p. 94).

Both cholesterol and cholestenone can be converted into a saturated alcohol cholestanol ($C_{27}H_{48}O$) on reduction with sodium and boiling alcohol (Neuberg and Rauchwerger). This saturated alcohol is

not the same as coprosterol which is formed in the intestine from cholesterol.

Cholesterol is held to contain a saturated cyclic system, with an isopropyl group attached so as to give it affinity to the terpenes. The isopropyl group can be removed as acetone by the action of chromic acid, the cholesterol being at the same time oxidised at other points as well. If, however, it be first converted by hydrogenation into cholestane $C_{27}H_{48}$, and then oxidised by chromic acid, the removal of acetone then leaves an acid which is isomeric with the cholanic acid $C_{24}H_{40}O_2$ that can be obtained from cholic acid. For cholic acid on dry distillation *in vacuo* at 300° C. loses 3 molecules of water, and the product saturated with hydrogen is cholanic acid. If instead of artificially hydrogenated cholesterol, the natural coprosterol (*vide infra*) be used for preparing cholestane, by reduction of the hydroxyl group, the cholestane obtained is a different one (a cis-trans isomeride), and it on oxidation with chromic acid gives the same cholanic acid as is obtained from cholic acid (Windaus and Neukirchen). Lifschütz has given other less direct evidence for the relationship between cholesterol and bile acids; after oxidation with benzoyl peroxide they both give with acetic sulphuric acid mixture and ferric chloride the same green colour and absorption of red rays in the spectrum, which he can use for the estimation of either substance.

The cholesterol contained in the bile after it reaches the intestine is mostly reabsorbed (Dorée and Gardner).

Lifschütz gives evidence for the existence of another form of cholesterol that crystallises in ellipses and melts at 140° C. He found it in the brain, the blood and in the kidney of the ox; in the kidney it is the predominating form. He also describes as occurring in the body an oxidation product, which he calls oxycholesterin, that can be obtained from cholesterol by the action of permanganate in acetic acid solution. Strong sulphuric acid gives with this, but not with cholesterol, a green colour and an absorption band in the red of the spectrum. Oxycholesterin is present in the blood, more in the portal than hepatic blood; it disappears in the liver, where it is supposed to undergo further oxidation. It has been found also in the human brain, partly in the form of esters, by M. C. Rosenheim, who describes another reaction by which its presence may be recognised. The relation of oxycholesterin to cholesterol has not been defined.

Coprosterol, $C_{27}H_{48}O$, a reduced cholesterol formed in the intestine from the cholesterol of the food, was described by Bondzyncki and Humnicki. It is soluble in cold absolute alcohol, and dissolves easily

in ether, chloroform, or benzene. It crystallises in fine needles melting at 95° to 96° C. : α_D = + 24°. It gives most of the colour reactions of cholesterol, though with differences; the Salkowski reaction at first gives only a yellow colour that gradually turns brown and finally red. With Liebermann's reaction the colour is blue from the first, turning green later. For the relation of the cholestane formed from coprosterol to bile acids, see the paragraph above, on the constitution of cholesterol.

Coprosterol does not absorb bromine, and can be separated from cholesterol by bromination of the latter, and extraction with petroleum ether.

Hippocoprosterol, $C_{27}H_{54}O$.—A still further reduced cholesterol is found in the fæces of herbivora (horse, ox, rabbit, sheep: Dorée and Gardner), but appears to be contained in grass and to be excreted unchanged, and is therefore not a product of the reduction processes in the intestine acting on cholesterol. It crystallises out of boiling alcohol in fine needles, melting at 74° to 75° C., which are dextrorotatory. It gives Liebermann's reaction (*cf.*, too, Gittelmacher, Wilenko).

Isocholesterol occurs together with cholesterol in wool fat. It is said to occur also in vernix caseosa (Ruppel). It is obtained by treating wool fat with cold alcohol which dissolves out cholesterol. The insoluble remainder is heated with alcoholic potash in a sealed tube or an autoclave at 100° C., the alcohol evaporated, the residue mixed with water, and shaken with ether. The residue, after evaporating the ether, is heated with four times its weight of benzoic acid to 200° C., or with benzoyl chloride for a few minutes to 160°. The benzoates, after washing with potash, are taken up in ether, the ether evaporated, and the residue after boiling out with alcohol is crystallised from ether. The benzoate of cholesterol crystallises in plates, m.p. 150° to 151° C., that of isocholesterol in minute needles, m.p. 194° to 195° C., and they can be separated mechanically. The benzoate when saponified yields the alcohol.

Isocholesterol is less soluble than cholesterol in cold alcohol, dissolves in hot alcohol, and separates as a jelly on cooling. It crystallises from ether, in which it dissolves readily, in needles, m.p. 137° to 138° C.

It is dextrorotatory, α_D = + 60° (Schultze).

It behaves differently from cholesterol when tested by the colour reactions. (1) In chloroform solution with strong sulphuric acid the solution slowly turns brown, not crimson (Salkowski's reaction); (2) with acetic anhydride and strong sulphuric acid it gives a yellow coloration which turns reddish-yellow with a green fluorescence; (3) the yellow stain left when isocholesterol is heated to dryness in nitric acid turns yellowish-red with ammonia.

Isocholesterol undergoes a change when kept for years exposed to light resulting in a lowering of the melting point.

Phytosterol.—In oils derived from vegetable sources a substance occurs that was taken for cholesterol till certain differences were noted by which it was distinguished, and given the name phytosterol. On analysis phytosterol appeared to be an isomeride of cholesterol, but differed from it in its melting point and its crystalline form, and it could be separated and distinguished too after acetylation.

The melting point of phytosterol from different sources was, however, given very various values, from 135° to 144° C., and similar irregularities were observed in the physical properties of its derivatives. These facts seemed to point to the presence of more than one substance in the preparations.

The crystalline form of phytosterol was given as that of thin flattened needles, pointed at either end, which under the polarising microscope appear dark when the plane is parallel to or at right angles to the long axis of the crystal. With cholesterol the extinction planes run approximately parallel to the diagonals.

The most certain means of distinguishing phytosterol from cholesterol and therefore for detecting adulteration of animal with vegetable oil is to acetylate the unsaponifiable cholesterol or phytosterol or mixture of the two by boiling the crystals with a little acetic anhydride for one minute in a basin covered with a watch-glass, and then evaporating the excess on a water-bath. The residue is crystallised from a small quantity of absolute alcohol, and the crystals, after repeating the crystallisation several times if a mixture is suspected, will give the melting point 125° C. if it be pure phytosterol acetate, 114·5° C. if pure cholesterol acetate. The admixture of phytosterol is indicated by a melting point higher than this latter figure after several recrystallisations even when, according to Bömer, as little as 1 per cent. of a vegetable oil is present in the original oil from which the alcohols were obtained.

The facts cited above, however, suggest that there should be some doubt about the chemical individuality of phytosterol. And as a matter of fact in the phytosterol of Calabar beans Windaus showed that two substances were present, sitosterol and stigmasterol (Windaus and Hauth).

Sitosterol, $C_{27}H_{44}O + H_2O$, was first obtained from the oil of wheat and rye. Burian prepared it by extracting the lime salts of the fatty acids with acetone, and recrystallising the extract from methyl alcohol. The crystals obtained from dilute alcohol contain 1 molecule of water, and are plates; from ether anhydrous needles are obtained. The melt-

ing point is 137·5° C. (uncorr.) (Burian). A preparation from maize oil gave the same melting point; the crystals sometimes had re-entrant angles like cholesterol, more often bifid ends compared to swallow tails. It is lævorotatory, α_D – 26·7.

It gives the colour reactions of cholesterol.

It forms a dibromide when treated in solution in carbon bisulphide with bromine. This dibromide is, unlike that of cholesterol, soluble in a mixture of ether and glacial acetic acid. The acetate is obtained by heating with acetic anhydride in the form of white scales, m.p. 127° C. This can be brominated, and the product crystallised from dilute alcohol.

Stigmasterol, $C_{30}H_{48}O$ or $C_{30}H_{50}O$, was obtained by Windaus by treating the crude phytosterol of the oil of Calabar bean with acetic anhydride, and then the acetylated product in ethereal solution with bromine dissolved in acetic acid. An insoluble compound containing four atoms of bromine was thrown down. This, crystallised from a solution in hot chloroform by the addition of alcohol, forms four or six sided plates, melting at 211° to 212° C. It is very slightly soluble in ether or acetone, easily soluble in chloroform. The bromine can be removed with sodium amalgam or zinc dust and acetic acid, and the resulting acetate saponified; the alcohol so obtained crystallises in needles or elongated thin plates with pointed ends, which contain 1 molecule of water of crystallisation, melt at 170° C., and give Salkowski's and Liebermann's reactions.

Dissolved in chloroform stigmasterol exhibits the rotatory power, α_D = – 45·0°.

Stigmasterol has been found to be present also in the "phytosterol" of rape oil and cacao butter, but not in that of linseed oil. These facts, therefore (Windaus and Hauth), suggest an explanation of the discrepancies concerning the physical properties of "phytosterol" obtained from different sources.

Other phytosterols have been found, having, as a rule, the general formula, $C_nH_{2n-10}O$: alcornol (C_{32}), Hartwich and Dünnenberger, 1909, amyrin (C_{30}), Windaus and Welsch, 1908; brassicasterol (C_{28}), in rape oil, Windaus and Welsch, 1909; one in the South African plant, Hyenanche globosa (C_{28}), Hervey, 1920; ergosterol, Tanret, 1889, and 1908, in ergot and other fungi, fongisterol (C_{25}), Tanret, 1908 associated with the last in some fungi (*cf.* M. T. Ellis, 1918).

Glucosides of phytosterol occur in many plants (Power and Salway, 1913); these compounds are referred to under the name phytosterolins.

Esters of cholesterol and isocholesterol occur in certain fats of animal

origin, but the isolation and identification of them has seldom been successfully carried out.

An alcohol extract of blood serum was evaporated and extracted with ether; the residue obtained on evaporating the ether was heated with ethyl acetate and a solution of cholesterol esters was obtained, from which lecithine separated out as the solution cooled. From this solution on concentration the esters crystallised out, and by subsequent crystallisation from alcohol the oleate was separated from palmitate and stearate (Hürthle).

A silicic acid ester of cholesterol was found by Drechsel and Winogradoff in the feathers of birds.

The esters of cholesterol and isocholesterol which are believed to exist in wool fat have not been isolated. Nor were the esters of octadecyl alcohol in the extract of the anal glands of geese and ducks successfully isolated.

For an account of the two compounds of fatty acids with phosphoric acid and bases in the same glyceride molecule, lecithine, and cephaline, reference should be made to the monograph in this series by Dr. H. MacLean on "Lecithin and Allied Substances," and to papers by Levene in the "Journal of Biological Chemistry."

CHAPTER II.

EXTRACTION AND ESTIMATION OF FAT.

THE fat or oil in animal or vegetable tissues is obtained for commercial purposes by methods which are seldom suitable in physiological investigations. The material is heated by itself or with water, and the melted fat poured or skimmed off; or, especially in the case of vegetable matter, seeds, nuts, etc., the oil is expressed by means of hydraulic presses. In the physiological examination of animal tissues these methods cannot generally be employed. The fat of adipose tissue can, of course, readily be obtained by melting it and straining it off from the connective tissue, vessels, etc. But when the connective tissues from those parts in which fat is usually deposited in the largest amount happen to contain but little fat, or when an exact determination of the amount of fat is necessary, recourse must be had to extraction by solvents or some other method.

The extraction of fat from the body or the organs of an animal with solvents, without change in the nature of the compounds in which the fatty acids are present, cannot be done quantitatively. The tissue must either be dried and powdered, in which case drying at high temperatures in the air results in the partial oxidation of the more unstable unsaturated acids, as well probably as in other changes in the nature of some of the more complex fatty substances; or else the drying may be effected by means of alcohol, in which case the subsequent extraction with ether can hardly lead to exact quantitative results.

The extraction of fat, therefore, in a condition as little altered as possible, must be dealt with as a problem distinct from that of the estimation of the amount of fat in animal tissues.

A. *The Extraction of Fat.*

The most efficient solvents for animal fats, *e.g.*, ether, chloroform, benzene, petroleum ether, are immiscible with water. Consequently it is necessary that the material to be extracted should be dried before it is treated with these liquids, if the extraction is to be at all complete,

and the dried material must then be powdered so as to give the solvent the best chance of reaching the soluble fats enclosed in the great mass of insoluble substances composing the tissue.

The methods used for drying the material to be extracted are :—

1. Drying in air or neutral gas.
2. Drying by means of alcohol.
3. Drying by means of salts, anhydrous sodium, or calcium, sulphate.

1. *Drying in Air or Neutral Gas.*

The readiness with which the unsaturated acids undergo oxidation when heated in the air makes it necessary to avoid the procedure that suggests itself most readily and has been actually most used, that is to say, to *avoid heating the material in open vessels on water-baths or in ovens*, if it is desired to obtain the fatty substances unaltered and as they exist in the animal body. The unsaturated acids and the combinations in which they are found in animals are partially converted when so treated into substances insoluble in petroleum, with difficulty, if at all, soluble in ether, and easily soluble only in alcohol.

The lower the temperature, of course, the less change will be effected by drying in the air, and therefore it may be sometimes practicable to make use of the plan of *drying in a current of air at a low temperature.* By means of an electric fan a current of warmed air can be directed over glass plates on which the finely minced tissue is spread out in a thin film. It is generally necessary to keep the surface constantly renewed. It is possible to reduce the weight of large amounts of, for instance, liver pulp 50 per cent. by drying before a fan for 6 hours with air warmed to about 30° to 35° C. It is, of course, not possible to dry the material completely in this way; nor is it in all cases possible to obtain it in a condition in which it can be powdered, especially if it contains much fat. But the greater part of the remaining water can be removed by the use of alcohol, and then the material may be reduced to a condition of finer subdivision by appropriate means.

Small quantities of material may be dried under reduced pressure at a raised temperature. And an apparatus for this purpose can be easily constructed in which a framework supporting tiers of dishes each resting on a flat coil of a pipe through which steam is directed by an inlet in and to an outlet in the metal plate on which the framework and the exhaustible bell jar rests. A rubber washer with a luting of soft soap will make a tight joint between metal and glass.

2. *Drying by Alcohol.*

Minced or pulped animal organs may be treated with an equal volume of alcohol, and after some hours the fluid strained and pressed off. This first alcoholic liquor removes a large part of the water and very little of the fatty substances. Thus in one case 840 grms. of liver pulp containing fatty substances, estimated as fatty acids soluble in petroleum ether after saponification, amounting to 8·8 per cent., was treated with 900 c.c. of spirit and the expressed fluid contained in all 1·13 grms. of fatty substances estimated in the same way, or 0·13 per cent. of the pulp, or less than 1·5 per cent. of the total fat that it contained. The fatty substances that are thus removed will clearly, from their solubility in such aqueous alcohol, be phospholipines not simple glycerides, and the iodine value of the fatty acids they contain is high.

The undissolved mass is then treated repeatedly with fresh quantities of alcohol, and the expressed alcoholic liquors mixed, filtered and evaporated *in vacuo* at a low temperature with a fine, slow stream of carbonic acid or some inert gas passing through a capillary tube beneath the surface. When the alcohol and water have boiled off, the residue can be taken up in ether.

After the tissue has been in this way dried with alcohol it can be reduced to powder and extracted with ether. The ether extracts are freed from solvent at a low temperature and pressure, and the residue again taken up in ether can be mixed with the ethereal solution of the alcoholic extracts.

After four successive extractions with alcohol followed by two with ether there remained unextracted in the liver pulp, in the experiment referred to above, 2·3 grms. or 3·1 per cent. of the total fatty substance of the liver.

The first portion of alcohol with which the tissue is treated necessarily contains after expression much water. And on evaporation *in vacuo* this gives trouble from frothing. It is convenient, therefore, not to mix it with the subsequent alcoholic liquors, but either to disregard it or evaporate it separately.

It would probably be wise where it is desired to obtain the most sensitive fatty substances from a tissue with the least possible alteration, to use for drying the tissue alcohol that has been recently freed from dissolved oxygen by boiling: for the coefficient of absorption of alcohol for oxygen is about twenty times as great as that of water for this gas. And in any case the drying under alcohol should be carried out in closed vessels.

If the risk of change from contact with air at the temperature of a hot water-bath is in any case immaterial, and the drying of the tissue is to be effected in the old way in an open vessel at a high temperature, then too the practice of adding alcohol to the tissue in small portions at a time, and as each portion evaporates of rubbing the material down with a pestle in the evaporating basin greatly facilitates the final pulverisation of the dried substance.

3. *Drying by Means of Anhydrous Salts.*

Anhydrous sodium sulphate rubbed into an intimate mixture with moist tissues is converted into the hydrated salt at the expense of the water of the tissue, and in this way a dry, easily pulverisable mass can be obtained after a few hours (Pinkus, Schryver). In the case of the brain, yolk of egg and of blood, this method is particularly convenient; 100 grms. of the anhydrous sodium salt during complete conversion into the hydrated crystals take up about 125 grms. of water.

Calcium sulphate has been similarly used (*cf.* Rosenheim); but since 100 grms. of the anhydrous calcium salt take up only about 25 grms. of water the bulk of material for extraction becomes very great. If the removal of water is effected entirely by this means, at least 3 grms. of plaster of Paris must be taken for 1 grm. of most animal tissues, whereas 0·6 grm. of anhydrous sodium sulphate should be sufficient for this same amount of the same tissue.

When the material is dried, the pulverisation of small quantities in a mortar by hand presents no great difficulty; but where large quantities have to be dealt with the mills used for grinding coffee or pepper may sometimes serve, but for the liver or any material that contains at all large amounts of fat, a mechanical mortar or a "drug mill" is better, and, in this case, the grinding may be done under alcohol.

But if the original mincing of the fresh tissue was efficiently carried out, then it is questionable whether the labour of pulverising the dried material to the finest state possible will be repaid in the additional yield of material. Finely minced liver at any rate easily gives up to alcohol and ether, without any laborious powdering of the dried material, all but 2 or 3 per cent. of its compounds of fatty acids.

Material that has been dried with sodium sulphate can be easily pulverised if it was originally finely minced, even without the use of mechanical mortars, but a motor driven ball mill may save labour.

B. *The Estimation of Fat in Animal Tissues.*

The estimations were for long always done by extraction of the dried and powdered tissues with fat solvents. For long, too, the

different procedures in doing this were compared one against the other, merely as to the amount of extract soluble in ether that was obtained: a high yield meant a good method. It was then realised that much that was soluble in ether might not be fat, and more attention was paid to the "purity" of the extract. But, though the fat stored in the connective tissues is almost entirely composed of simple glycerides of fatty acids soluble in ether and easy to obtain "pure," the fat in the cells of the liver and other organs is largely composed of complex phospholipines containing nitrogen and phosphorus, and much less fatty acid than the simple fats; and, moreover, these phospholipines are able to carry with them into solution in ether substances not in any way chemically related to fats; so that it is impossible to obtain a complete extraction of the fat in these organs without obtaining an "impure" extract containing nitrogen and phosphorus, and giving a low percentage of fatty acid on saponification.

In view of this inherent difficulty, methods were introduced in which, instead of attempting to determine the gross weight of substances soluble in ether, the amount of higher fatty acids which they were capable of yielding after saponification was estimated. In this way the common component of simple and complex fats alike, was determined; the component which gives to both their physiological significance, and which accounts for their high value in the economy of the organism.

The methods, therefore, for estimating fat may be dealt with in two groups, extraction methods and saponification methods.

1. *Extraction Methods.*

All such methods present certain inherent defects: (1) The tissue must be dried; and if this is done by heating in the air unsaturated acids are partially oxidised, their weight thus on the one hand increased, their solubility on the other hand altered. This will make most difference in organs like the heart, liver, kidney, etc.

(2) The extract will have a varying composition according to the organ from which it is obtained, and its condition at the time. The estimation, therefore, of fat will mean different things at different times.

The different methods employ different solvents, and generally more than one, *e.g.*, alcohol or acetone followed by ether or chloroform, and the extract is generally, after removing the solvent used for extracting it, taken up in ether or petroleum. They also differ in the way in which the attempt is made to ensure that the fat solvents have free access to the fat. With this in view at first, attention was specially

paid to the drying and powdering that preceded extraction with ether. Pflüger showed, however, that this could not give complete extraction however long it was continued, and suggested the digestion of the proteins in the tissue with pepsin, filtering off fat, drying the filter and extracting it, and, at the same time, shaking out the filtrate with ether. The use of alcohol for the first stage of the extraction has the advantage of removing water, and leaving the tissue in a condition to give up its fat more completely to ether; but, besides this, alcohol seems effectually to liberate fat from cells in a way not clearly understood, possibly counteracting the tendency for some fatty substances to adhere to surfaces in the presence of water, much as alcohol will remove a dye that has adhered to charcoal suspended in water. Alcohol extraction as a first step in the estimation of fat is still of great value in certain cases; for instance, in the case of the blood (*vide infra*, p. 61), and also in the case of fæces. For this latter material acetone may be preferred to alcohol. Solid matter can be placed in a cartridge of grease-free paper, wrapped and tied in fine grease-free silk, and this, suspended from the cork of a flask containing alcohol or acetone and fitted with a condenser; from the condenser solvent falls on the cartridge which is kept near the boiling point by the vapour.

2. *Saponification Methods.*

Liebermann introduced the practice of heating a known amount of the substance with strong alkali and alcohol so as to saponify all forms of fat, then making acid and shaking the fluid with a known volume of petroleum ether, and weighing the fatty acids present in a fraction of this after it has separated clear. Liebermann himself titrated the acids with phenolpthalein and then dried and weighed the soaps, allowing for the weight of alkali and indicator used.

In some form this method is now greatly used in estimating fat for physiological investigations; that which for many years we have employed may be described in some detail.

From 10 to 20 grms. of the fresh moist material is weighed out to the second place of decimals in a basin. The amount to be taken should be determined roughly by the amount of fatty acids likely to be obtained from it; this amount should be from 0·3 to 0·7 grm. Of a liver or heart, therefore, yielding the usual amount of fatty acids, about 3 per cent. of its fresh weight, from 10 to 20 grms. is to be desired; but when this amount is not available an estimation can still be carried out, with 5 grms. or perhaps even less. Into the basin is then measured roughly a corresponding amount, 10 to 20 c.c., of a solution of caustic

potash made by dissolving potash sticks in two-thirds of their weight of water, and the basin is heated under a watch-glass on the water-bath till the tissue is disintegrated. If it is stirred occasionally with a glass rod, and the bath be boiling, this takes half an hour. Then 10 to 20 c.c. of spirit are stirred in, the watch-glass removed, and the heating continued with occasional stirring for an hour. Sometimes, if drops of unsaponified fat are visible, more alcohol may be necessary. The soap is then washed with hot water into a stoppered flask with a neck 8 inches long and 1 inch internal diameter and a bulb having the capacity of about 200 c.c. The flask and contents are cooled under the tap, and 15 to 30 c.c. of 40 per cent. sulphuric acid are cautiously added, with constant cooling, so as to avoid frothing over. When this has been done it is convenient to heat the flask in the water-bath for a few minutes to drive off gas. Then, after again cooling, either 25 or 50 c.c. of petroleum ether, exactly measured, are added according to the amount of fatty acids expected to be present, the object being to obtain a solution of between 0·5 and 1·5 per cent., stronger solutions than this involving an appreciable error from change of volume. The volume of fluid in the flask is, by the addition of water, so adjusted that the column of petroleum lies entirely in the neck of the flask and in the lower part of it.

The stopper moistened with glycerine is then put in, and the flask shaken some thirty times at intervals of a minute, each time for about 10 seconds. Steps must be taken to ensure the stopper not being driven out by the pressure in the flask, which, however, will be slight if the flask was heated as directed after adding the acid and then sufficiently cooled. The flask is then left for an hour or more till a layer of clear petroleum has separated, when an aliquot part is removed with a pipette, such an amount as will contain not less than 0·15 grm., preferably 0·2 or more, and run into a weighed round-bottom flask with a short, wide neck and a capacity of 150 to 200 c.c. From this flask the petroleum is distilled off in a water-bath while a current of CO_2 is kept passing through. When the distillation is completed the receiver is connected with the water-pump, the CO_2 stream reduced, and the apparatus exhausted with the flask immersed in the bath for half an hour. The flask is then removed to a desiccator and, when cold, weighed.

In some cases the petroleum solution tends to separate as an emulsion. This can generally be broken up, after it has stood for a while, by appropriate agitation, so as to give a clear layer that is at least four-fifths of the whole. But occasionally the emulsion is more obstinate and

it can then be cleared by adding a few c.c. of a solution of bile salts.

The crude fatty acids so obtained will contain cholesterol and possibly other unsaponifiable matter. And since this is present in varying amounts, and may in some cases form nearer 20 than 10 per cent. of the crude fatty acids, it is clearly desirable that it should be removed, and its amount determined. This will only be possible with any degree of accuracy when somewhat larger quantities of the crude fatty acids are obtainable, 1·0 grm. or more. In that case the mixture may be dissolved in hot alcohol and made alkaline (after being titrated if desired) with potash, and the solution transferred to a separating funnel so as to contain about equal amounts of alcohol and water. It is then shaken with petroleum ether, the lower layer removed and shaken with fresh petroleum, and the upper layer shaken with fresh alkaline 50 per cent. alcohol. The combined petroleum solutions contain the unsaponifiable substances, which can be weighed after removing the petroleum.

Such a method as that here described is at almost every point a compromise. But in order to make the estimation of fat a practicable operation compromises are necessary, and they are necessary too for the approximation to accuracy. Complications that are theoretically desirable may defeat themselves not only by making an estimation impracticable in the time available, but also by introducing fresh sources of error with every fresh operation.

In preparing animal organs for estimation of fat care must, of course, be taken to remove adipose connective tissue. In the case of the liver the large vessels and the gall-bladder should be removed; in that of the heart the auricles and even the right ventricle must as a rule be sacrificed entirely; the basal portion of the ventricle, the valves, and the entire epicardial surface must be cut away, especially along the course of the vessels; also the endocardial surface should be similarly cleared of all that is not pure myocardial substance; the kidney should be split longitudinally by three parallel cuts exposing the ramifications of the pelvis and vessels so that they can be pursued into the substance of the kidney and cut entirely away.

When the total fat in an animal's body is to be estimated, either without or after removing any of the organs, the carcase can be heated without subdivision in a covered basin with the requisite amount of strong potash, 30 to 50 c.c. for 100 grms., till entirely disintegrated, the bones readily rubbed to a fine powder with a pestle, and, after the saponification has been completed by the addition of alcohol, the

whole washed into a measuring flask, and an aliquot part taken for treatment, after acidification, with petroleum ether.

Modifications of the saponification method have been described by Kumagawa and Suto, and by Mottram. The following is the method of Kumagawa and Suto, with slight modifications introduced by Mayer and Schaeffer.

The finely divided tissue is placed in a beaker with 25 per cent. caustic soda solution, using 40 c.c. of this for every 10 grms. of tissue. A little alcohol (about 5 to 10 c.c.) is added, and the whole heated on a water-bath for an hour and a half. When saponification is complete the contents of the beaker are added gradually to a solution of hydrochloric acid in a decantation flask, the amount of acid in the flask being in excess of the amount required to neutralise the soda used in the saponification. The mixture is allowed to cool. Ether, free from alcohol, is now poured into the flask until a layer about 3 cms. thick lies above the aqueous portion of the contents. The contents are well shaken; the two phases are allowed to separate and the ether decanted into a distillation flask. The residual liquid in the decantation flask is shaken out with ether several times and the whole of the extracts poured into the distillation flask. The ether solution is then distilled under reduced pressure and finally warmed to 100° for 5 minutes, the pressure being maintained at about 10 mm. of mercury. The residue is taken up in dry ether, the solution filtered into a beaker and evaporated on an electrically heated bath at 80°. The residue is heated at 50° in an oven for 8 to 12 hours. To the dry ether extract, while still warm, 30 to 40 c.c. light petroleum are added, and after gentle agitation the contents of the flask are allowed to stand for 3 hours. In this period of time the small amount of resinous matter which is insoluble in light petroleum settles to the bottom of the flask. The solution is filtered into a tared beaker, the precipitate well washed with light petroleum and the solution and washings evaporated. The residue, which should be practically colourless, is dried for an hour at 50° and weighed. It consists of fatty acids and unsaponifiable matter. To remove the latter the procedure described above in the modified Liebermann method may be used (A. Mayer and G. Schaeffer, 1913).

The saponification method cannot be applied directly to the estimation of fat in blood (Shimidzu, 1910), but with certain modifications in the procedure satisfactory results may be obtained. The serum or blood, of which 20 to 30 c.c. should be taken, is first poured into five times its volume of alcohol with constant stirring, the precipitate is allowed to settle for 24 hours and then filtered off through an

extraction thimble. The precipitate in the thimble is now extracted with hot, absolute alcohol, by suspending it in the neck of a flask in which the alcohol is boiled, condensing the alcohol vapour in a reflux condenser, and allowing the condensed alcohol to drip through the thimble on its way back to the main bulk of boiling liquid. The extraction is continued for 6 hours. The first and second alcoholic extracts are now united, 10 c.c. of 50 per cent. sodium hydroxide solution are added and the liquid is boiled under a reflux condener for an hour. The condenser is now removed and the alcohol allowed to evaporate, while the saponification continues. When the alcohol has been removed and the saponification is complete the estimation of the fatty acids may be proceeded with by the Liebermann or Kumagawa methods. The precipitate remaining in the extraction thimble still contains combined fatty acids, and they may be recovered and estimated by the ordinary saponification method, or, after saponification, the alkaline liquid may be added to that obtained by the saponification of the alcoholic extracts and the estimation carried out in the usual way. If the estimation of the fatty acids in the precipitate after alcohol extraction be neglected the error is not more than 5 per cent. (Mayer and Schaeffer). It is sometimes convenient to do this when dealing with whole blood or corpuscles because of the troublesome emulsions obtained when making the ether extraction.

A micro method for the estimation of fat in blood has been worked out by Bloor (1914), Bloor, Pelkan and Allen (1922). It has the advantage that only a small amount of blood is required, but it is open to the general criticisms that may be directed against all nephelometric methods. Bloor's procedure is as follows: Five c.c. of blood are run with stirring into 75 to 80 c.c. of an alcohol-ether mixture (alcohol 3, ether 1), in a 100 c.c. flask. The liquid is raised to boiling in a water-bath, and then cooled. The volume is made up to 100 c.c. with the alcohol-ether mixture, the contents well mixed and filtered. For the determination, a volume (10 to 20 c.c.), containing about 2 mgms. of fatty acid, is measured into a small Erlenmeyer flask, 0·1 c.c. of concentrated NaOH made from sodium is added, and the mixture evaporated on the water-bath. When the evaporation is nearly complete, the flask is rotated to distribute the liquid evenly over the bottom of the flask. The drying is stopped when only 2 or 3 drops of liquid remain. The alkali is then partially neutralised by the addition of 0·1 c.c. of dilute sulphuric acid (1 vol. conc. acid to 3 vols. water), and the liquid well mixed and distributed over the bottom of the flask. The drying is then continued until the residue is dry and the moisture has disappeared from

the sides of the flask. After cooling, the cholesterol is removed by extraction with cold chloroform. For this purpose the contents of the flask are allowed to stand for 10 minutes with 10 c.c. of chloroform, which is then poured off and the extraction repeated with two successive portions of 5 c.c. each. The combined extracts may be used for the estimation of cholesterol if desired by a method described by Bloor, Pelkan and Allen. For the estimation of the fatty acids after the removal of the cholesterol, the residue in the flask is boiled with 10 c.c. of redistilled alcohol for 10 minutes. The hot alcohol is then poured through the filter used for the filtration of the chloroform extract into a 100 c.c. Erlenmeyer flask. The extraction is repeated once using 5 c.c. of alcohol, and the combined extracts are evaporated to about 2 or 3 c.c. The solution is transferred quantitatively to a small stoppered cylinder, and the flask is rinsed out with just enough alcohol to bring the total volume up to 5 c.c. One hundred c.c. of distilled water are next measured into a 200 c.c. beaker, and the alcoholic extract is added with stirring through a small funnel with the stem drawn out to form an opening about 1 mm. in diameter, and extending nearly to the bottom of the beaker. The cylinder is rinsed with the solution in the beaker, and the rinsings poured back into the beaker through the funnel. To another beaker containing 100 c.c. of water are added through a pipette with stirring, 5 c.c. of the alcoholic standard containing 2 mgms. of a mixture of oleic and palmitic acids in 95 per cent. alcohol (redistilled). The mixed fatty acids contain 60 per cent. oleic and 40 per cent. palmitic acids. Ten c.c. of dilute hydrochloric acid (1 part conc. acid to 3 parts water) are added to each beaker with stirring, and after standing not less than 3 or not more than 10 minutes the solutions are compared in the nephelometer.

CHAPTER III.

DETERMINATION OF THE CHARACTER AND PROPERTIES OF FATS.

WHEN quantities of a specimen of fat are obtainable, which for analytical purposes are unlimited, as is the case in the technical examination of commercial fats, many properties can be, and are, profitably determined which, in physiological investigations, owing to the comparatively small amounts available, often cannot be determined, and at the same time, great as their practical significance may be in technical chemistry, do not convey to the biologist the information which he most requires. Much fuller accounts, therefore, of the determination of such properties as these will be found in technological books than would be in place here.

The methods that may be employed for examining and determining the properties and character of a fat will be dealt with here under the three following heads :—

A. Methods for determining physical properties.
B. General chemical methods in use in technical analysis of fats.
C. Methods for the separation, identification and estimation of the constituents of a fat.

A. PHYSICAL PROPERTIES OF FATS.

1. *Specific Gravity.*

The specific gravity of fats and oils can be determined with different forms of pycnometer or with small amounts by suspending drops in different mixtures of alcohol and water of known graded specific gravities. The pycnometer used by Ubbelohde, with capillary side tube rising from low down in the bottle, can be used for liquid oils and also for solid fats; in the latter case the bottle is filled with water and weighed, and then a weighed amount of the solid is introduced, the stopper inserted, and the diminution of weight plus the weight of the solid fat gives the amount of water displaced and the volume of the fat. It may be noted that the presence of glycerides of lower fatty acids raises the specific gravity of a fat. Butter has at 100° C. the specific

gravity 0·868 (water at 15° C. being 1); tallow, 0·860, and cocoa-nut oil, 0·873; whereas cacao butter gives the figure 0·857. Rancidity is accompanied by an increase in the specific gravity; in the case of olive oil, in six months' exposure to light and air the specific gravity at 15° was observed to rise from 0·9168 to 0·9246. Remarkably low specific gravity values are characteristic of the liquid waxes, *e.g.*, sperm oil, 0·830 at 100° C.

2. *Melting Point.*

The melting point of fats has been frequently used in physiological experiments for characterising a fat, and sometimes to good purpose. It has been noted, for instance, that cows fed on certain kinds of oil yield butter with so low a melting point as to be unmarketable, and the inference drawn that the fat secreted by the mammary gland is the same fat that the animal has had in its food, not fat that has been made by the gland expressly for its secretion, a conclusion for which, of course, there is now other and stronger evidence. Again, Rosenfeld states that geese fed on potatoes yield a fat with a considerably higher melting point than goose fat generally exhibits, and cites this in support of his belief that fat synthesised from carbohydrate food in the animal body is comparatively rich in the glycerides of the saturated acids.

For technical purposes the melting point is determined very frequently, and in some cases this determination is of considerable service. The melted fat is drawn up into a capillary tube, which is then sealed so as to give a column of about 1 cm. in length. The tube should be of about 1 mm. bore, and after it has been filled at least a day should be allowed to pass before it is used for a determination; or a piece of the fat is placed on mercury in a vessel suspended in water that is heated, and the thermometer immersed in the mercury gives the temperature at which the fat spreads out over the mercury.

But since fats are not single substances but mixtures of different glycerides, the melting points are not sharp. Attempts are made to determine the point at which fusion begins and separately the point at which it is complete. For this purpose a capillary is made with a bulbar enlargement on it. In this enlargement with the capillary in a horizontal position a drop of the melted fat is allowed to solidify, and then after one or two days with the capillary vertical in the usual way the temperature is gradually raised. When the drop slides from its lateral position to the bottom of the bulb the point of incipient fusion is taken; when the melted fat runs down into the lower part of the capillary another reading is taken, the point of complete fusion.

In all determinations of the melting point of fats it is necessary to wait some time, not less than one day, before fat which has been melted—for the purpose of filling a capillary, for instance—is used for such a determination. For even pure glycerides of fatty acids that are single chemical entities melt at a much lower temperature if they have been recently melted than that at which they melt if they have been kept in the solid state for some time. The explanation of this phenomenon is not clear, but it has been attributed to the existence of the fat in two forms—an unstable and a stable. The passage from the unstable to the stable form occurs spontaneously but slowly, unless accelerated by the addition of a crystal of the stable glyceride (*cf.* Grün and Schacht, Bömer, Guth).

It is clear from all this that the melting point is not a constant that can be relied upon for characterising a fat in biological work except under special conditions.

3. *The Solidification Point and Titre Test.*

When a melted fat or oil is cooled down the temperature falls gradually to a variable degree, then rises rapidly to a certain constant temperature, at which it remains steady for a time before it begins to fall again. This steadily maintained temperature is the solidification point.

The determination of this point can be made with a considerable degree of accuracy, and in commercial analysis is very largely used. It is generally carried out on the fatty acids obtained by saponification of the fats, and is then officially known as the Titre test. In different countries somewhat different prescriptions are given for the carrying out of this test, and the results obtained vary according to the method followed. The method described by Dalican is in one form or another in use in Great Britain, the United States and France. The form prescribed by the Association of Official Agricultural Chemists in the United States is briefly as follows: 75 grms. of the fat are saponified in a metal dish with 60 c.c. of 30 per cent. caustic soda solution and 75 c.c. of 95 per cent. alcohol or 120 c.c. of water. It is evaporated to dryness, dissolved in 1 litre of water, and boiled to remove alcohol. The fatty acids are separated by adding 100 c.c. of 30 per cent. sulphuric acid and heating till clear. They are washed with hot water till free from soluble acids, and then filtered through a dry filter on a hot-water funnel, dried for 20 minutes at 100°, and cooled down to within 15° to 20° C. of the solidification point. They are then poured into a test tube 25 mm. in diameter and 100 mm. long, which

is suspended by a cork in the mouth of a jar 70 mm. wide and 150 mm. high. A thermometer graduated in tenths of 1° C. between 10° and 60° is used, the bulb of which is 3 cm. long by 6 mm., and this serves also as a stirrer; the determinations should not vary by more than one-tenth of 1° C.

It is important that the fatty acids should be efficiently dried. Overcooling does not always occur, but in that case the same steadily maintained temperature can be observed, not preceded by a rise of the mercury.

A method that requires such a large amount of material is not likely to be much used in purely physiological investigations.

4. *The Refractive Index.*

The refractive index of fats and oils varies pretty widely, and its determination is made use of in the detection of adulteration of butter, but has not as yet found use in purely scientific physiology.

5. *The Rotatory Power.*

The rotatory power of fats and oils is often due to the presence of cholesterol and other impurities in them. But a triglyceride containing different fatty acids is capable of stereoisomeric modifications, and some of the fatty acids—in Chaulmoogra oil for instance—may themselves be asymmetric substances. Lecithine and glycerophosphoric acid are optically active. Certain optically active glycerides have been synthesised (Abderhalden and Eichwald), but such glycerides have not been proved to occur as might be expected in nature. The rotatory power is small.

B. GENERAL CHEMICAL METHODS IN USE IN THE TECHNICAL ANALYSIS OF FATS.

There are several well-recognised chemical methods of examining and characterising a fat in common use in technical chemical analysis which are also of service in the study of fats in physiology.

1. *The Acid Value.*

The acid value of a fat is obtained by titrating any free fatty acid that it contains and determining the number of milligrammes of caustic potash required to neutralise a gramme of the fat. It is therefore a measure of the degree of hydrolysis of the fat, which may be due to rancidity or to ferment action. A weighed amount of the fat is dissolved in neutral alcohol or alcohol ether, and a few drops of a 1 per

cent. solution of phenolphthalein in alcohol added, and the titration effected with a tenth or half-normal aqueous alkali, according to the amount of fat available and the amount of free acids it contains. Under ordinary circumstances animal fats have a very low acid value, and therefore 5 to 10 grms. must be taken for an estimation of this value. The end point is a pink colour that lasts 2 minutes, though after that it will probably disappear.

In the case of difficulty in seeing the end point owing to the darkness of the solution it may be necessary to adopt the method of Bödtker, who shakes 5 c.c. of the oil with 25 c.c. of alcohol, 50 of water, sufficient N/10 soda to give a strong red colour with phenolphthalein, and then titrates back. In such cases, too, a fluorescent dye such as eosin can be used as an indicator.

2. *The Saponification Value.*

The saponification value of a fat is given by determining the number of milligrammes of caustic potash that are neutralised during saponification of 1 gramme of the fat by the total fatty acids that it contains, whether originally combined with glycerol or other alcohol or free.

For this determination an alcoholic potash solution is required which should be about half-normal. The exact titre of this alkali, as it is liable to change, should be determined shortly before use by titration with a half-normal hydrochloric acid solution.

A weighed amount, usually from 1·5 to 2 grms. of the fat, is heated in the flask in which it is weighed with 25 c.c. of the alcoholic potash on a boiling water-bath for half an hour. In the neck of the flask, which should be of some glass that does not give off alkali, is a cork with a long straight glass tube for a condenser passing through it. Another similar flask similarly fitted, containing the same amount of alcoholic potash and heated side by side with the first, serves as a blank control in case the titre should be altered by carbonic acid or in any other way during the heating. The saponification of the fat having been completed, the alkali in each flask is titrated with half-normal hydrochloric acid and phenolphthalein, and the difference between the amounts of acid required by the two flasks gives the amount of alkali neutralised by the fatty acids contained in and liberated during saponification from the amount of fat taken; from this the saponification value can be calculated.

In the titration of fatty acids it must always be remembered that soaps are hydrolysed by water and therefore react alkaline. This hydrolysis is prevented if sufficient alcohol is present. A simple and safe rule is to see that there should be about as much alcohol present

in the solution as there is water when the titration is completed. According to Kanitz, hydrolysis of soap does not occur in 40 per cent. alcohol, so that to aim at equal parts of alcohol and water is to be on the safe side. If, therefore, during a determination of the saponification value of a fat there has been much evaporation of alcohol, it is necessary to add neutral alcohol before titrating, the amount to be added being determined by a rough estimate of the amount of aqueous hydrochloric acid that will be required for the titration.

If the oil is dark the weighed amount may be boiled for an hour with 25 c.c. benzene and 25 c.c. of N/2 alcoholic potash; then 30 c.c. of water added, and the alkaline solution drawn off from a separator, the benzene washed twice with water and the mixed liquids titrated with phenolphthalein (Meyer).

The saponification value is clearly a measure of the mean molecular weight of the fatty acids entering into the composition of a fat. Fats such as lard or tallow give figures about 195, whereas butter and oils containing glycerides of lower fatty acids, such as cocoa-nut oil or palm-nut oil, give much higher figures, butter 220 to 233, cocoa-nut oil about 250. Similarly, the low saponification values of liver fat and fish-liver oils (cod-liver oil 171 to 189) indicate the presence either of unsaponifiable substances such as cholesterol or of glycerides of acids of higher molecular weight than stearic, mainly the latter. In the oils of most cruciferous seeds the saponification value is less than 180 (colza oil sometimes as low as 170) owing to the presence of large quantities of erucin.

The saponification of waxes, with the exception of spermaceti, is not so readily effected as that of fats, and many suggestions have been made for the facilitation of the process in such cases. The alcoholic potash used should contain as little water as possible, and the heating be prolonged for at least an hour or as much as three hours, and be carried out over a flame on wire gauze and with an efficient condenser. This is said to suffice in the case of beeswax and carnauba wax, but for wool wax other more drastic measures must be taken. Lewkowitsch heats in a sealed tube with twice normal alcoholic potash for 3 hours at 105° C. Kossel and Obermüller proposed the use of sodium ethylate (5 grms. of sodium dissolved in 100 c.c. of absolute alcohol, freshly prepared). Henriques proposed a method of saponification in the cold: the fat is dissolved in petroleum ether and a solution of soda or potash in absolute alcohol added and the mixture left for 12 hours. Winkler uses potash dissolved in propyl alcohol, the higher boiling point of which is effectual in saponifying waxes.

The saponification values of waxes are low, because the molecular weights of the alcohols as well as of the fatty acids of which they are composed are high. The figure for beeswax is 90 to 98, for wool wax 102, and for spermaceti about 130.

When in the estimation of fat in animal organs a method is employed such as that described on page 58, the fatty acids obtained may be dissolved in alcohol and titrated, and the mean molecular weight of the acids insoluble in water determined. If the figure obtained is high, as is often the case, it may be partly or entirely due to the presence of cholesterol or similar unsaponifiable substances. The removal of these by shaking the soap solution with petroleum ether in the way described on page 93, and the determination of their amount by weighing the residue left after distilling off the petroleum from the washed solution, makes it possible to make a correction that brings the molecular weight of the fatty acids down, sometimes from as much as 320 to 280.

The *ester value* is nothing more than the difference between the saponification and acid values, and therefore the number of milligrammes of caustic potash required to neutralise the fatty acids present in a fat in the form of neutral esters.

3. *The Iodine Value.*

The iodine value of a fat gives the amount of halogen reckoned as iodine that the unsaturated acids entering into its composition will take up, expressed in percentage of the weight of the fat. Thus triolein (M.W. 884) will absorb six atoms of iodine ($6 \times 127 = 762$) or 86·2 per cent., and its iodine value is 86·2. The iodine values of fatty acids may be obtained similarly, that of oleic acid being 90·1. The saturated acids and their glycerides of course absorb no iodine, and therefore the iodine value of a fat or of a mixture of fatty acids is an index of the proportion of unsaturated and saturated acids present. The acids with unsaturated linkages in more than one place absorb of course proportionately more iodine, so that the iodine value 86·2 which is given by triolein may also be given by many mixtures of glycerides in which saturated glycerides and at the same time glycerides of acids less saturated than oleic acid are present.

For this determination the most convenient method is that of Wijs, for which the solutions required are a titrated solution of iodine monochloride obtained by mixing solutions of iodine trichloride and iodine in glacial acetic acid, a titrated solution of sodium thiosulphate, a solution of potassium iodide about 10 per cent.

9·4 grms. of iodine trichloride are weighed out into a flask of about

300 c.c. capacity, into which is then poured about 200 c.c. of glacial acetic acid; the flask fitted with a cork through which passes a calcium chloride tube is heated on the water-bath till the whole is dissolved. 7·2 grms. of iodine rubbed to a fine powder in a mortar are then washed with glacial acetic acid into another similar flask and similarly heated. The contents of the flasks are poured into a stoppered litre flask and the undissolved iodine heated again with further quantities of acetic acid till all is dissolved. The solution is then stoppered, allowed to cool down, and made up to a litre with acetic acid and titrated on the following day. For this purpose 10 or 20 c.c. of the solution, measured exactly with a pipette, is treated in a large Erlenmeyer flask with 5 or 10 c.c. of the potassium iodide solution, diluted with about 200 or 400 c.c. of water, and a sodium thiosulphate solution of known strength run in till the fluid is pale yellow, when some starch solution is added and thiosulphate again run in till the blue colour disappears. From the amount of standardised thiosulphate used the amount of iodine in the measured amount of Wijs is calculated. The strength of the iodine chloride solution is likely to alter a little in the first 24 hours, but after that should remain very nearly constant for some weeks. It should be restandardised from time to time in any case, but the intervals will depend on the stability of the particular preparation used. In preparing the solution it is necessary to attend especially to the acetic acid. This should be recrystallised, the mother liquor poured off, the crystals melted and again crystallised. By attending to the preparation of the solvent and preventing subsequent absorption of water a solution is obtained that keeps its titre well.

The thiosulphate solution is prepared by dissolving 48 grms. of the salt in 2 litres of water, and after allowing it to stand for a day standardising it by Volhard's method. For this purpose a solution of 3·8657 grms. of potassium bichromate in 1 litre is prepared. Then 10 c.c. of the potassium iodide solution is treated in a 750 c.c. Erlenmeyer flask with 5 c.c. of strong hydrochloric acid and exactly 20 c.c. of the bichromate solution. These fluids are well mixed, and then diluted with about 300 c.c. of water. The strength of the bichromate solution is such that 20 c.c. acidified with hydrochloric acid liberates exactly 0·2 grm. of iodine from the iodide. When titrated, therefore, with the thiosulphate the number of milligrammes of iodine corresponding to each cubic centimetre of thiosulphate is obtained. The thiosulphate solution should alter very slightly, but should be restandardised from time to time.

The estimation of the iodine value is carried out as follows. Some

of the fat or fatty acid mixture is weighed in a stoppered flask of 50 to 150 c.c. capacity. The amount taken should depend upon the probable iodine value and the amount of Wijs' solution used. There should be between two and three times as much iodine in the latter as the fat can absorb. If 25 c.c. of the Wijs' solution be used (= about 640 mgrs. iodine), the amount of iodine absorbed by the fat should be about 250 mgrs. So that if the fat be connective tissue fat, with a low iodine value, about 0·4 grm. should be taken; if the fatty acids obtained from an organ are being investigated about 0·2 to 0·25 grm. should be taken for 25 c.c. of the Wijs' solution. The weighed fat is dissolved in 10 c.c. of carbon tetrachloride, which has been proved not to absorb iodine, and when dissolved the measured amount of Wijs' solution added and the flask stoppered. After standing in the dark for from 1 to 2 hours, the contents of the flask are poured into a 750 c.c. Erlenmeyer flask, 10 c.c. of potassium iodide run into the former to dissolve the traces of iodine left in it, and the contents then washed quantitatively into the Erlenmeyer flask with water. The volume of fluid obtained should be about 300 c.c. This is then titrated with thiosulphate, and the calculation made as in the following example:—

Iodine contained in 25 c.c., Wijs	= 645·7 mgrs.
Thiosulphate used in titration = 34·4 c.c. . . .	
1 c.c. Thiosulphate = 12·2 mgrs. iodine . . .	
Unabsorbed iodine = 34·4 × 12·2	= 419·7 mgrs.
Iodine absorbed . . .	= 226·0 mgrs.
Fat taken = 0·378 grm. ,, ,, . . .	= 59·7 % = iodine value.

The amount of fatty acids or of fat required for this estimation is about the amount obtained in the method of estimation of fat described in an earlier section, and it is convenient when the two estimations are to be carried out to arrange that this should be so; then the sample of fat weighed in the first estimation can be used directly for the second, that of the iodine value, without having to be transferred to another vessel.

The estimation of the iodine value of fats is very largely used in commercial analysis, and indeed the classification of fats and oils in use in commerce is based upon this value. The drying oils are those that, owing to their containing glycerides of acids of the linoleic and linolenic series, readily undergo oxidation in the air so as to "dry" to a varnish, and therefore till they have undergone such oxidation, can absorb large amounts of iodine, and have high iodine values. According to their iodine values, oils are divided into drying, semi-drying and non-drying oils. Certain animal fats and oils, however, have high iodine values

without the property of forming varnishes on exposure to air; salmon oil, for instance, and cod-liver oil have iodine values about 160 and do not dry.

Most of the iodine values given in technological handbooks have been estimated by the older method of von Hübl. In this method the operation lasts much longer, and the iodine solution used is a solution of 25 grms. of iodine in 500 c.c. of 96 per cent. alcohol, mixed the day before being used with an equal quantity of a solution of 30 grms. of mercuric chloride in 500 c.c. of 96 per cent. alcohol. Waller recommended a solution that keeps its titre better than von Hübl's, obtained by adding to 1 litre of the mixed iodine and mercuric chloride solutions 50 c.c. of strong hydrochloric acid. The hydrochloric acid prevents the reaction between iodine monochloride and water

$$(ICl + H_2O = HCl + IOH).$$

The values obtained with Wijs' solution are in some cases higher than those obtained with von Hübl's or Waller's, in the case, for instance, of linseed oil, certain neutral oils, and remarkably so in that of cholesterol. (See p. 94.)

It will be noticed that the amounts of iodine trichloride and of iodine used in preparing Wijs' solution are not in molecular proportion. Lewkowitsch prepares the solution by dissolving 7·9 grms. of iodine trichloride and 8·7 grms. of iodine in glacial acetic acid, that is to say, molecular proportions, as required by the equation $ICl_3 + I_2 = 3ICl$.

4. *The Hehner Value.*

The Hehner value gives the percentage of fatty acids insoluble in water that are yielded on saponification by a fat.

A weighed amount, from 1 to 4 grms., of the fat is saponified with alcoholic potash, the soap solution washed with hot water into a beaker and made acid with dilute sulphuric acid. The beaker is kept on a hot bath till the subjacent aqueous layer is clear, and then the contents are filtered through a weighed filter. The filter should be carefully selected, but most of the better-class filter papers serve. It should be folded so as to insure rapid filtration, and be half filled with water before the fatty acids are poured on. The beaker is washed with a jet of hot water on to the filter and the fatty acids washed on it similarly so long as any acid reaction can be detected in the washings. The washing should be carried out continuously, so that the filter is kept filled with water. The funnel and filter can then be immersed in a beaker of cold water, so that the fatty acids solidify and then allowed to drain dry; the filter is then dried and weighed in a weighing bottle, or when dried may be

extracted with petroleum ether in a soxhlet apparatus, the petroleum evaporated and the residue dried and weighed.

In many cases this operation presents no difficulties; but the fatty acids obtained from some fats are apt to pass through even the most carefully selected and most carefully handled filter, and then special measures must be adopted. The drops that have passed through may be allowed to solidify and filtered through another filter; the two filters dried and extracted in a soxhlet with some solvent with which the funnel or other vessels to which particles of the fat have adhered have been washed. The solvent can then be evaporated in a weighed flask and the dried residue weighed.

The Hehner value of triolein would be 95·7, that of lard and most oils or fats is about 95, while that of butter is between 86 and 88, and that of cocoa-nut oil is sometimes lower still, in both cases because of the solubility in water of the lower fatty acids which occur in the glycerides present in these fats.

The fats that occur in animal organs yield relatively small Hehner values, because they contain phospholipines.

Lecithine and cephaline yield only about 70 and 76 per cent. respectively of their weight of fatty acids. The estimation, therefore, of the total insoluble fatty acids in the fat extracted from an organ or tissue may serve to give an idea of the nature of its composition. For this purpose about a gramme of the extract weighed in a small flask can be saponified in the flask; if it be desired it can first be titrated, and then saponified with a titrated amount of alcoholic potash so as to obtain its "acid" and "saponification values" at the same time; and then the soap dissolved and washed with water into a flask, such as that described above (p. 59), acidified and shaken with petroleum ether, and finally an aliquot part of the petroleum solution taken as in the quantitative method there described. The extract of the liver of normal animals ordinarily yields from 60 to 70 per cent. of fatty acids insoluble in water, whereas that of a fatty liver may yield more than 90 per cent.

5. *The Reichert-Meissl Value.*

The Reichert-Meissl value is a measure of the amount of lower fatty acids entering into the composition of a fat which volatilise in a current of steam. The value is expressed by the number of cubic centimetres of 0·1N alkali required to neutralise the volatile fatty acids liberated under certain prescribed conditions from 5 grms. of the fat or oil. The conditions prescribed in different countries are different. In England the Wollny modification has been adopted

by the Government laboratories and the Society of Public Analysts. In this operation 5 grms. of the fat are introduced into a flat-bottomed flask, with a neck 2 cm. in diameter and 7 to 8 cm. in length, and treated with 2 c.c. of a solution of 98 per cent. caustic soda in an equal weight of water, which should be kept protected from carbonic acid, and 10 c.c. of 92 per cent. alcohol. The flask is heated on a boiling bath for 15 minutes under a reflux condenser, and then the alcohol allowed to evaporate off completely by further heating for at least half an hour without condenser. Then 100 c.c. of water that has been kept boiling for at least 10 minutes to remove carbonic acid are added and the flask heated till the soap dissolves. Forty c.c. of normal sulphuric acid and some bits of pumice or porous clay plate are added, and the flask is then at once connected with a condenser tube 8 mm. in diameter, surrounded by a water jacket 35 cm. long, by means of a bent tube 15 cm. long from the cork of the flask to the bend of the tube and 7 mm. in diameter, on the middle of which a bulb 5 cm. in diameter is blown. The flask is heated on an asbestos board 12 cm. in diameter, which has an opening in its centre 5 cm. in diameter, at first with a small flame, till the insoluble acids are melted, and then more strongly. In the course of half an hour 110 c.c. of distillate should be collected and the distillation is stopped. The distillate is well mixed; 100 c.c. measured off, titrated with 0·5 c.c. of 1 per cent. alcoholic solution of phenolphthalein and decinormal soda or baryta solution. A blank test carried out with the same quantities of everything, but without the fat, gives a correction which should not amount to more than 0·2 to 0·3 c.c. The number of cubic centimetres used multiplied by 1·1 gives the Reichert-Meissl-Wollny number.

Leffmann and Beam's modification of the method is more expeditious, and consists in using 20 c.c. of glycerol instead of the alcohol used by Wollny. After heating with the glycerol and soda for 8 minutes the fluid becomes clear and is allowed to cool to 80° C.; 90 c.c. of water, at about the same temperature, and 50 c.c. of 2·5 per cent. sulphuric acid are added. The rest of the process is as in the description given above. This is the official method in Germany.

Clearly such estimations have a comparative value only, and even that only if the same conditions are always observed.

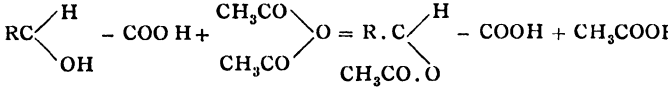
By sending a current of steam through the acid mixture figures that are 25 per cent. higher are obtained, but owing to the decomposition of non-volatile acids by prolonged heating a source of error is introduced if the attempt be made to repeat the distillation, by adding more water till the distillate no longer reacts acid.

Most fats and oils contain only traces of volatile acids or their glycerides in the fresh state, and give values therefore that are less than 1. Butter is the most notable exception, with the value 26 to 32. Other exceptions are cocoa-nut oil, Reichert-Meissl-Wollny value 5 to 8, and dolphin and porpoise oils, the Reichert-Meissl-Wollny value of which may rise above 60. On the other hand, rancidity is characterised by a rise of this value, as also is the change that accompanies the process of "blowing" oils.

6. *The Acetyl Value.*

The acetyl value gives the number of milligrammes of caustic potash required to neutralise the acetic acid liberated when 1 grm. of acetylated fat or fatty acids is saponified.

If in a fat or oil some of the fatty acids are hydroxy acids, they will when heated with acetic anhydride become acetylated—



The number of acetyl groups taken up by a fat or mixture of fatty acids will clearly depend on the amount of hydroxy acids present and the number of hydroxyl groups they contain. When by saponification the acetyl groups are split off again as acetic acid, the determination of the amount of acetic acid so liberated by a given quantity of the acetylated product will be a measure of the amount of these hydroxyl groups.

The determination is carried out as follows: 5 to 10 grms. of the fat or mixture of fatty acids is boiled with twice its weight of acetic anhydride in a round flask with an inverted condenser for 2 hours. The contents are then transferred to a beaker of 1 litre capacity, and 500 to 600 c.c. of boiling water added. It is heated for half an hour, while a slow stream of carbonic acid is led through to prevent bumping; salt may be added if the oily matter does not separate readily. Separation is then allowed to take place, and the aqueous layer syphoned off, more water added and syphoned off till the acetic acid formed from the excess of anhydride has been all removed. The oily acetylated product is then filtered through a dry filter in a drying oven to remove water.

A weighed amount, 3 to 5 grms., is then saponified with a known amount of alcoholic potash, and the saponification value determined. The soap is then freed from alcohol by evaporation, and the acetic acid estimated by either of the following methods:—

(1) *Distillation Method.*—An excess of 10 per cent. sulphuric acid is added, and the liquid distilled in a current of steam. The distillate collected till 100 c.c. requires no more than 0·1 c.c. of decinormal alkali to neutralise it; about 600 to 700 c.c. of distillate will generally be obtained. The total amount of decinormal alkali used to neutralise the distillate, with phenolphthalein as indicator, multiplied by 5·61 and divided by the amount of acetylated product taken, gives the acetyl value.

(2) *Filtration Method.*—The soap solution is treated with the amount of standardised sulphuric acid that exactly corresponds to the alcoholic potash fixed during saponification of the acetylated product, as shown by the determination of the saponification value; the mixture is heated on the bath till complete separation has taken place, and then filtered and washed with hot water till the aqueous filtrate no longer comes through acid; the filtrate then titrated using phenolphthalein gives the acetyl value.

Animal fats do not as a rule contain hydroxy acids that would give an acetyl value. But the presence in a fat of free cholesterol or other alcohols insoluble in water would enable acetylation to occur and cause the fat to have an acetyl value. Certain vegetable oils, however, notably castor oil, contain glycerides of hydroxy acids. Ricinolein, which occurs in castor oil, would itself, if pure, have the acetyl value 159; castor oil commonly shows the value 153 to 156, and grape-stone oil the value 144.

Besides free insoluble alcohols, other substances present in a fat may account for an acetyl value that is not due to hydroxy acids. Partial hydrolysis may have caused the liberation of mono- or di-glycerides which would be acetylated by the acetic anhydride and give products insoluble in water. In a fat, therefore, that has been partially hydrolysed an acetyl value may be obtained which is not due to hydroxy acids. Similarly, a fat that contains glycerides of those lower fatty acids which are soluble in water would, when saponified in the operation of determining the acetyl value, give off the soluble fatty acids to the wash water, and they would not be distinguished from acetic acid liberated from acetylated hydroxy acids. If these lower fatty acids are known to be present, and their amount is known, a correction may be made, and the "true acetyl value" obtained, according to Lewkowitsch, as distinct from the "apparent acetyl value," which is obtained when this correction is not made.

C. SEPARATION, IDENTIFICATION AND ESTIMATION OF THE SEVERAL CONSTITUENTS OF A FAT OR OIL.

The methods that are available for the separation, identification and estimation of the several constituents of a fat or oil depend very largely on those simple properties which underlie the six methods by which the characteristics of a fat or oil are commonly determined in technical analysis, which form the subject of the last section. But there are other methods besides these that must be resorted to when the examination of a fat is to be complete, and indeed at the present time a complete determination of the composition of many kinds of fat is not yet practicable by any of the methods that have been devised.

It is, for instance, in many cases easy to separate neutral fat from free fatty acids by dissolving 5 to 10 grms. of the fat in 50 to 100 c.c. of petroleum ether, adding 25 to 50 c.c. of absolute alcohol and titrating with alcoholic potash and phenolphthalein; then adding as much water as alcohol, so as on shaking to get a separation of a solution of soap in 50 per cent. alcohol from a solution of neutral fat in petroleum. If these solutions are washed by shaking, the soap solution with fresh petroleum, and the neutral fat solution with fresh 50 per cent. alcohol and adding the washings to the corresponding solutions, the whole of the fatty acids originally present in the fat in the free state or in the form of soap can be obtained from the alcoholic solution by acidifying and shaking out with petroleum ether.

Similarly, in the petroleum solution, which contains the neutral fats and any cholesterol or other unsaponifiable substances that may be present, these latter are obtained by saponifying the fat with alcoholic potash, dissolving the product in equal parts of alcohol and water, and shaking the solution with petroleum ether. The saponified fats will be contained in the alcoholic solution and the unsaponifiable matter in the petroleum ether. If, however, the original fat contains phospholipines a solution of it in petroleum is likely to give up to 50 per cent. alcohol some of these phospholipines as well as any soap that is present in the mixture.

The subject-matter, therefore, of this section resolves itself primarily into two divisions—the separation, identification and estimation of—

1. The various fatty acids; and
2. The various alcohols, that enter into the composition of the fat.

1. *Separation of the Fatty Acids.*

The fatty acids that enter into the composition of a fat are obtained by saponifying the fat with alcoholic potash. For 100 grms. of the fat

it is convenient to take the equivalent of about 40 grms. of potash (80 c.c. of a solution obtained by dissolving 500 grms. of potash sticks in 500 c.c. of water) and 100 c.c. of alcohol. The mixture is heated for half an hour at or near its boiling point on the water-bath, and then after most of the alcohol has evaporated dissolved in a large beaker in hot water, of which about 2 litres should be taken. The soap solution heated on the bath will lose the remainder of its alcohol if that is desired, and then 100 c.c. of 40 per cent. sulphuric acid is stirred in and the heating continued till the fatty acids separate to the top, leaving the aqueous acid solution clear below. This is then syphoned off, or if on cooling the fatty acids solidify, can be poured off. The fatty acids are then heated again with fresh changes of water till the water shows no reaction with congo paper and the mineral acid has been removed.

When there is reason to suppose that volatile or soluble fatty acids are present in the fat, when, *i.e.*, the saponification value is high, the sulphuric acid should be added in a flask so that the volatile acids can be distilled off in a current of steam.

The non-volatile acids left after the distillation of the volatile acids can then be washed repeatedly with water to remove sulphuric acid and dried for further examination. When there is reason to suppose that the insoluble acids so obtained contain unstable unsaturated acids, when, *i.e.*, the iodine value is high, the fatty acids should be dissolved in petroleum, the solution dried with anhydrous sodium sulphate, filtered, and the fatty acids kept in petroleum solution in stoppered bottles till further steps are taken for examining them.

The fatty acids so obtained will necessarily contain, as mentioned before, cholesterol and any other unsaponifiable matter which is soluble in petroleum, and before attempting a further separation and characterisation of the acids such substances should be removed by converting the acids into soaps and extracting either the dried soaps or a solution of them with some solvent such as ether or petroleum ether according to the methods described later.

The *mean molecular weight of the acids* may be taken when this has been done. As much of the petroleum solution as will contain about 0·5 grm. of fatty acids is measured off into a weighed round flask with a short, wide neck and of about 150 c.c. capacity. The flask is fitted to a condenser with a bent tube, and has leading through its cork another tube connected with an apparatus for delivering carbonic acid. It is filled with carbonic acid and the petroleum distilled off on a water-bath. When the distillation is apparently finished the receiver is connected with a pump and exhausted, and the heating continued in a good

vacuum for half an hour. The flask is then removed, allowed to cool in a desiccator and weighed. The fatty acids are dissolved in the flask in about 20 to 25 c.c. of alcohol, and after adding a few drops of 1 per cent. alcoholic solution of phenolphthalein are titrated with 0·1 N alkali. The amount of alcohol taken for dissolving the acids should be not less than the amount of aqueous alkali used for the titration, so that the final concentration of the alcohol should be sufficient to prevent hydrolysis of the soaps, *i.e.*, about 50 per cent. The weight of fatty acids taken in milligrammes divided by the number of cubic centimetres of decinormal alkali used and multiplied by ten gives the molecular weight (*e.g.*, 0·284 grm. stearic acid would require 10 c.c. decinormal alkali to neutralise it, and the molecular weight of stearic acid is 284). If high values for the molecular weight are obtained this should lead to the suspicion that cholesterol and unsaponifiable substances are present in considerable quantities, and after complete removal of these lower values will in that case be observed.

In this way the acids will have been separated into two portions, one volatilised by a current of steam contained in the aqueous distillate, the other non-volatile and insoluble in water.

Solubility of Volatile Fatty Acids in Water.

	100 c.c. dissolve at 15° C.	At 100° C.
Acetic and Butyric .	∞ grms.	— grm.
Valeric . . .	3·5 ,,	— ,,
Caproic . . .	0·882 ,,	— ,,
Caprylic . . .	0·079 ,,	0·250 ,,
Capric . . .	— ,,	0·1 ,,

The volatile acids may consist of the acids soluble in water—formic, acetic, propionic and butyric—and of acids of higher molecular weight, more or less insoluble in water, up to and including traces at any rate of lauric and myristic acid, if they are present. When caproic or higher volatile acids are present they will be seen floating on the aqueous distillate as oily drops, or even as solid flakes. As a general principle it is the first portions of the distillate that contain the less soluble volatile acids of higher molecular weight.

Duclaux distilled ten fractions of 10 c.c. each from 110 c.c. of saturated solutions of butyric, caproic and caprylic acid respectively, and found that, while 75 per cent. of the caprylic was contained in the first two fractions, three fractions had to be collected before this amount of the caproic acid was recovered, and six fractions before the same amount of the butyric acid could be obtained. The total yield of caproic and caprylic acids in the ten fractions was 100 per cent., of butyric 97·5 per cent.

No separation of the volatile acids can be effected by collecting the distillate in fractions, and if more than two or three acids are present in the mixture no analysis.

Gillespie and Walters found that when 110 c.c. of a solution containing formic, acetic, propionic and butyric acids was distilled, and the distillate collected in 10 c.c. fractions at the indicated stages of the operation, the following percentages of the acids had come over—

	Formic.	Acetic.	Propionic.	Butyric.
10 c.c.	4·2	6·4	11·2	16·4
30 ,,	13·2	19·7	32·7	44·8
60 ,,	29·3	41·6	62·6	76·2
100 ,,	65·5	79·9	93·6	97·8

The total amount of acid in the distillate can be titrated and the solution obtained evaporated down. To attempt to determine the mean molecular weight of the acids by evaporating down a neutral solution to dryness and weighing the residue would lead to no certain result owing to hydrolysis of the salts in dilute solution and loss by evaporation of some of the acids, so that it is best to add excess of alkali before concentrating the distillate and to attempt to identify and separate the acids subsequently. The alkaline salts when dry may be treated with a small excess of 20 per cent. sulphuric acid. Caproic and higher acids will then separate as an oil. The subjacent aqueous solution may be treated with salt, and butyric acid will then separate out from the salt solution also as an oil. And the salt solution shaken with ether will give up its lower acids to the ether. Silver, calcium or barium salts of the several fractions of acids so obtained may be made, and from the amount of metal these salts contain the composition of the acids in each fraction determined. The amides of the several acids may also be prepared and identified by their melting points.

When volatile acids higher than caproic are present it may be worth while to collect the first portion of the distillate separately, dissolve the insoluble acids that it contains in ammonia and precipitate the silver salts with silver nitrate, collect the precipitate, dry it and estimate the silver in it. The later fractions of the distillate containing only soluble acids may be made to yield silver salts by heating the solution with silver carbonate, filtering hot and crystallising out in the cold.

When the amount of volatile acids obtained as sodium salts is sufficient to allow it, the best method of separating them is of course to shake out the acidified mixture with ether, evaporate off the ether and fractionate the residue by distillation. Each fraction is weighed, its boiling point noted, and salts or amides prepared and analysed.

In his study of the products of fermentation Nencki made use of the formation of guanamines, on heating guanidine salts of volatile fatty acids to 220° to 230° C., for the identification of these acids. The guanamines crystallise readily and can be identified by their crystalline form.

The separation of the non-volatile acids into the two groups of *the solid saturated acids and the liquid unsaturated acids* is generally effected by the conversion of the acids into their lead salts and the extraction of these with ether. Broadly speaking, the lead salts of unsaturated acids are soluble, those of saturated acids insoluble, in ether. The method may be used for the purpose of quantitatively determining the approximate proportions in which these two groups of acids occur in a fat, but is also of value for the separation of the groups of acids with a view to the further study of the individual components of each of the two groups.

The method was first proposed by Gusseroff in 1828, but is generally known as Varrentrapp's method, as he was the first some years later to use it for the preparation of oleic acid. For quantitative purposes it has undergone many slight modifications since that time.

From 2 to 4 grms. of fat are saponified in a 300 c.c. flask with 50 c.c. of about half-normal alcoholic potash, and the solution having then been acidified with acetic acid in the presence of phenolphthalein, alcoholic potash is run in till the neutral point is reached, and water added so as to make about 100 c.c. in all. The lead salts are precipitated by adding gradually and with continual shaking a boiling mixture of 30 c.c. of 10 per cent. lead acetate solution with 150 c.c. of water. The lead soaps will adhere to the sides of the flask, and after cooling down the water can be poured off clear, if necessary through a filter. Any loose particles on the filter are returned to the flask, which is then again filled with boiling water and allowed to cool; the water is then poured off and the washing repeated. Finally, adherent drops of water are removed with filter paper, and without any further drying of the soaps 150 c.c. of ether are poured on, and with continual shaking the ether is warmed under a condenser till they are loosened from the flask and the insoluble part settles as a powder beneath the ethereal solution. After cooling down the solution is poured through a filter into a separating funnel, and the undissolved soaps washed with ether on to the filter; the filtrate and washings are then shaken with dilute hydrochloric acid to decompose the lead salts. When separation has taken place the lead chloride and acid water are drawn off and the ethereal solution of fatty acids washed with small quantities of water till the washings are neutral. Finally the ether is evaporated from a weighed round-bottom flask filled with carbonic acid gas and the last traces removed *in vacuo* on a hot water-bath.

From the insoluble lead soaps the solid saturated fatty acids can be similarly recovered. If the iodine value of these acids be taken an idea of the success of the separation can be formed. Theoretically it should be zero; practically it may be as low as 3 or 4, but often is higher.

The separation as a matter of fact is not exact. The lead salts of even the higher saturated acids are not entirely insoluble in ether, and in the presence of unsaturated acids salts are formed in which the dibasic lead combines with an atom of saturated and an atom of unsaturated acid. Moreover, the lower saturated acids give lead salts which are soluble in ether, so that when these acids are present, as, for instance, in cocoa-nut oil, the iodine value of the acids obtained from the soluble lead salts is low—as low as 36 it may be. It is also to be remembered that some unsaturated acids, erucic and iso-oleic, form salts with lead which are very slightly soluble in ether.

Another method proposed by Farnsteiner, and recommended by some on account of its convenience, effects a separation of the same sort as Varrentrapp's method with a similar degree of accuracy. The lead soaps prepared in the same way are dissolved in warm benzene, of which 50 c.c. are required for the acids obtained from 1 grm. of oil. In this solvent they all dissolve, but the salts of the saturated acids separate out on cooling to 10° C. in crystalline form. The supernatant benzene solution is drawn off by means of a small inverted thistle funnel with close-meshed silk tied over the mouth, the other end being bent so as to pass through the cork of a filtering flask connected with a water-pump; the crystals are washed a few times with 10 c.c. of benzene cooled to 10° C., and then redissolved in 25 c.c. of warm benzene and recrystallised. The whole process is repeated, and the crystalline lead salts finally dried and weighed; the benzene solution is evaporated, and the soluble lead salts also dried and weighed. The results obtained by Farnsteiner showed that it was possible to get figures correct within 2 or 3 per cent. of the theoretical.

The principles of the lead salt ether or lead salt benzene method can be used for the separation of solid and liquid fatty acids on a larger scale when the examination of the components of either of these groups of acids is contemplated. But as a rule the ether method, requiring a smaller volume of solvent, will be preferable. In any case the iodine value of the fractions so obtained should be taken and compared with that of the original mixture of acids so as to see how far the separation has been successful.

A method for the separation of the highly unsaturated acids from those which are less unsaturated and from saturated acids has been

described by Tsujimoto. It depends on the ready solubility of the lithium salts of the highly unsaturated acids in acetone containing a little water (95 per cent. acetone by volume). The lithium salts of the saturated and less unsaturated acids are either insoluble or only sparingly soluble in this solvent.

The saturated acids obtained from the lead salts insoluble in ether or benzene can be resolved into their constituents by use of the following methods :—

(1) Fractional precipitation of magnesium or barium salts (Heintz). The acids are dissolved in sufficient alcohol to prevent separation on cooling, and while hot a solution of magnesium acetate in alcohol is added which contains only one-fifth or one-tenth of the amount calculated to be necessary for complete precipitation. The precipitate is separated and the filtrate treated with sufficient ammonia to neutralise the liberated acetic acid, and then again with the same amount of magnesium acetate. In this way a series of fractions is obtained from which the free acids are separated by means of petroleum ether and dilute hydrochloric acid. The melting point and molecular weight of the acids from each fraction is taken, and it will be found if more than one solid acid was present that the acids of higher molecular weight and melting point occur in the earlier fractions. By this method, at any rate when only two solid acids are present, a separation and identification of the acids is possible, especially if the acids are crystallised from alcohol a few times.

For acids of lower molecular weight barium acetate is used in preference to magnesium acetate.

(2) Fractional distillation of the acids or their methyl or ethyl esters *in vacuo* has frequently proved useful as a means of separating and isolating pure substances. The boiling points that have been observed with the corresponding pressure readings will be found in the tables of the saturated and the unsaturated acids on pages 16 and 32.

In addition to the above methods, which are necessarily long and laborious, and require large quantities of material, certain methods have been devised for detecting or estimating individual acids that should be mentioned.

Arachidic acid, of which pea-nut oil contains about 5 per cent., can be detected in olive oil that has been adulterated with not less than 10 per cent. of the former oil, and contains therefore 0·5 per cent. of this acid, if the fatty acids from 10 grms. of the oil are dissolved in 50 c.c. of hot 90 per cent. alcohol (specific gravity, 0·818 at 20° C.), and cooled down to 15° C. The crystals obtained are washed with measured quantities of 90 per cent. alcohol, and then with 70 per cent. alcohol,

and finally dried and weighed; a correction being made for the amount of 90 per cent. alcohol used on the basis of the solubility of arachidic acid or rather of the mixture of this acid with lignoceric acid that occurs in pea-nut oil (100 c.c. 90 per cent. alcohol dissolves 22 mgrs. at 15°, 45 mgrs. at 20° C.). The acid may be identified by its molecular weight.

Stearic acid is estimated by the method of Hehner and Mitchell, which consists in crystallisation from a previously prepared solution of pure stearic acid in 95 per cent. alcohol saturated at 0° C. For this 3 grms. of stearic acid are heated with a litre of the alcohol in a stoppered bottle, which is then placed in ice water at 0° C. for 12 hours. Without removing the bottle the saturated solution is drawn off with the help of a pump through a thistle funnel covered with fine silk or linen. About a gramme of the solid acids to be examined is weighed out exactly, and dissolved in 100 c.c. of the stearic acid solution, and kept similarly in ice water for 12 hours. The flask is then agitated and left for an hour or so longer in the ice water. The solution is then sucked off, as above, with the pump; the flask washed with small amounts of the stearic acid solution at 0° C., and the washings removed as completely as possible. Finally the crystals adhering to the funnel or its covering are dissolved into the flask with a stream of hot alcohol, and after evaporating off the alcohol are weighed in it. A small correction should be made for the stearic acid dissolved in the film of solution unavoidably left after washing the flask and crystals.

In this way the amount of stearic acid contained in certain fats and oils, as given below, was ascertained:—

Butter contains	0·5 per cent.	stearic acid.
Mutton tallow contains	16 – 22 p.c.	,,
Beef tallow ,,	51 p.c.	,,
Lard ,,	6 – 16 p.c.	,,
Cacao butter ,,	39 – 40 p.c.	,,
Cocoa-nut oil ,,	1 p.c.	,,
Palm oil ,,	0·5 – 0·7 p.c.	,,
Olive oil ,,	0 p.c.	,,
Almond oil ,,	0 p.c.	,,

Palmitic acid can be estimated from the mean molecular weight of a mixture of this acid with stearic acid, if it is known that no other acid is present. And it can be isolated from such a mixture, if the mixture is dissolved in enough alcohol to hold in solution at 0° C. the palmitic acid known to be present (100 c.c. 95 per cent. alcohol at 0° C. dissolves 0·56 grm. palmitic acid and 0·12 grm. stearic acid). On cooling the

solution to 0° C. for some hours the crystals formed will be principally stearic acid and by recrystallisation can be purified. The filtrate, concentrated to half its volume and treated with enough magnesium acetate solution to precipitate the stearic acid that it contains, will give a filtrate from which the greater part of the stearic acid has been removed, and if a second precipitation with half the first amount of magnesium acetate be carried out the filtrate will yield after recrystallisation from alcohol pure palmitic acid.

For the detection and isolation of lauric and myristic acids, a low molecular weight after removal of volatile acids on the one hand, and fractional precipitation of barium salts on the other, are the only methods other than the more satisfactory fractional distillation.

The unsaturated acids obtained from the lead soaps soluble in ether or benzene may be examined by the following methods :—

(1) *Bromination.*—On treating a cooled solution of the unsaturated fatty acids in glacial acetic acid or chloroform with bromine they undergo bromination and the unsaturated carbon atoms become saturated. The dibromostearic acid formed from oleic acid is soluble in the ordinary solvents for fat, including petroleum ether; the tetrabromostearic acid from linoleic acid is only slightly soluble in petroleum ether but dissolves readily in diethyl ether, while the hexabromostearic acid from linolenic acid is almost insoluble in ether but dissolves in hot benzene. A partial separation at any rate of the bromination products can be effected by means of these solvents, and the composition of a mixture of unsaturated acids approximately ascertained in this way. Thus if the mixed acids have the iodine value 120 and yield no bromination product that is insoluble in ether, *i.e.*, no product with more than four atoms of bromine, it would be safe to conclude in most cases that the mixture was composed of two-thirds oleic acid (iodine value 90 to 91) and one-third linoleic acid (iodine value 181 to 184). For unsaturated acids containing more or less than eighteen carbon atoms have been shown to exist in very few fats or oils.

The *hexabromide test*, which is used in commercial analysis, is an application of this principle of separation. For this test 0·3 grm. of the liquid fatty acids or 1 to 2 grms. of the unsaponified oil is dissolved in glacial acetic acid by itself or mixed with ether, and cooled down to 5° C. Bromine is added drop by drop till the coloration persists. The mixture is allowed to stand at 5° C. for 3 hours and then filtered. The precipitate is washed with 10 c.c. of ether at the same temperature several times and what is undissolved is dried and weighed.

This residue, insoluble in ether, may consist in some cases of a

mixture of hexabromo and octabromo derivatives. Hexabromostearic acid melts at about 180° C., whereas the octabromide from marine animal oils decomposes at about 200° C. without giving a true melting point. Estimation of the amount of bromine it contains will show this, or else the precipitate may be subjected to long extraction with hot benzene. If it does not dissolve in this solvent entirely the presence of an octabromo derivative is indicated, and its amount may be approximately estimated. The fatty acids obtained from linseed oil yield from 30 to 40 per cent. of their weight of "hexabromide," corresponding to from 11 to 15 per cent. of linolenic acid. Octabromides have been obtained from cod-liver oil, herring oil and other marine animal oils, and from the fatty acids of the pigs' liver. The bromination products insoluble in ether obtained by Hartley seemed to consist entirely of an octabromoarachidic acid; hexabromostearic acid could not be detected.

The bromination products soluble in ether may similarly, by the use of petroleum ether, be resolved into their constituents since tetrabromostearic acid dissolves in this solvent only when heated and crystallises out in needles, m.p. 114-115, almost quantitatively on cooling; 100 c.c. of petroleum ether at 12° C. dissolves from 14 to 21 mgrs. of the tetrabromo derivative of linoleic acid. Dibromostearic acid is a liquid substance easily soluble in all fat solvents, including petroleum ether. It may be identified by its molecular weight, 442, as may also tetrabromostearic acid, M.W. 600, and hexabromostearic acid, M.W. 758. In the case of this last determination the substance is dissolved in 50 parts of benzene at about 75° C., an equal volume of hot absolute alcohol is added, and the titration carried out in the hot liquid (Farnsteiner).

The recovery of the original unsaturated acid from a bromination product was carried out by Hazura in the case of tetrabromostearic acid: 17 grms. of the tetrabromide were boiled for 36 hours with 600 c.c. of alcohol and 150 c.c. of strong hydrochloric acid and some tin-foil. The mixture was then diluted with water and shaken with ether, which took up the ethyl ester of linoleic acid.

(2) *Identification of unsaturated acids by oxidation and isolation of the acids so formed.*—When oleic acid is oxidised at a temperature a little above zero with alkaline permanganate, it takes up two hydroxyl groups and is converted into the saturated dihydroxystearic acid. Similarly, under these conditions linoleic acid takes up four hydroxyl groups and linolenic acid six; sativic or tetrahydroxystearic, and linusic or hexahydroxystearic acid, are formed respectively.

The isolation of these oxidation products reveals therefore the

presence of the corresponding unsaturated acids. The procedure first devised by Hazura is as follows: 30 grms. of the liquid fatty acids are dissolved in 2 litres of water with the requisite amount of potash, and the solution cooled down to near zero. Into the vessel containing the soaps and standing in a freezing mixture 2 litres of 1·5 per cent. potassium permanganate also cooled to 0° C. is run slowly in a fine stream, while the mixture is mechanically stirred. After standing for a short time the manganese peroxide can be filtered off and a clear filtrate obtained, which is then acidified with sulphuric acid. (Hazura dissolved the peroxide and acidified at the same time by means of sulphurous acid, which is not so convenient if a complete examination of the oxidation products is contemplated.) When the reaction becomes acid unaltered fatty acids and some of the oxidation products are precipitated. These are filtered off, pressed out and dried on porous plates. They are then extracted with petroleum ether, which dissolves out and removes unaltered fatty acids but not the hydroxy derivatives. The insoluble part is then treated with large quantities of ether (2 litres for 20 grms.). In this the dihydroxystearic acid dissolves, and from the solution it may be recovered and recrystallised from alcohol till its melting point is no longer changed by the process. The tetra- and hexahydroxy acids are insoluble in ether, and the former slightly soluble in boiling water (1 grm. in 2 litres), the latter comparatively easily soluble in cold water. By boiling out therefore the products which do not dissolve in ether repeatedly with large volumes of water and filtering in a hot funnel a solution is obtained from which sativic acid if present will crystallise out as it cools. The large volumes of mother liquor are concentrated down after being neutralised, and when sufficiently concentrated to give a precipitate on acidification made acid; the precipitate collected and dried is extracted with ether to remove lower oxidation products and then crystallised from alcohol. Linusic and isolinusic acid so obtained may be separated by crystallisation from small volumes of water in which the latter is more soluble than the former.

The separation of tetrahydroxystearic from dihydroxy and hexahydroxy acids can be effected in this way more easily than the isolation of individual acids. The melting points given for the acids from linseed oil when isolated are:—

Dihydroxystearic acid	. . . . .	136·5
Sativic	,, ...	173
Linusic	,, ...	203-205
Isolinusic	,, ...	173-175

But it is not always easy to obtain products that melt true to these data, and even in Hazura's experiments there are indications that the sativic acid fraction contained two acids of different melting points (*cf.* Hartley). In some cases the dihydroxystearic acid appears, if obtained from other sources than those drawn on by Hazura, to be a different acid with different crystalline form, different solubilities and different melting point. The acid obtained from iso-oleic acid melts, for instance, according to Saytzeff at 77° to 78° C.

Where therefore it is desired to identify the particular oleic or linoleic acid that is present in a fat, oxidation with permanganate at low temperatures will probably fail. Information may, however, be obtainable if the further oxidation of the products obtained in cold oxidation be carried out at higher temperatures.

Oleic acid, which yields a dihydroxystearic melting at 136·5 when oxidised at 0° C., yields on heating with permanganate on the water-bath, as does also the isolated dihydroxystearic acid itself, pelargonic and azelaic acid. As mentioned above (p. 21), the group $-C\overset{H}{\underset{OH}{}}-C\overset{H}{\underset{OH}{}}-$ in the centre of the chain in this dihydroxystearic acid is the seat of cleavage resulting from its further oxidation and the formation of two carboxyl groups. In this way two new acids are formed—monobasic, nonylic or pelargonic acid on the one hand, and dibasic azelaic on the other.

To obtain this result oleic acid or the dihydroxystearic acid formed from it is dissolved in the requisite amount of potash, and heated with permanganate solution on the water-bath.

(3) *Fractional distillation of the methyl or ethyl esters* of the fatty acids effects a partial separation of these acids.

By this means Bull isolated from cod-liver oil palmitoleic acid $C_{16}H_{30}O_2$, gadoleic acid $C_{20}H_{38}O_2$, and erucic acid $C_{22}H_{42}O_2$, three unsaturated acids of the oleic series not previously found in animal fats. The methyl esters from 2 kgrms. of cod-liver oil were distilled in fractions at 10 mm. pressure at temperatures from 160° to 240°, the acids prepared from the several fractions were separated, and their properties and those of some of their salts determined (Bull).

The methyl esters of the unsaturated fatty acids from the livers of pigs were prepared by Hartley, and fractionated by distillation at 3 mm. Hg pressure. In this case the results seemed to indicate that the boiling point rose with an increase in the number of doubly bound carbon atoms. Several fractions were collected boiling between the temperatures 150° and 190° C. The lowest boiling fraction had the iodine value 127·5,

and as the boiling point went up the iodine value rose correspondingly, and the fraction boiling at 190° showed the value 249. But the true explanation of this phenomenon probably is to be found in the fact that the most highly unsaturated acid present in this case, the acid from which the octabromide referred to above was obtained (p. 87), was an acid of higher molecular weight than the others with a lower iodine value, and for this reason, and not because it was less saturated, boiled higher. Lewkowitsch, in distilling the methyl esters of the acids from cotton-seed oil, could not detect any such relationship between boiling point and iodine value, the acids with differing iodine values having in this case probably the same, or nearly the same, molecular weight.

Fractional distillation separates unsaturated acids of different molecular weights, but not acids of similar molecular weights but different iodine values. This fact is illustrated, too, by the fractionation effected by Bull. The boiling point of stearic acid in absolute vacuum is 155°, that of oleic acid under these conditions is given as 153°.

(4) Farnsteiner proposed a method by which he maintains it is possible to separate not only saturated from unsaturated but oleic from less saturated acids. The acids are converted into the barium salts and dried, and then heated with benzene to which 5 per cent. of 95 per cent. alcohol has been added. The unsaturated soaps dissolve, while the saturated do not. On cooling to 11° C. for one and a half hours, barium oleate separates out in crystals almost quantitatively, while the salts of linoleic and linolenic acids remain in solution. This method has not, however, given satisfactory results in the hands of others. Farnsteiner himself gives among his results the following :—

	Saturated acids, per cent.	Oleic acid, per cent.	Other unsaturated acids, per cent.
Olive oil . .	10·0	70·9	14·9
Pig fat . .	42·2	39·2	13·9
Cacao butter .	59·7	31·2	6·3

The presence of *hydroxy acids* is revealed by the acetyl value. They occur in rancid fats and oils, and in such as have undergone oxidation from exposure to the air. In nature castor oil is composed mainly of the glyceride of ricinoleic acid ; wool fat contains lanopalmic and lanoceric acids.

Attempts may be made to separate hydroxy acids from other fatty acids by means of petroleum ether ; but though an isolated pure hydroxy acid such as ricinoleic acid may be insoluble in petroleum ether, when mixed with other acids it is liable to remain in solution in petroleum, so

that the acids obtained from the filtered petroleum solution may have an acetyl value.

Anhydrides or lactones of fatty acids or hydroxy fatty acids are detected by comparing the amount of potash required for neutralising the acids with the amount neutralised after boiling them with alcoholic potash, by comparing, that is, the acid and saponification values of the fatty acids. Anhydrides will, of course, react neutral on titration unless they have been reconverted into the acids by boiling with alcoholic potash.

2. *Identification, Isolation and Estimation of Alcohols in Fats.*

(*a*) *The Estimation of Glycerol.*

When a fat is saponified, the soap taken up in water and acidified, the glycerol remains in the acid aqueous fluid; and if this is neutralised and evaporated down to a small volume the presence of glycerol can be qualitatively recognised by the acrolein reaction. On heating some of the concentrated solution with acid potassium sulphate the pungent fumes of acrylic aldehyde, acrolein, are given off, the glycerol having lost 2 molecules of water.

(1) *The Acetin Method.*

The estimation of glycerol is not infrequently carried out in technical analysis, and is sometimes necessary in biological investigations. Many methods have been proposed, the most generally approved of which is the acetin method. In this procedure the glycerol is converted into triacetin, and the amount of acetic acid so bound determined by its saponification value. If the problem is to determine the amount of glycerol in a fat or oil it is customary to take as much as 20 grms. for the purpose, corresponding to about 2 grms. of glycerol. The fat is saponified in the usual way with alcoholic potash, the soap solution acidified with sulphuric acid and filtered. The filtrate is treated with barium carbonate in excess and evaporated down to a small volume. The residue is treated repeatedly with a mixture of 1 part of ether and 3 parts of alcohol, and the filtered alcohol ether evaporated at a low temperature. The crude glycerol, in a round-bottom 100 c.c. flask, is dried in a desiccator, and its weight approximately determined. It is then heated for one and a half hours under a condenser with about 10 c.c. of acetic anhydride and about 4 grms. of sodium acetate that has been well dried in an oven. After cooling down somewhat about 50 c.c. of water is poured down the condenser to wash into the flask traces of adherent triacetin that has volatilised and condensed on the sides of the condenser

tube. The triacetin is dissolved in this water with slight warming if necessary, but without detaching the condenser. The solution is filtered into a 600 c.c. flask when cold, and neutralised to phenolphthalein with half-normal aqueous soda which is free from carbonate. The flask must be shaken well while the soda is added and an excess carefully avoided to prevent saponification of the triacetin, the first change in colour being taken as the end reaction rather than the development of a definite pink. 25 c.c. of a 10 per cent. soda solution is then added, and the same amount of the same soda measured with the same pipette introduced into another control flask. Both flasks are heated for a quarter of an hour with condensers on a water-bath and titrated with standard hydrochloric acid (normal or half-normal). The difference between the two titrations gives the amount of acetic acid set free during saponification : 1 c.c. normal acid = $\frac{1}{3}$ mgr. mol = 30·67 mgr. glycerol (Benedikt, Cantor and Lewkowitsch).

(2) The Benedikt Zsigmondi method, as modified by Herbig, consists in the conversion of glycerol by strongly alkaline permanganate at the ordinary temperature into a molecule of oxalic and a molecule of carbonic acid. The oxalic acid is separated as lime salt and titrated. From 2 to 3 grms. of fat is saponified with potash and pure methyl alcohol, the alcohol evaporated off and the soap taken up in water, treated with hydrochloric acid and warmed. If the fatty acids remain liquid on cooling paraffin may be added to facilitate filtration. The filtered aqueous solution and wash water is neutralised with potash and 10 grms. of potash added in excess, and then a solution of about 5 per cent. permanganate run in till the fluid is no longer green but blue or almost black. The mixture is allowed to stand for half an hour, and enough hydrogen peroxide then added to decolorise the fluid, and no more. It is poured into a litre flask and made up to 1 litre, and 500 c.c. are filtered off. This filtrate is acidified with acetic acid and the oxalic acid precipitated with calcium chloride ; the washed oxalate of lime dissolved in excess of warm dilute sulphuric is then titrated at about 60° C. with decinormal permanganate : 1 c.c. = 4·5 mgr. oxalic acid, or in the total amount used at the outset, 9·2 mgr. glycerol. If the fat, however, contained unsaturated acids which might have undergone oxidation, this method cannot be used, as the products of such oxidation may yield oxalic acid when treated with permanganate.

Another method that is used in some laboratories in preference to the above is oxidation with bichromate in acid solution and titration of the excess of bichromate (Legler and Hehner). A method has been proposed similar to Zeisel's method for estimating methoxyl or ethoxyl

groups, in which by treatment with hydriodic acid glycerol is converted into isopropyl iodide and distilled into silver nitrate and the silver iodide weighed (Zeisel and Fanto). It is possible also to estimate glycerol directly, as done by Shukoff and Schestakoff. The glycerol solution is made slightly alkaline with potash and concentrated at a temperature not above 80° C. till syrupy. The amount taken should be calculated to yield about 1 grm. of glycerol and the evaporation should be carried only so far as to have about the same amount of water present as glycerol. Then 20 grms. of anhydrous sodium sulphate are added, and the mass extracted with acetone in a soxhlet apparatus for 6 hours. The acetone must be pure and dry and all connections must be of glass. The acetone solution, washed with petroleum ether if oily drops float on it, is evaporated and the glycerol dried at 75° to 80° C. for 4 hours and weighed in the flask fitted with a stopper. The product is generally coloured.

Fats that have undergone partial hydrolysis may contain *mono-* or *diglycerides*. These should be revealed by the following procedure: The fat is freed from free fatty acids in the usual way, and its acetyl value determined. The fat must of course contain no glycerides either of hydroxy acids or of soluble fatty acids, or if it does, the amount of these must be determined separately and a correction made.

(*b*) *Isolation and Estimation of Other Alcohols.*

The principle on which the separation of cholesterol from fats is carried out was indicated above (pp. 60 and 63).

The fat is saponified and the soaps, dissolved in 50 per cent. alcohol, are shaken with petroleum ether. The petroleum solution is separated and washed with 50 per cent. alcohol, and the alcohol solution is washed with petroleum ether, each two or three times. The combined petroleum solutions are evaporated and the residue after carrying it through the whole process a second time with smaller volumes of the solvents is finally weighed (Hönig and Spitz).

Cholesterol is less soluble in petroleum ether than in ordinary ethyl ether, so that there are advantages in using the latter solvent for the extraction,[1] and in this case it may be found more convenient to extract

[1] When ether is used Bömer recommends, in order to avoid delay from the formation of emulsions, that the following proportions should be closely adhered to: 100 grms. fat, saponified with 200 c.c. of a solution of 200 grms. KOH in 1 litre of 96 per cent. alcohol, is dissolved in 600 c.c. of water and shaken with 800 c.c. of ether, and then two or three times each with 400 c.c. of ether. The combined ethereal solutions are evaporated to dryness, and the residue heated again with 10 c.c. of the same alcoholic potash for a quarter of an hour. It is then dissolved in 30 c.c. of water and shaken twice with 100 c.c. of ether.

the soap solution in a continuous extraction apparatus. One of the simplest and most convenient forms of apparatus for this purpose consists of a cylinder about two-thirds filled with the soap solution, through the cork of which two tubes pass, one reaching down to near the bottom of the cylinder, the other only just through the cork; the former of these tubes is continued straight up and just projects through the upper end of a cork in the mouth of a vertical condenser: the other tube is bent at an angle of about 60° after leaving the cylinder and is then carried by another bend vertically through the cork of a flask on a water-bath in which ether is boiled. The ether vapour passes up from this flask through another bent tube leading from the flask into the condenser from which it is returned to the bottom of the cylinder by the long straight tube.

When the unsaponifiable matter has been separated it should be examined as to its complete freedom from soap by burning a portion and testing for an alkaline ash. If this is detected the material must be dissolved again in petroleum and washed. It should then be crystallised from alcohol, the crystals examined for those forms that have been described as characteristic for cholesterol and the related alcohols. The iodine value is not a certain guide as to purity of sterols.

Holde found that with the original Hübl solution cholesterol gave the iodine value 73 to 77 (65·7 theoretical), phytosterol 41 to 76 according to the time given for reaction, but with Wijs' they both gave the value 135, approximately double the theoretical.

An investigation of the iodine absorption from Wijs' solution and Hübl's solution, of a wide range of substances, has led Maclean and Thomas to the conclusion that the acid present in Wijs' solution acts in certain cases as an enolising agent on carbonyl compounds and that the addition of halogen then takes place. The Hübl reagent, being neutral has little or no enolising action. In other compounds that give anomalous results like the sterols, probably substitution takes place with Wijs' solution and to a much smaller extent with Hübl's.

The identification of cholesterol, isocholesterol and the kindred alcohols depends upon the recognition of the properties of these substances and their derivatives (described above, p. 45 *et seq.*).

The separation, isolation and identification of *other alcohols*—those of the aliphatic series—also present but few points for discussion. The separation is, in the first instance, effected, as in the case of other unsaponifiable matter; only it must be remembered that in the case of esters of the highest alcohols and the highest fatty acids

the solubility in alcohol is low, and saponification takes much longer than is the case with the common glycerides. Röhmann recommends heating in a pressure-bottle, with a solution of baryta in methyl alcohol; Kossel and Obermüller recommend treatment with sodium ethylate, obtained by dissolving 5 grms. of metallic sodium in 100 c.c. of absolute alcohol.

Sometimes, if large amounts of higher aliphatic alcohols are present, these will separate out from the alcohol in which the saponification has been carried out; and in the case of beeswax, for instance, with the separating myricyl alcohol, the potassium salt of cerotic acid also comes out of solution. If these are filtered off they can be separated by means of ether. Small quantities of higher aliphatic alcohols will remain dissolved in the alcoholic soap solution, and these can be obtained in the usual way by shaking a solution of the soap in 50 per cent. alcohol with petroleum ether. When the alcohols are obtained free from soap they should be crystallised from alcohol and the melting point taken, the acetate prepared, and the acetyl value determined. If cholesterol or such alcohols are present, as shown by the reactions for these substances, they may be separated by the operation by which at the same time steps are taken for identifying the particular aliphatic alcohol present, that is to say, by heating the mixture with soda lime. This converts aliphatic alcohols into the corresponding fatty acids. Cholesterol, of course, yields no fatty acid when thus treated, and indeed is but little changed. Octadecyl alcohol heated in this way with four times its weight of soda lime to 270° to 280° C. for 20 hours yields stearic acid (Röhmann). If a mixture of aliphatic alcohols were used, the resulting acids could be separated according to the methods given for separating saturated fatty acids. Hell has worked out a method for determining the amount of higher aliphatic alcohols in beeswax by measuring the amount of hydrogen given off on oxidation of the alcohol with potash lime: $RCH_2OH \rightarrow RCOOH + H_2$.

The hydrocarbons which are present in some waxes can be separated from the alcohols after these latter have been oxidised in this way by shaking the mixture with petroleum ether and 50 per cent. alcohol.

CHAPTER IV.

THE ORIGIN AND OCCURRENCE OF FATS IN NATURE.

The Distribution of Fats in Plants and Animals.—In the vegetable kingdom fats are found principally in spores, seeds and fruits, but they also occur in the vegetative organs, more especially the leaves. In the former situations the fat undoubtedly serves as a store of reserve material which can be called upon when germination takes place, but in the latter, its function as well as its composition is uncertain. Owing to the unsatisfactory methods which have been used for its extraction and separation it is still doubtful whether the fatty substances present in the vegetative organs consist of neutral fat or lipines or mixtures of the two. In animals, fats are widely distributed in the various organs of the body, but certain special regions apparently serve as reserve storehouses, for in these, large accumulations of fat are normally found. Examples of such are seen in the adipose tissue of mammals and the liver and muscles of certain species of fish. Although in these situations the fat is laid aside as a store it may have functions of a physical nature, for when deposited in the subcutaneous tissues it serves as a barrier protecting the organism from cold, and when laid down around a sensitive organ such as the kidney or eyeball, protects it from injury by forming a semi-fluid cushion in which it lies. In mammals the fat found in active organs, such as the liver, heart or kidney, is more unsaturated than that found in adipose tissue, and as will be indicated later, this difference has been made use of in investigating the transport of fat from the fat depots when it comes into use. Owing to the lack of good quantitative methods for the separate estimation of neutral fats and lipines, very little information is available as to the distribution of neutral fats in the various organs of the body. On the other hand, accurate methods exist for the estimation of fatty acids, whether free or combined, in animal tissues, and many data have therefore been collected which enable us to form a fairly accurate picture of the distribution of fatty acids in the body.

Mayer, Schaeffer, Terroine, and their colleagues, at the Collège de France, have carried out a laborious and careful investigation of the

total fatty acid content of many species of animals in various states of nutrition, and of certain organs of the larger animals. As a result of these studies the French observers divide the fatty acids which are found in the body into two parts, an *élément constant* and an *élément variable*. The former represents the fatty acid content of animals that have died of inanition. It is independent of the individual and of the fat content of the animal at the beginning of the inanition period. Within narrow limits it is constant for the species when expressed as a percentage of the body weight. The *élément constant* does not vary greatly in homœothermic or poikilothermic animals, each considered as a class, but in the former it has a value about five times that found in the latter. The *élément variable*, which is the difference between the total fatty acid content and the *élément constant*, exhibits great individual variations even in animals of the same species. The following figures given by Terroine (1919) illustrate these results :—

Homœotherma.			Poikilotherma.		
Animal.	Élément Variable.	Élément Constant.	Animal.	Élément Variable.	Élément Constant.
Mammal—					
Mouse	6·2-64·1	23·4	Perch .	8·5-28·6	5·28
Aves—					
1. Hypochera chalybeata .	13·2-66·1	21·6	Tench .	1·3-13·5	5·00
2. Sporoeginthus melpodus	17·9-42·0	26·9	Frog .	9·8-13·0	4·70
3. Chicken . . .	9·6-26·2	25·6			

Note.—The figures represent the percentage of fatty acids reckoned on the dry weight of the animal.

The *élément variable* consists of those fatty acids that are present in the form of neutral fat and form the true reserve fat of the animal ; the *élément constant* is that part of the total fatty acid which cannot be diminished in amount without causing death. It is probably an essential component of the animal's protoplasm (Chap. X.). The potential energy of the reserve fat enormously outweighs that of the glycogen which may be found stored in the body. In mice the fat reserve may have twenty times the calorific value of the glycogen reserve and in chickens, fifty times its value.

To investigate the fatty acid content of the separate organs of animals in various states of nutrition, dogs, rabbits and pigeons were used. Strikingly little difference is revealed between over-fed, normal and starved animals except in the liver and muscles. In the dog, for instance, the fatty acids found in the kidney were 11·1, 11·9 and 13.4

per cent. in the over-fed, normal and starved animals respectively. On the other hand the corresponding figures for muscle were 17·6, 11·3 and 4·06 per cent., and for liver 12·9, 10·5 and 11·3 per cent. The French authors thus conclude that muscle may be regarded as a tissue in which a considerable amount of reserve fat may be stored but the liver does not accumulate fat to anything like the same extent, any excess of fat found there having only a transitory existence. In other organs such as the lung, kidney, spleen and heart the fatty acid content does not vary with the state of nutrition. They contain no *élément variable*, only an *élément constant*.

The form of combination of the fatty acids which represent the *élément constant* is uncertain. Mayer and Schaeffer (1914) have estimated the ether soluble phosphorus in several organs of animals which had been subjected to starvation in order to exclude the presence of the *élément variable* and have then determined the ratio of fatty acids to ether soluble phosphorus. The values obtained were often lower than those for well-known lipines which may be isolated from various tissues but also lower than those for any known lipines. The nature of these fatty acid complexes, rich in phosphorus, is therefore very uncertain and awaits further investigation.

In addition to the constancy of the *élément constant*, a relation has been shown to exist between the cholesterol and fatty acids present in various tissues. This ratio, termed by Mayer and Schaeffer the lipocytic coefficient, is characteristic for a given organ but varies according to the species. If in several species the organs are arranged in accordance with the value of this coefficient, they always fall into the same order. The following table given by Mayer and Schaeffer (1913) illustrates this. The values given are those for the coefficient,

$$\frac{\text{cholesterol}}{\text{fatty acids}} \times 100.$$

Animal.	Lung.	Kidney.	Liver.	Muscle.
Dog	20·0	10·5	6·8	2·2
Rabbit . . .	17·1	13·3	8·4	7·3
Guinea pig . .	15·5	7·8	6·5	1·6
Pigeon . . .	24·1	9·1	7·9	1·6
Eel	11·2 (gills)	7·1	3·8	0·7

A generous diet, rich in fat, causes no change in the value of the lipocytic coefficient of any of the organs examined, with the exception of liver and muscle. The changes in the former are only slight, and are due to a temporary increase in its fatty acid content, but in the

latter a considerable increase of fatty acids may occur without a parallel change in cholesterol and so cause a diminution of the ratio. These observations have confirmed the conclusions referred to above, that the essential role of the liver in fat metabolism is not that of storing fat, but that muscle is a tissue in which a considerable reserve of fat may be stored.

On the other hand, Mottram (1909) has shown that in rabbits and guinea pigs, starvation of a few days' duration may cause a two to three-fold increase of fat in the liver. It is also a common experience of those who have had to determine the fatty acids in the liver of cats that large amounts may be found in apparently normal animals [Leathes and Meyer-Wedell (1909), Coope and Mottram (1914), Raper (1913)]. It may be that such increases are purely temporary, probably they are, but they are of considerable magnitude, and suggest that fat may accumulate in the liver of certain species of animals under certain physiological conditions to a much greater extent than the French authors have observed in their experiments.

The Fat of Adipose Tissue.—In mammals, adipose tissue forms the chief store of reserve fat, but in the lower animals fat may be stored in other regions such as the liver or muscles or special glands. Adipose tissue contains from 88 to 93 per cent. of fat, the amount varying slightly with the species. The fat consists almost entirely of triglycerides, and may be taken to be made up partly of fat that has been synthesised by the animal from substances of a different nature, and partly of fat which has been taken as food in excess of its requirements. It is thus likely to vary in composition with the fat in the food, and there are many observations on record which show that this is so. Lebedeff (1882), for instance, starved two dogs until almost the whole of their reserve fat had been used up, and then fed one on linseed oil, and the other on mutton fat. After a time the dogs were killed, and the melting point of their adipose tissue fat determined. The dog that had received linseed oil contained adipose tissue fat with a much lower melting point (it was liquid at 0° C.) than that of normal dogs, whereas the dog that had received mutton fat gave a melting point higher than normal, above 50° C. The melting point of the connective tissue fat thus tended to approach that of the fat contained in the food. This same type of experiment has also been used to show that fatty acids not normally found in the body may be stored there if a sufficiently large amount is given in the food. In this way Winternitz (1898) has obtained a storage of so-called iodised fat, *i.e.*, unsaturated fat that has had its unsaturated linkages saturated by treatment with iodine, and

Munk (1884), has succeeded in getting a deposition of erucin (the triglyceride of erucic acid, $C_{22}H_{42}O_2$) on feeding a dog with rape-seed oil. These experiments were of an extreme type. The animals were practically freed from reserve fat before the fat was given, the deposition of which was awaited. They do not tell us to what extent the adipose tissue fat of a normal animal is modified by the type of fat in the food. Henriques and Hansen (1901) have carried out experiments which bear upon this point, and which indicate the great effect of the fat in the food on the kind of connective tissue fat which is laid down. Two pigs were fed on different cereals, one on barley, the other on maize. The fat from the animal fed on barley had an iodine value of 57·7 and a melting point of 27·4°, that from the one fed on maize had an iodine value of 75·6 and melting point 23°. These differences correspond to differences in the same constants of barley fat and maize fat respectively. Notwithstanding variations of this kind which may be produced in the same species of animal under normal conditions, it is usual to find that the adipose tissue fat of any one species is approximately constant in composition, and has an iodine value that does not vary greatly from a mean value. This being so, it is very probable that this constancy is due more to similarity of diet in a given species than to the synthesis in the animal of a fat of constant composition.

In the same animal, the composition of the adipose tissue fat varies. It is common knowledge that the fat which lies just under the skin has a lower melting point than that found more internally. These differences in melting point are associated with corresponding differences in iodine value. The lower melting fats have a higher iodine value, and therefore contain a greater proportion of unsaturated fats. Henriques and Hansen (1901) have investigated this phenomenon, and have shown that there is a regular gradation in iodine value in a downward direction as the melting point of the adipose tissue fat increases. Even in the subcutaneous fat of the pig these gradations can be observed as one passes inwards from the outermost layer. The following figures given by Henriques and Hansen for a pig fed on barley illustrate this:—

Source of Fat.			Iodine Value.	Solidifying Point.
Subcutaneous fatty tissue	Outer half	Outer layer	60·0	—
		Inner layer	57·1	26·4°
	Inner half	Outer layer	51·8	28·0°
		Inner layer	50·6	27·7°
Perirenal fat			47·7	29·6°
Omental fat			46·5	29·4°

Jaeckle (1902) has examined the subcutaneous fat from new-born infants and compared it with that of adults. From an infant 3 days' old the iodine value was 47·0; from others 2 to 3 weeks' old, 58·1; and from adults, 62 to 73. The fat of the new-born infant has also a higher melting point than that of adults, and this is no doubt to be correlated with the internal situation of the fœtus. Henriques and Hansen attribute these differences in composition to differences in temperature of the regions where the fat is laid down. They determined the temperature of the subcutaneous tissue in a pig at different depths from the surface, and found that 1 cm. deep it was 33·7°, 2 cm. 34·8°, 3 cm. 37·0°, and 4 cm. 39·0°. The rectal temperature was 39·9°. In confirmation of this view of the influence of the temperature in the immediate neighbourhood on the composition of the fat deposited, three young pigs from the same litter were kept under identical conditions as regards diet, but the external temperature was varied. One was kept at 30° to 35° C., one about 0° C., and the third at 0° C. but covered with a sheepskin coat. After two months the pigs were killed and the constants of their adipose tissue fat determined. The lowest iodine value, 67·0, was given by the third pig; the highest, 72·3, by the pig kept at 0° C. and unprotected, whereas the pig kept at 30° to 35° gave the figure 69·4. These figures are for the outermost layers of the subcutaneous connective tissue fat, and the figures for the other layers correspond with them closely. There appear to be grounds, therefore, for attributing these well-marked differences in the composition of adipose tissue fat, to the fact that it is deposited in regions of different temperature. Now it is generally held that the cells of adipose tissue do not synthesise from substances, such as protein or carbohydrate, the fat which they store up, but rather that they take up this fat from the blood stream.[1] In what form it is taken up, whether as neutral fat, fatty acid or soap is uncertain, but the process seems to be one which favours the storage at lower temperatures of the more unsaturated fats. It would be mere speculation to suggest any physical or chemical cause for this phenomenon, but it appears to be an example of the process of adaptation which might well have such a basis. It is possible, for instance, that the permeability of the adipose tissue cell to neutral fat, fatty acid or soap varies with temperature, and that at lower temperatures it is more permeable to the unsaturated fats, fatty acids or soaps than to the saturated ones. An adequate explanation based on experimental evidence is, however, still wanting, but it does

[1] Rosenfeld (1902) does not, however, assent to this view.

not appear unlikely that it will be found in an unequal variation with temperature of some property common to the saturated and unsaturated fats, fatty acids or soaps.

The Synthesis of Fat in Plants.

It may be stated in general that the investigations so far carried out on the mode of formation of fat in plants have been directed to a determination of the site of fat synthesis, and to the discovery of the parent substance from which fat is made. Many of the observations on record are of no great value on account of the methods used for estimating the fat. They have consisted too often in weighing the ethereal extract of the part under investigation, and taking the weight obtained to represent the amount of fat present. Such an extract might contain many organic substances which are not fat. In other cases, an extract made with light petroleum has been substituted for the ethereal extract, and, whilst it is true that this procedure would limit the substances dissolved to comparatively few, it is still open to the objection that higher alcohols, lipines and some resins, to mention only the more obvious substances, would be extracted along with the fat. In spite of these deficiencies of method, however, certain data have been obtained which indicate that the fat stored in seeds and fruits is made in the situation in which it is found and not brought there from other regions of the plant. Thus Rousille (1878) was unable to show any change in the fat content of the leaves of the olive during the ripening period when a large increase in the fat content of the fruit takes place, and Funaro (1880) has shown that the fat extracted by ether from the leaves is very different in composition from that found in the fruit. There is, however, as yet, no direct proof that fat may be formed in the fruit or seed when it is separated from the plant, though old observations by Pfeffer (1872) that the seeds of the pæony when separated from the plant before maturity, when they contain no fat, show a definite fat content after a time, indicate that such proof should be obtainable.

The mode of origin of the fat in seeds and fruits has been diligently studied, but almost the whole of the work which can be recorded in this field has been concerned with the finding of the precursor from which fat is formed, and not with the method of its formation. In 1861, de Luca put forward the view that mannitol was the parent substance of the oil formed in olives. This was based on his discovery of mannitol in quantity in the very early fruit, and its complete absence in the ripened fruit, coupled with an increase of mannitol in the leaves

from November to January, and then a disappearance towards the end of April. De Luca's view did not bear the test of further investigation, for Funaro, in 1880, stated that mannitol can only be detected in olives at a time when a large amount of oil has already been formed, and Hartwich and Uhlmann (1902) stated that they had been unable to isolate mannitol with certainty from olives at any time. There is not much ground, therefore, for regarding mannitol as the parent substance of the oil in olives. On the other hand, a series of studies by Leclerc du Sablon (1896 and 1897) have shown clearly that, in certain nuts, the appearance of fat during ripening is accompanied by a decrease in their carbohydrate content (starch, sucrose and glucose). The following table illustrates this :—

Date.	Walnut.			Date.	Almond.			
	Oil, Per Cent.	Glucose, Per Cent.	Sucrose, Per Cent.		Oil, Per Cent.	Sucrose, Per Cent.	Glucose, Per Cent.	Starch, Per Cent.
July 6 .	3	7·6	0·0	June 9 .	2	6·7	6·0	21·6
Aug. 1 .	16	2·4	0·5	July 4 .	10	4·9	4·2	14·1
,, 15.	49	0·0	0·6	Aug. 1 .	37	2·8	0·0	6·2
Sept. 1 .	52	0·0	0·8	Sept. 1 .	44	2·6	0·0	5·4
Oct. 4 .	62	0·0	1·6	Oct. 4 .	46	2·5	0·0	5·3

These observations have been confirmed and extended to other nuts by Valée (1903) and Ivanov (1912). There is thus satisfactory ground for the belief that carbohydrates are the precursors from which fat is formed in plants. The respiratory quotient has been measured by Gerber (1897) during the ripening of olives and castor oil seeds. In the early stage of development, when little or no oil is present in the seed or fruit, the quotient is less than 1. Later, in the stage of ripening, when oil is being produced the quotient rises above unity and this is also true for a short time if the fruit be separated from the tree. Although Gerber interpreted his results as indicating a formation of oil from mannitol, the wider view that they support the idea of a formation of fat from carbohydrate is more satisfactory.

A less probable explanation of the formation of fat in plants, but one which demands further examination by modern analytical methods is that put forward by Scurti and his colleagues (1910 and 1911). It has been shown by Power and Tutin (1908) that the leaves of the olive contain a higher alcohol, oleanol, $C_{31}H_{50}O_3$. Scurti and Tommasi have discovered also that the ether soluble substance of the green olive fruit consists almost entirely of this same alcohol and that later in the process of development it is still present but in addition, higher fatty acids,

both saturated and unsaturated, are found. Finally, when the ripening process is progressing rapidly, the ether extract consists almost entirely of the neutral oil but a trace of the alcohol is probably still present. Scurti suggests therefore, that the oleanol is produced in the leaf and transported to the fruit where it undergoes transformation into the neutral oil. Similar observations on other kinds of fat-containing seeds and fruits have been made; in all cases an alcohol closely related in composition to oleanol has been isolated from the leaves and the fruit. In the latter it diminishes in amount as the fat content increases. It is unfortunate that these observations are not based on separate estimations by saponification methods of the higher fatty acids and unsaponifiable substances. The absence of data which such methods would furnish prevent satisfactory conclusions being arrived at as to the probability of Scurti's views of fat formation. They are not in accord with the high respiratory quotients observed by Gerber during the ripening of olives, since the conversion of oleanol into fatty acids would be accompanied by a respiratory quotient less than 1.

Very few observations have been recorded concerning the composition of the fat present in seeds or fruits at various stages of the ripening process, and this is unfortunate, since studies of this kind might throw some light on the nature of the reactions by which fats are formed. Ivanov (1912) has observed an increase in the iodine value of the oil present in linseed during ripening. Seeds collected on July 5th contained oil with an iodine value of 120·6 and this had risen to 175·3 in the oil obtained from seeds collected 7 weeks later. On the other hand, colza oil, hempseed oil, and poppy oil showed no change during the ripening of the seed. When fatty acids are first formed in the seed or fruit they are not immediately combined with glycerol to produce a neutral fat, so that in the early stages of growth, seeds and fruits contain an oil in which notable amounts of free fatty acids are present. These become proportionately less as ripening proceeds. The explanation of this delay in the synthesis of the neutral fat is at present lacking. It may conceivably be due to one of several causes such as an inequality in the rate of formation of fatty acids and glycerol so that insufficient glycerol is present, or absence of the synthetic agent, lipase, or to an undue richness in water of the early seed inhibiting the synthetic action of lipase (Terroine, 1920). That the synthetic agent is a lipase has been proved experimentally by several observers (Dunlap and Gilbert 1911, Welter 1911, Ivanov 1911). The synthesis of neutral fat from fatty acid and glycerol is observed *in vitro* only when the concentration of water is small. Dunlap and Gilbert, by making a mixture of oleic acid and

glycerol and adding castor oil seeds from which the outer skin had been removed and the pulp extracted with ether, were able to show that within 11 days, 26 per cent. of the fatty acid had been converted into neutral fat. If dilute glycerol be used, synthesis does not take place. On the contrary in such a mixture, neutral fat will be hydrolysed to produce fatty acid and glycerol.[1]

The Synthesis of Fat in Animals.

One of the much debated questions amongst physiologists during the latter half of last century was the origin of the connective tissue fat of animals. In 1840 it was held by Dumas that the fat of adipose tissue was a remnant of that taken in the food, and that this source and this alone was the one from which animals drew the reserve fat which they stored. Liebig, about ten years later, was the first to suggest that animals must synthesise fat from some substance present in the food, and he regarded the carbohydrates as the most likely raw material for this purpose. He supported this theory by reference to the large amounts of fat which milch cows secreted daily in their milk—amounts surpassing greatly those present in the food. He also pointed out that the fat of animals receiving a very similar diet was different according to the species, beef fat, for instance, being different in composition from mutton fat. Liebig's view was the predominant one until about twenty years later, when Voit, and Voit and Pettenkofer, in a series of investigations using the "balance-sheet" method, claimed to have demonstrated conclusively that proteins were the raw material from which fat was synthesised in animals. Voit's work was remarkably accurate and raised no enduring criticism, so that for many years his point of view remained the one most acceptable. The gradual accumulation of evidence obtained in other, and more direct ways than those used by Voit, together with a searching criticism by Pflüger (1892), not of Voit's results, but of his method of calculation from them, finally led to the overthrow of the theory of fat formation from protein. Voit had shown that on feeding a dog with large quantities, 2·5 kg., of lean meat, the whole of the nitrogen in the meat was recoverable in the urine and fæces, but not the whole of the carbon. The carbon retained, 41·9 gm., was too much to be accounted for by a storage of glycogen, and hence Voit concluded that some or all of it was stored as fat, thus constituting a formation of fat from protein. The mode of calculation

[1] For a general discussion of this question see Bayliss, "The Nature of Enzyme Action," pp. 55-57.

of the carbon balance in this experiment was attacked by Pflüger, who pointed out that the ratio of carbon to nitrogen in lean meat was 3·23 : 1, and not 3·68 : 1, as used by Voit. Recalculating from this value, Pflüger showed that the carbon balance in Voit's experiment was only 3·0 gm., which was within the experimental error, or could easily be accounted for by a storage of glycogen. In this way, Voit's monumental proof fell to the ground. Experiments by other workers following the same lines as Voit were no more successful (E. Voit, Cremer, Kumagawa), and it may be concluded, therefore, that by "balance-sheet" experiments of this kind, a formation of fat from protein has not been proven. Evidence that protein may be converted into fat has also been sought for in several phenomena which at first sight appeared to indicate that such a process occurs. Amongst these the chief have been: the formation of adipocere in corpses after long immersion in water; the increase of fat content in certain kinds of cheese during the ripening process; the growth of larvæ of the blue-bottle fly on lean meat, blood, or solutions containing protein; and the fatty degeneration of organs in certain forms of disease. Only in the case of the fly larvæ investigated by Weinland (1908) has convincing evidence of fat formation from protein been obtained. In these experiments fat formation was demonstrated in mixtures of Witte's peptone and bacteria free larvæ which had been squeezed to a pulp. The process is anærobic, and its velocity is increased by an increase of temperature. The formation of adipocere may now be attributed to the slow hydrolysis of body fat by water (Ruttan and Marshall, 1916-17). Adipocere consists almost entirely of the insoluble saturated fatty acids which are left when the hydrolysis of the pre-existing fats is complete. It contains but little oleic acid, this being replaced by two monohydroxy-stearic acids produced by its slow hydration. Alterations in the fat content of cheese in the sense that the amount of fat increases, have been shown in most cases to be purely relative and due to loss of water, or the oxidation processes associated with bacterial action whereby the protein content of the cheese is diminished. In one instance, Jacobsthal (1893) has shown that the absolute amount of fat may increase, but he ascribes this to the growth of moulds. It may be concluded, therefore, that the formation of fat from proteins is very difficult to demonstrate.

Carbohydrates are the other main food substances which come into question in considering the raw materials from which fat may be made in animals. It has been pointed out above that upwards of seventy years ago, Liebig put forward the view that these substances are chiefly

used in fat synthesis, but he did not furnish any direct proof. This was first given satisfactorily by Lawes and Gilbert in their classical investigations on the feeding of farm stock. Oxen, sheep and pigs were the animals used, but only the last gave indubitable proof of the synthesis in question. The chief results were published in 1860, but, owing no doubt to the work of Voit, a further publication was made in 1866, containing additional and direct data. The most satisfactory experiment was the following: Two pigs were taken from the same litter. One was killed, and the fat and protein it contained were estimated. From the figures, the fat and protein content of the other was calculated. This was fed on a food mixture of known amount and composition, and after several weeks was killed and analysed. From the results, the amounts of fat and protein put on during the experimental period were obtained. It was found that the increase in fat was greater than could possibly be accounted for by the fat and protein in the food consumed, assuming that the whole of the carbon in the protein not stored or excreted as urea had been converted into fat. The excess of fat must, therefore, have been formed from the other main constituent of the food, namely, the carbohydrate. The following are among the more important figures which Lawes and Gilbert give :—

Total fat increase .	71·2 lbs.	Protein in food	64·0 lbs.
Fat in food . .	12·4 lbs.	Protein put on	6·5 lbs.
Fat synthesised .	58·8 lbs.	Protein available for fat synthesis	57·5 lbs.

Carbon in fat synthesised	45·3 lbs.
Carbon in available protein, less carbon excreted as urea	27·4 lbs.
∴ *Carbon in fat which must have come from carbohydrate*	17·9 lbs.

It is remarkable that Voit was aware of these results but would not allow himself to consider that they afforded satisfactory proof that carbohydrate could be transformed into fat. It is true that some of the experiments of Lawes and Gilbert were negative when the above rigorous method of calculation was used, but they were not devised specifically to prove that fat could be synthesised from carbohydrate, and consequently many of the diets used contained too much protein to produce rapid fattening. Oxen and sheep also showed themselves to be less suitable than the pig for the purpose in view.

The same kind of proof as that furnished by Lawes and Gilbert was subsequently obtained by other workers. Pigs have given the most satisfactory results in all cases, but Rosenfeld (1899) has had some measure of success with geese, and Rubner (1886) with dogs. In the latter animals, Morgulis and Pratt (1913) have shown that the process of fat formation is accompanied, as would be expected, by a high

respiratory quotient. There is thus no occasion to doubt that carbohydrates may be converted into fat in the animal body.

The formation of fat being a process of fundamental importance to animals and plants, it does not seem unlikely that the process in its essentials may be the same in both. This proof of the rôle of carbohydrates in the animal process strengthens, therefore, the less direct results which have been obtained with plants, which, it may be recalled, also indicate that, in the vegetable kingdom, carbohydrates form the starting point in the synthesis of fats.

The carbohydrates and fats differ so widely in their chemical characters and composition that it is unlikely that the whole of the processes of transformation of the former into the latter would be easily demonstrated experimentally. It is not surprising, therefore, that direct evidence of a transformation of carbohydrate into fat has so far not been obtained in any isolated organ. Leathes and Hildesheim (1904 and 1908) however, have shown definitely that the liver participates in the process and certainly in its later stages. Their experiments were carried out with the minced liver from the rabbit, dog or pig. It was found in many cases, but not in all, that the liver pulp after incubation at 37° for 24 to 48 hours showed a definite increase in its content of higher fatty acids, in some instances as much as 35 per cent. These increases were noted in experiments in which the liver was removed aseptically and kept sterile during the experiment and also when bacterial growth was inhibited by the addition of antiseptics. Additions of glycogen or glucose or other substances which seemed likely to be intermediate products in the transformation of carbohydrate into fat were unsuccessful in producing more marked increases in fatty acid than occurred without such additions. We are, as yet, unaware of the primary conditions which determine in an animal the formation of fat from carbohydrate, whether for instance, it is merely an excess of the latter beyond the animal's needs which sets the process in motion or whether other conditions are necessary, but Leathes' experiments indicate that in the liver, at any rate, a simple excess of carbohydrate, is not effective and that other factors which operate in the process have still to be discovered. Leo and Bachem (1913) have shown that the aseptic incubation of rabbit's liver in the presence of phosphorus leads, in some cases, to an increase in the amount of ether soluble substances present in the organ. In their work however, the fatty acids were not separately estimated so that the results cannot be taken as indicating a definite new formation of fat. According to Shibata (1911), autolysis of the liver at 32° for periods of 6 to 46 days does not result in an increase of its fat content.

The mammary gland, which might be expected to be a site for the new formation of fat, has so far not been shown to be such. The experiments of Foà (1913) indicate that milk fat is derived from the fat of the blood by the intervention of the cells of the gland and is not made there from carbohydrate. It is also well known that unusual fats present in the food of lactating animals may be detected in the milk [Winternitz (1897-98), Henriques and Hansen (1899), Caspari (1899)].

The Chemistry of Fat Synthesis.

Admirable as many of the researches have been which were planned to find out whether protein or carbohydrate was the parent substance of fat in the organism, they fail to-day to excite much interest apart from their historical value. Compared with the time when they were carried out, our understanding of the processes of digestion, absorption and intermediate metabolism is now vastly greater. There is good reason to believe, for instance, that certain of the amino-acids contained in the protein molecule are convertible into glucose and that the amino acids themselves, at least some of them, may be synthesised from ammonium salts of hydroxy- or keto-derivatives of the simpler fatty acids. This has shifted the centre of interest from the complex protein or starch molecule to the much simpler amino-acid or hexose molecule as possible sources of fat. The question now is, how may glucose or other hexoses yield fat by a series of chemical reactions and why, under ordinary circumstances, are the amino acids, which may be converted into glucose in the body under special conditions, not utilised for fat formation? If the more credible hypotheses that have been put forward to explain the mechanism of fat synthesis from carbohydrates afford a satisfactory explanation of the process, there is no reason to believe, from what is known of the oxidation of amino acids in the body, that a few of them, notably alanine, might not serve like the hexoses, as parent substances from which fatty acids may be synthesised.

The synthesis of fat presumably takes place in two stages, the first being the building up of the fatty acid; the second, the union of 3 molecules of the fatty acid with glycerine to form a triglyceride. There is ample evidence that the latter process may be brought about by the agency of the enzyme lipase, which, according to the conditions in which it acts, can either catalyse the hydrolysis of fat or its synthesis (see page 104). Bradley (1912) has shown that lipase is widely distributed in the body, and the reversibility of its action renders it easy to understand how the ready transformation of fatty acids and glycerine into fat may take place when they have been synthesised. The first

stage of fat synthesis, involving the formation of fatty acids and glycerine from carbohydrate is still a matter for chemical speculation. Fortunately, experimental proof exists, that the reactions involved in such speculation are known to take place under appropriate conditions. The matter has therefore advanced beyond the stage of hypothetical reactions, which take place only in the imagination of their authors, and never, in the experience of the chemist.

A study of the composition of the naturally occurring fatty acids is suggestive as to the lines on which their synthesis runs. In all those containing six or more carbon atoms these are found to be present in even numbers. This suggests that the long chains of carbon atoms are built up two at a time, and possibly by the union of several molecules of some substance containing two carbon atoms. Further, some are unsaturated in varying degree, whereas others are fully saturated. The process must therefore be one which may yield a saturated or unsaturated product with slight changes of conditions. A mere condensation of glucose or other hexose molecules, followed by oxidation and reduction processes, as suggested some years ago by Emil Fischer, does not furnish a satisfactory explanation. Hexoses do not condense in this way, so far as chemical experience shows, nor could the fats present in butter, for instance, containing as they do acids with an even number of carbon atoms from four to twenty, be formed from hexose molecules or from trioses, pentoses, and hexoses. One substance containing two carbon atoms, which the above considerations indicate as being a likely intermediate substance between the carbohydrates and the fatty acids, is acetaldehyde. Hoppe-Seyler (1879) and Nencki (1878) were the first to suggest this, and they regarded it as being formed from lactic acid, a reaction which may be brought about easily in the laboratory by warming with mineral acids—

$$\underset{\text{Lactic acid.}}{CH_3.CH(OH).COOH} = \underset{\text{Acetaldehyde.}}{CH_3.CHO} + \underset{\text{Formic acid.}}{H.COOH}.$$

Hoppe-Seyler (*loc. cit.*) also showed that when lactic acid was heated with caustic alkalies to about 220°, a series of fatty acids was produced, including butyric and caproic acids as well as higher fatty acids. He pointed out the significance of this for the understanding of certain fermentation processes in which Pasteur and Fitz had shown that butyric and caproic acids were produced from lactates. Hoppe-Seyler's experiments have been confirmed by Raper (1905) with the exception of the formation of the higher fatty acids which were not found to be produced in this reaction. They represent the first experimenta

demonstration of the synthesis of fatty acids (lower members of the series, it is true), from a substance itself easily derived from the carbohydrates. Nencki suggested that the acids were formed from acetaldehyde by condensation, and this view was developed later by Magnus-Levy (1901) and Leathes (1906), to embrace the formation of the higher fatty acids as well. The acetaldehyde is supposed to condense first to aldol, a reaction which takes place readily at the ordinary temperature in the presence of traces of alkali. The aldol then undergoes oxidation at the -CHO group and simultaneous reduction at the -CH(OH) group, both at the expense of 1 molecule of water, and is thus converted into butyric acid—

$$\underset{\text{Acetaldehyde.}}{2CH_3.CHO} = \underset{\text{Aldol.}}{CH_3.CH(OH).CH_2.CHO}$$

$$\underset{\text{Aldol.}}{CH_3.CH(OH).CH_2.CHO} = \underset{\text{Butyric acid.}}{CH_3.CH_2.CH_2.COOH.} + H_2O$$

H . H . . . . O

If the aldol condenses further with acetaldehyde or undergoes autocondensation before the above change takes place, then compounds containing multiples of two carbon atoms would be produced which might be capable of transformation into fatty acids. In this way the long, straight carbon chains found in the naturally occurring fatty acids might well have their origin. The formation of butyric acid from aldol by the mechanism represented above is not known to occur *in vitro ;* moreover, chemical experience shows that aliphatic hydroxy compounds are difficult to reduce. It seems more probable therefore that the aldol would lose water and be converted into crotonaldehyde, a change which takes place very readily, and that the crotonaldehyde would be reduced to butaldehyde by hydrogen from the initial reaction in which lactic acid breaks up into acetaldehyde and formic acid, the latter yielding hydrogen and carbon dioxide. The butaldehyde would then be oxidised to butyric acid by oxygen supplied from external sources. Similarly, the products of condensation of several molecules of acetaldehyde, by loss of water would form higher unsaturated aldehydes and these in turn would be reduced[1] to form more or less completely saturated aldehydes, the final stage being always the oxidation of the terminal -CHO group to form the corresponding fatty acid. This would prevent further condensation.

[1] Knoop and Oeser (1914), have supplied experimental proof that unsaturated linkages can undergo reduction in the body. Benzal-lævulinic acid administered to dogs results in the excretion of phenyl-α-hydroxy-butyric acid,

$$C_6H_5.CH:CH.CO.CH_2.CH_2.COOH \rightarrow C_6H_5.CH_2.CH_2.CH(OH).COOH.$$

As an example, the formation of octoic acid by the above scheme may be represented as follows :—

$$\underset{\text{Lactic Acid.}}{CH_3.CH(OH).COOH} \rightarrow \underset{\text{Acetaldehyde.}}{CH_3.CHO} + H_2 + CO_2$$

$$4CH_3.CHO \rightarrow \underset{\text{Unsaturated Aldehyde.}}{CH_3.CH:CH.CH:CH.CH:CH.CHO} + 3H_2O$$

$$CH_3.CH:CH.CH:CH.CH:CH.CHO + 3\,H_2 + O \rightarrow \underset{\text{Normal Octoic Acid.}}{CH_3.CH_2.CH_2.CH_2.CH_2.CH_2.CH_2.COOH}$$

The only serious objection to this view on the grounds of chemical experience is that almost without exception, when aliphatic aldehydes condense with acetaldehyde the resulting compounds possess a branched and not a straight chain structure. Lieben (1883 and 1901) and his pupils have shown, for instance, that when acetaldehyde and butaldehyde condense β-hydroxy iso-caproic aldehyde is produced and not the normal compound :—

$$\underset{\text{Butaldehyde.}}{CH_3.CH_2.CH_2.CHO} + \underset{\text{Acetaldehyde.}}{CH_3.CHO} = \underset{\text{Hydroxy iso-caproic aldehyde.}}{\begin{matrix} CH_3-CHOH \\ | \\ CH_3-CH_2-CH-CHO \end{matrix}}$$

It is now known, however, that this objection does not hold in the case of acetaldehyde condensing with itself, for Raper (1907) has shown that aldol undergoes auto-condensation to form a straight chain compound, and later Smedley (1911) showed that the same was true of crotonaldehyde.[1]

There are thus no serious hindrances to the view that the higher fatty acids may be produced by the auto-condensation of acetaldehyde

[1] The auto-condensation of aldol and crotonaldehyde to give straight chain compounds is not without parallel. Lapworth (1901) showed that ethyl oxalate condensed with ethyl crotonate at the γ carbon atom to give a straight chain compound,

$$CH_3.CH:CH.COOC_2H_5 + (COOC_2H_5)_2 = C_2H_5.OOC.C(OH):CH.CH:CH.COOC_2H_5.$$

In a later publication, Lapworth (1920) has advanced a hypothesis based on these and other phenomena, which attributes to the carbon atoms in organic compounds a positive and negative polarity. This polarity is induced by a "key atom" (in the above cases, this is the oxygen atom of the -CHO group), and alternates from carbon atom to carbon atom. In crotonaldehyde the oxygen atom of the -CHO group is the "key atom," and the polarities may be represented thus :—

$$\begin{matrix} C.C:C.C:O \\ -\ +\ -\ +\ - \end{matrix}$$

The presence of double bonds appears to extend the influence of the "key atom," and in crotonaldehyde the negative polarity of the γ carbon atom is thereby enhanced. It thus reacts in condensations of the "aldol" type like the α carbon atom of saturated aldehydes, so that condensation with a second aldehyde molecule may take place at the γ carbon atom and result in the production of a compound with a straight chain structure. The same is probably true of the behaviour of aldol, which may also be considered as having negative polarity developed at the γ carbon atom.

to form higher hydroxy or unsaturated aldehydes, which by loss of water, reduction and oxidation could be converted into fatty acids.

Observations by Friedmann (1908) indicate that the liver is able to bring about the aldol condensation. Aldehyde ammonia on perfusion through the surviving liver gives rise to an increased formation of acetoacetic acid and acetone; the same is true of aldol, so that the formation of this is probably a stage in the reaction. Further evidence in support of this hypothesis is provided by the butyric acid producing bacteria. Certain of these organisms when grown in a medium containing calcium lactate produce from it both butyric and caproic acid. Small amounts of octoic acid and decoic acid are also formed [Béchamp (1894), Raper (1907), Neuberg and Arinstein (1921)]. The organisms are anaerobes, and hydrogen is given off during the fermentation. It might well be, that the processes taking place in this fermentation are those suggested above as being responsible for the formation of higher fatty acids in animals and plants.[1]

The production of acetaldehyde from lactic acid, although easily brought about in the laboratory, has not so far been demonstrated as a biological process, and this therefore presents a difficulty in the acceptance of the above hypothesis as it stands. There is another possible source of acetaldehyde for which satisfactory experimental proof exists. Neuberg and Karczag (1911) have shown that yeast contains an enzyme, "carboxylase," which causes the decomposition of pyruvic acid into acetaldehyde and carbon dioxide, thus:—

$$CH_3 . CO . COOH = CH_3 . CHO + CO_2.$$

This reaction may, therefore, serve to explain the origin of the aldehyde in the plant kingdom, since Zaleski (1913) claims to have found carboxylase in higher plants, but, so far, carboxylase has not been discovered in animal tissues. It is also uncertain, in the animal organism, to what extent, if at all, pyruvic acid is produced from carbohydrates as an intermediate product in their metabolism. Another possible source of pyruvic acid would be the oxidation of lactic acid. There is, as yet, no direct evidence that this takes place in the body. Embden and Schmitz (1910, 1912) have, however, provided some indirect evidence. The ammonium salts of both lactic and pyruvic

[1] In a recent investigation, Neuberg and Arinstein (1921) have suggested that in the butyric fermentation, pyruvic acid is the substance which condenses to give products containing longer chains of carbon atoms, and that acetaldehyde or aldol are not intermediate products in the formation of butyric acid. The bacillus butylicus ferments "pyruvic aldol" (α keto-γ carboxy-γ valerolactone) and produces butyric acid. The formation of acetaldehyde may, however, be detected when this organism ferments sugar solutions.

acids give rise to the formation of alanine when they are added to blood which is being perfused through a surviving liver. Since ketonic acids in general give rise to the corresponding amino acid in this type of experiment, the presumption is that lactic acid first yields pyruvic acid by oxidation, and this is then converted into alanine. Although this view of the possible origin of pyruvic acid and hence of the acetaldehyde necessary for fat synthesis may appear, at first sight, satisfactory, it is open to the objection that no hydrogen is provided for the extensive reduction process that has to take place in order that saturated fatty acids may be produced. There is, however, another possible mode of origin of pyruvic acid which does not present this difficulty. Pyruvic aldehyde (methyl glyoxal) is believed to be produced as an intermediate product in the alcoholic fermentation of glucose. In the presence of acetaldehyde it is oxidised to pyruvic acid, and the aldehyde is reduced to ethyl alcohol (Neuberg and Reinfurth, 1919)—

$$CH_3.CO.CHO + CH_3.CHO + H_2O = CH_3.CO.COOH + CH_3.CH_2OH.$$

There is considerable evidence[1] that pyruvic aldehyde is a product of the decomposition of glucose in alkaline solution. It is possible, therefore, that it is produced in the body, and, by a coupled reaction, oxidised to pyruvic acid, some other substance concurrently undergoing reduction as in the alcoholic fermentation. The unsaturated aldehydes which the hypothesis we are considering represents as being produced by the condensation of acetaldehyde, might well act as the acceptors for hydrogen in this reaction, and become reduced to saturated aldehydes.

Although this suggestion overcomes the difficulty of accounting for the reduction process that occurs in fat synthesis from carbohydrate, there is still another to encounter. Dakin and Dudley (1913) have shown that an enzyme is widely distributed in the body which converts pyruvic aldehyde rapidly into lactic acid by the addition of the elements of water. If pyruvic aldehyde arises, therefore, as an intermediate product in the decomposition of glucose, it would, in all probability, be converted into lactic acid and not pyruvic acid.

In the light of our present knowledge of animal metabolism, therefore, it is not yet safe to conclude that pyruvic acid arises as a degradation product of carbohydrates in the body, and consequently it does not appear at present to be the precursor of the acetaldehyde required in the hypothesis of fat synthesis which we have been considering.

[1] See Dakin's Monograph in this series, "Oxidations and Reductions in the Animal Body," pp. 86-87.

An alternative hypothesis to explain the formation of fatty acids in the living organism has been put forward by Smedley (1912-1913). It was shown in 1903 by Erlenmeyer, Junr., that benzaldehyde and cinnamic aldehyde will condense with pyruvic acid in the presence of strong caustic soda. Lubrzynska and Smedley (1913) extended this observation, and showed that both aliphatic and aromatic aldehydes will condense with pyruvic acid in dilute alkaline solution. An unsaturated keto-acid is produced, which, on oxidation with hydrogen peroxide, loses carbon dioxide, and yields an α-β unsaturated acid, thus—

$$R.CHO + CH_3CO.COOH = R.CH:CH.CO.COOH + H_2O$$
$$R.CH:CH.CO.COOH + H_2O_2 = R.CH:CH.COOH + CO_2 + H_2O.$$

Smedley's hypothesis is that pyruvic acid is formed in the body as a decomposition product of carbohydrate and is the starting point for the synthesis of fatty acids. One molecule of pyruvic acid is supposed to decompose yielding acetaldehyde and carbon dioxide and the aldehyde so produced condenses with a second molecule of pyruvic acid. The keto-acid which results from this reaction may then undergo one of two processes. It may lose carbon dioxide and yield crotonaldehyde or it may be oxidised and yield crotonic acid. In the former case the crotonaldehyde may condense with another molecule of pyruvic acid again yielding a keto-acid which alternatively may lose carbon dioxide or be oxidised to give an unsaturated aldehyde or acid, respectively. By a continued formation of unsaturated aldehydes (all containing an even number of carbon atoms) and condensation with pyruvic acid, the long, straight carbon chain structure of the higher fatty acids would be produced. By reduction of the unsaturated linkages, either partially or completely, and final oxidation of the terminal aldehyde group the process would be completed. The following equations illustrate the formation of caproic acid by this method :—

$$CH_3.CO.COOH = CH_3.CHO + CO_2$$
$$CH_3.CHO + CH_3.CO.COOH = CH_3.CH:CH.CO.COOH + H_2O$$
$$CH_3.CH:CH.CO.COOH = CH_3.CH:CH.CHO + CO_2$$
$$CH_3.CH:CH.CHO + CH_3.CO.COOH = CH_3CH:CH.CH:CH.CO.COOH + H_2O$$
$$CH_3.CH:CH.CH:CH.CO.COOH = CH_3.CH:CH.CH:CH.CHO + CO_2$$
$$2CH_3.CH:CH.CH:CH.CHO + 4H_2 + O_2 = 2CH_3.CH_2.CH_2.CH_2.CH_2.COOH$$

Caproic acid.

In order to overcome the supposed difficulty of the toxicity of acetaldehyde to living tissues,[1] Lubrzynska and Smedley postulate that

[1] This does not seem serious in view of Neuberg's work on the production of acetaldehyde by yeast.

in the above process, "Free acetaldehyde is not liberated but that the decomposition of the keto-acid is in some way regulated by the pyruvic acid with which the nascent aldehyde combines."

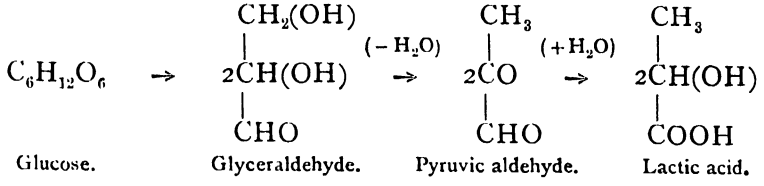
This hypothesis, like the first, would account for the formation of fatty acids with an even number of carbon atoms and it also indicates how partially or completely saturated acids could be produced according to the extent to which reduction took place. The chief difficulties in its acceptance are, (1) that carboxylase or any substance exerting a similar action has so far not been found in animal tissues and such a substance would be required to bring about the decomposition of the α-keto-acids in the above scheme; and (2), the uncertain place of pyruvic acid as a normal intermediate product of carbohydrate metabolism in animals.

In conclusion, it may be said of both these hypotheses, that a survey of the known reactions of organic chemistry has so far yielded none more likely to take place under the conditions found in living organisms and none which provide so satisfactorily for the wide variation in constitution of the naturally occurring fatty acids. Both await further experimental verification and extension and until this is forthcoming it is not possible to make a definite choice between them.

Before passing to the discussion of the origin of glycerol one other matter awaits consideration, namely, the possible formation of fat from amino acids. Since certain of the naturally occurring amino acids have been shown to be capable of transformation into glucose in diabetic animals, it is reasonable to suppose that they may also be converted into fatty acids because glucose is capable of this change. Nevertheless, as indicated earlier in this chapter, protein, which is built up from amino-acids, has, so far, not been proved to give rise to fat formation in the body. The reason is probably to seek in two directions. Firstly, only a certain number of the amino acids present in the protein molecule have been shown to be convertible into glucose, and then not always quantitatively; only three out of the five carbon atoms of glutamic acid, for instance, are so converted. Thus only a portion of the protein taken as food could be made into carbohydrate, and become available for fat formation even when all the conditions were favourable for this change. Secondly, the "balance sheet" method has its limitations. Animals will not endure unlimited amounts of protein, and the quantities which would be required to provide for the claims of growth, the liberation of energy, and an excess from which the formation of fat would be awaited, are sufficient to upset the ordinary processes of nutrition, and so thwart the experiment. There is every reason to

believe on chemical grounds that fat may be formed from certain of the amino acids, namely, those which give rise to glucose formation in diabetic animals, but the biological proof is beset with experimental difficulties.

The Origin of Glycerol.—There is a strong presumption on chemical grounds that glycerol is produced from carbohydrates. It is well known that a solution of glucose in the presence of caustic alkalies undergoes complex changes, and one of the main products produced is lactic acid. There is good evidence[1] that the precursor of lactic acid under these conditions is pyruvic aldehyde and the changes may be represented as follows :—



In this scheme, glyceraldehyde is represented as an intermediate product between glucose and pyruvic aldehyde, but its production has not been proved experimentally. It is, however, very probable, since, on treatment with caustic alkalies, glyceraldehyde like pyruvic aldehyde gives rise to lactic acid. On reduction, it would yield glycerol. That this process can be brought about in the body was proved by Embden, Schmitz and Baldes (1912), who found that glyceraldehyde perfused through the surviving liver or even incubated with the mashed organ was converted into glycerol. In vegetable organisms the production of glycerol has been most diligently studied in the alcoholic fermentation of sugar by yeast. Pasteur, in 1858, was the first to point out that glycerol was produced in this process, the yield being about 3 per cent. of the sugar fermented. Its origin was carefully studied by Buchner and Meisenheimer (1906) who came to the conclusion that it was derived from the sugar and not from proteins, fats or lipines. In 1918, Neuberg and Reinfurth discovered that, by the addition of sodium sulphite to a fermenting mixture of yeast and glucose, the yield of glycerol was largely increased, and, in addition, a considerable amount of acetaldehyde, produced during the fermentation, was "fixed" by the sulphite. In subsequent communications,[2] Neuberg and his colleagues have indicated the probable course of the reactions by which glycerol is

[1] Dakin, "Oxidations and Reductions in the Animal Body," pp. 85-87.
[2] See Harden, "Alcoholic Fermentation."

produced. The glucose is supposed to dissociate first to glyceraldehyde, and this, by loss of water, is converted into pyruvic aldehyde—

$$C_6H_{12}O_6 \rightarrow 2\,\begin{matrix}CH_2(OH)\\ |\\ CH(OH)\\ |\\ CHO\end{matrix} \rightarrow 2\,\begin{matrix}CH_3\\ |\\ CO\\ |\\ CHO\end{matrix}$$

In the normal fermentation process, when sulphite is not present, a coupled reaction then occurs in which 1 molecule of pyruvic aldehyde is oxidised to pyruvic acid, and 1 molecule of acetaldehyde (produced from pyruvic acid by carboxylase), is reduced to ethyl alcohol, the oxygen and hydrogen required for these processes being supplied from a molecule of water—

$$CH_3.CO.CHO + CH_3.CHO + H_2O = CH_3.CO.COOH + C_2H_5.OH.$$

In the presence of sodium sulphite the acetaldehyde, produced from pyruvic acid as above, is fixed in some way by the sulphite and cannot act as an acceptor for hydrogen, the latter is therefore taken up by glyceraldehyde with the production of glycerol—

$$CH_2(OH).CH(OH).CHO + H_2 = CH_2(OH).CH(OH).CH_2(OH).$$

Fermentation in the presence of sulphite thus leads to the production chiefly of glycerol, acetaldehyde and carbon dioxide. It is uncertain whether this mechanism for the formation of glycerol also operates in the higher plants, but the formation of acetaldehyde by moulds has been demonstrated by Cohen (1920). If this suggests no more than that in the plant kingdom, glucose may dissociate to yield glyceraldehyde, the formation of this substance enables us to understand how under suitable conditions glycerol may be produced from it by reduction.

It is safe to conclude, therefore, from what is known of the behaviour of glucose in the body and when attacked by lower vegetable organisms, that glycerol arises from it by the formation of glyceraldehyde, which subsequently undergoes reduction to glycerol.

The Influence of Environment on Fat Synthesis.—When animals or plants store carbohydrate as a reserve they are laid down as glycogen or starch respectively. Only these two substances are made use of. Glycogen prepared from different animal tissues and from different species of animals is always the same substance so far as chemical investigation has shown. The same is true of starch. It is remarkable, therefore, that when fats are stored in the plant or

animal, not one substance only, but a great variety of substances is found. This variety is due to differences in structure of the three fatty acid molecules, which are united to glycerol to form the naturally occurring triglycerides. The greatest variation is caused by differences in the degree of saturation of the constituent fatty acids, but differences in their molecular weight, due to the number of carbon atoms, is also a contributing factor. In a few instances, also, the fatty acids may contain hydroxyl groups. This diversity of composition gives rise to corresponding variations in physical properties, such as viscosity, solidifying and melting point. There is reason to believe that all the fats found in nature are fluid at the temperature at which they are normally formed in the animal or plant. The fats found in cold-blooded animals, or in plants growing in temperate or cold climates, are therefore always unsaturated to a considerable extent, since the unsaturated acids form triglycerides with low melting points. On the other hand, the saturated fats occurring in nature are only found to any marked extent in warm-blooded animals or in tropical plants, since the presence of saturated fatty acids, especially those containing 16 or 18 carbon atoms, gives rise to fats which are solid at the ordinary temperature. In the case of animals these differences are probably largely conditioned by the nature of the fat present in their food, but with plants they must be ascribed to variations in the synthetic processes by which the fats are produced. Pigulevski (1915) has examined the oils derived from a number of different genera of certain families of plants, and states that the capacity of plants to produce oil of a definite composition depends on conditions of climate and possibly of nutrition. Cultivated genera, occurring in more favourable circumstances than wild ones, produce fatty acids which are less unsaturated than those produced by the latter, and therefore have a lower iodine value. There is thus some relation between the composition of the naturally occurring fats and the conditions of the environment in which they are formed. Pigulevsky suggests that a cold climate induces in plants the necessity of accumulating in the seed a highly unsaturated oil and one, therefore, possessing great chemical activity, and that plants regulate their physiological apparatus to adapt themselves to the surrounding conditions. A simpler explanation of these facts would seem to be, however, that fats are formed in nature by a series of reactions, the end points of which vary according to the temperature at which they occur. At higher temperatures the chemical reactions leading to the formation of higher saturated fatty acids reach completion, but at lower temperatures, possibly because reduction processes do not take place so easily, the

same end point is not reached, the unsaturated fatty acids being in this case the final products. This conception of the process of fatty acid formation places the unsaturated fatty acids as intermediate between the carbohydrate, which may be regarded as the primary substance from which they are formed, and the higher saturated fatty acids, which are considered as end products of the reaction or series of reactions. Another view of the origin of these acids is that the unsaturated ones are produced from the saturated by a process of oxidation, whereby hydrogen atoms are removed and that the saturated acids are the intermediate and the unsaturated acids the final products of the reaction. Until we know more of the nature of the reactions by which fat is formed it is not possible to decide finally between these two views. It seems improbable, however, that the second view is correct, because the saturated acids first formed would have a higher melting point and be relatively more stable than the unsaturated acids. Further, the saturated acids contain a greater store of potential energy than the unsaturated ones, and it does not seem clear why a substance stored up as a source of energy should be made first with a maximum store and then partially degraded to one with a lower potential energy by an oxidation process. That this process of "desaturation" takes place when stored fat is called upon to yield up its energy is almost certainly proved (see Chap. IX.), but it appears unlikely as a process which operates in the synthesis of unsaturated acids previous to their storage as neutral fat.

CHAPTER V.

THE DIGESTION AND ABSORPTION OF FATS.

FAT in varying amount forms part of the diet of most animals. This fat, according to its source, shows marked differences in chemical constitution, at any rate so far as the fatty acids it contains are concerned, as is indicated in the earlier chapters. Nevertheless, it is found that once the fat has been absorbed through the alimentary system, such differences are not associated with differences of availability in the organism. One reason for this is probably that the fatty acids, since they do not possess asymmetric carbon atoms, are not capable of existence in two or more optically active forms. Such optical isomerism is a potent factor in the body tending to modify the availability of substances differing only in this particular, *e.g.*, the optically active amino acids. The unsaturated acids, however, may exist in more than one modification due to stereo-isomerism of the *cis-trans* type, but it is not known whether this affects in any way their rates of change in bodily reactions. Such differences in the degree of assimilation of the naturally occurring fats or fatty acids as have been observed are to be ascribed chiefly to variations in their physical properties of which the melting point seems to be the chief. The enzymes which hydrolyse fats in the alimentary canal to produce fatty acids and glycerol do not exert any marked selective activity. All the fats appear to be hydrolysed readily if presented to the enzyme in the same physical condition. The discovery that fats undergo this hydrolysis in the alimentary canal was made in 1850 by Claude Bernard, who showed that pancreatic juice was responsible for the change. Since that time much work has been done on the digestion and absorption of fat in the alimentary canal, but even now there are parts of the process of absorption which are still obscure.

Digestion of Fats in the Stomach.—Up to the year 1900 it was believed that fats undergo practically no digestive changes in the stomach. In this year, however, Volhard (1900) pointed out that under certain conditions a considerable hydrolysis of fat could be observed. Using the stomach contents obtained after the usual form of test breakfast and employing a finely emulsified fat as substrate, Volhard obtained hydrolysis

to the extent of 30 per cent. in a few hours. The method used for detecting the fat splitting action was to digest the gastric contents with an emulsion of egg yolk. The digestion mixture was then dried and extracted with ether; a little alcohol was added to the extract and the free fatty acids estimated by titration with alcoholic potash. The solution was then saponified and the total fatty acids estimated. This method was simplified and improved by Stade (1903), who extracted the digestion mixture without previous drying. Many observers have repeated Volhard's experiments or made modifications of them, and in general his results have been confirmed; Stade (1903), Fromme (1906), Laqueur (1906), Heinsheimer (1906), and others. When Volhard's experiments became known, questions arose as to the origin of this fat splitting agent in the stomach and many have tried to answer them. The possibilities are that the hydrolysis is due to the hydrochloric acid of the gastric juice, or to a definite lipase secreted by the stomach, or to a reflux of duodenal contents, containing pancreatic lipase, into the stomach. That the action is not due to hydrochloric acid is proved by the absence of fat digestion when boiled gastric contents are incubated with egg yolk emulsion. Further, Volhard observed that there is little or no action if the gastric juice is strongly acid. Experiments which have been carried out with pure gastric juice [Volhard, Heinsheimer, Laqueur, Pekelharing (1902)] or with juice obtained from the stomach of dogs into which a reflux of duodenal contents could not take place [London (1907)], have shown that the degree of hydrolysis is much less than that obtained when the products of a test meal are used. These results suggest that the considerable extent of fat splitting observed originally by Volhard was due to a reflux of duodenal contents. That this is not the sole explanation and that gastric juice in reality does contain a fat splitting enzyme has been shown by Hull and Keeton (1917). These observers used ethyl butyrate as substrate and found that the fasting and acid free juice collected from dogs with gastric fistulas or "miniature stomachs" always contained an appreciable but small quantity of lipase. The enzyme is sensitive to free acid and a 15 minutes' exposure to 0·02 per cent. hydrochloric acid destroys it. Consequently its presence in acid gastric juice can only be detected if the juice be neutralised whilst it is being collected, or in the case of highly acid juice, by lowering the acidity by the introduction of protein into the stomach. When the juice is collected under conditions which cause no deterioration of the enzyme its activity on fat is well marked. For instance, in an experiment in which 1 c.c. of juice from a fasting dog was incubated with 20 c.c. of an egg yolk emulsion, 22 per cent. of the fat

had been hydrolysed at the end of 2 hours. A comparison of the relative amounts of lipase present in gastric juice and intestinal juice was made by Hull and Keeton who found 5 to 6 times as much in the former as in the latter. Other confirmatory evidence of the identity of gastric lipase is found in the optimum H – ion concentration for its action, [Davidsohn (1912)]. For gastric lipase this is 2×10^{-6} whereas for pancreatic lipase it is 1×10^{-8}. Further, sodium fluoride inhibits the action of gastric lipase less than that of pancreatic lipase. The enzyme has been detected in the gastric mucous membrane and stomach contents of the human fœtus from 6 months onwards (Ibrahim and Kopec, 1910), and in glycerine extracts of the mucosa from the stomach of pigs and dogs (Fromme). It appears to be absent from the pyloric region of the stomach.

Some importance must be attached to the presence of this enzyme in spite of the limitations to its activity which must occur under normal conditions in the stomach. Volhard has suggested that the well-known inhibition of hydrochloric acid secretion in the stomach by fat is an adaptation to secure some digestion of fat, especially in the case of milk, and so relieve the intestine of some of its work. That this may occur in suckling animals in which the fat intake is relatively large, and the fat is present in an emulsified form, is probably true, but these conditions do not occur in adult animals. Here, the fat in the food is not, as a rule, emulsified, nor does it become so in the stomach. Conditions are not present, therefore, even in the early period of gastric digestion before the acidity has reached its normal value, in which the gastric lipase will be able to exert any marked action. Thus Levites (1906) found that practically no fat digestion had taken place in dogs with a pyloric fistula when 100 grms. of beef fat, butter or lard were given by the mouth, and the gastric contents examined after expression through the fistulous opening. It is possible, however, that, in pathological conditions in which there is little or no hydrochloric acid secretion, fat digestion may occur and be of great value to the individual, since, in the absence of acid, one of the normal stimuli to pancreatic secretion will be lacking.

Digestion of Fats in the Small Intestine.—Although, under certain very limited conditions, hydrolysis of fat may take place in the stomach, the main digestion takes place normally in the small intestine. This is due, in the first place, to the fine emulsification of the fat which occurs, thereby enormously increasing the surface area of the fat globules exposed to the action of the digestive juices, and secondly, to the presence of fat splitting enzymes, especially that of the pancreas.

Emulsification is necessary because fat is insoluble in the intestinal chyme, and any action of enzymes upon it must take place at the surface of the fat droplets. It is, therefore, of the first importance that the fat be converted into a fine emulsion as a preliminary to digestion. This is brought about in all probability by the action of soaps, formed at the surface of the droplets by the combined action of lipase and the alkaline pancreatic and intestinal juices. The great lowering of surface tension which is produced by the soap film, together with the mechanical agitation of the contents of the intestine by movements of its muscular wall, result in the production of a very fine emulsion of the fat which thereby becomes more amenable to the action of lipase. Although bile possesses some power of emulsifying fat, and the emulsification which occurs in the small intestine is often attributed to it, there is no doubt that the action of soaps formed at the surface of the fat droplets, in the manner just indicated, is by far the more potent factor.

Two fat splitting enzymes are present in the intestinal contents, one being provided by the pancreatic juice and the other by the intestinal juice. It has been shown by Boldyreff (1907) that the secretion collected from a Thiry-Vella fistula in dogs will hydrolyse monobutyrin and also neutral fat, and that the action is assisted by the presence of bile. Further, Lombroso (1912), working with a Vella fistula, discovered that whereas the juice secreted during the normal processes of digestion possessed a slight but definite fat splitting action, that secreted in response to the introduction of olive oil into the fistula was much more powerful in this respect. This suggests that there is a mechanism for the provision of lipase in the intestinal contents by the secretion of intestinal juice whenever the presence of undigested fat indicates the necessity for such. The action of intestinal juice is, however, overshadowed by that of pancreatic juice, which exerts a very powerful lipolytic action. The discovery of this property was due to Claude Bernard (1850), who showed that a solution of butter in ether quickly acquired an acid reaction when submitted to the action of pancreatic juice. A year later, Berthelot (1851) established the nature of the reaction by isolating the fatty acids produced, and its general character, by demonstrating the action of the juice on monobutyrin. It is not without interest at the present day to note this fruitful result of the co-operation of the chemist and physiologist 70 years ago. Bernard's discovery was hotly contested by many who possessed neither his skill in technique nor his faculty for scrupulously careful observation, but it has held its own, and the presence of lipase in pancreatic juice is now a commonplace fact of physiology. More recent investigations have been

concerned with the factors which influence the action of lipase. Some of these are common to the action of all enzymes, and are discussed elsewhere,[1] but certain of them are of specific interest, and may be mentioned appropriately here. In examining the properties of pancreatic lipase, various preparations may be used. Pancreatic juice, collected by inserting a cannula into the duct, is the most satisfactory, but extracts of the gland, made under special conditions, also possess a marked lipolytic activity [Kanitz (1905), Mellanby and Woolley (1914)]. The action of the enzyme on fat emulsions is usually followed by titrating at suitable intervals the free fatty acids which are liberated. In order to do this conveniently, alcohol is added, until, with the water present, a solution containing at least 50 per cent. alcohol is obtained. Hydrolysis of the soaps formed on titration is thus minimised, and a sharp end point may be obtained with phenolphthalein as indicator. The enzyme is rapidly destroyed by free trypsin [Terroine (1908), Mellanby and Woolley (*loc. cit.*)], so that activation of the trypsinogen in the fresh pancreatic juice or extracts of the gland must be avoided. Egg albumin exerts a protective action, and this, no doubt, partially explains how fat digestion can take place in the small intestine even in the presence of free trypsin. Terroine (1910), and Mellanby and Woolley (*loc. cit.*) working with pancreatic juice, have determined the influence of many factors on the activity of the enzyme. It acts in neutral solution, but the activity rapidly diminishes on the acid side of the neutral point. Increase in alkalinity increases the activity up to a certain point, but it then falls off. According to Terroine, the maximum action is observed in N/150 NaOH. Electrolytes increase the action, and each salt examined by Terroine (sodium salts, KCl, $MgCl_2$, $BaCl_2$, $CaCl_2$), has its optimum concentration for this effect. Heating the enzyme for 10 minutes at 60° causes its destruction, and the addition of bile salts markedly diminishes its stability on heating. It is also rapidly destroyed by N/50 HCl. Rosenheim (1910), working with a glycerine extract of pancreas, was able by dilution with water and filtration to separate the enzyme into two parts, neither of which was active without the other. The precipitate obtained on dilution he believed to contain the enzyme itself, since it was not thermostable.

The filtrate from this precipitate restored activity to the enzyme, and this property was not destroyed by boiling. On this account, Rosenheim considers it to be a co-enzyme. This separation of the enzyme into two parts has, so far, not been accomplished with pancreatic juice, and Mellanby and Woolley suggest that it is only possible

[1] Bayliss, "The Nature of Enzyme Action."

with glycerine extracts because the enzyme is in coarse suspension after dilution, and can be filtered off, whereas filtration of pancreatic juice, under the same conditions, has no effect on its activity. It is possible that the thermostable substance of the glycerine extract increases the degree of dispersion of the enzyme precipitate, and hence its activity. Bile enhances the activity of pancreatic lipase, a discovery first made by Rachford (1891). Von Fürth and Schutz (1906) have demonstrated that, in this way, a fourteen fold increase in action may sometimes be observed, and that this property of bile is due to the bile salts which it contains. Sodium cholate also exhibits the same action, but further degradation products of cholic acid do not possess the property. This action of bile is not due to changes in the degree of emulsification of the substrate, but to some action on the enzyme itself, since it is observed when lipase acts on soluble esters [Loevenhart and Souder (1906-7), Terroine (1919)]. Experimental and clinical evidence on the effects of the absence of bile from the intestine do not suggest, however, that bile is essential for the complete hydrolysis of fat, since, in cases of obstruction of the common bile duct, the "fat" appearing in the fæces is usually present chiefly as free fatty acids or soaps, and not as glycerides. A much more important influence of bile is exercised in the absorption of fat, and will be discussed later.

A comparison of the rate of hydrolysis of a homologous series of glycerides has been made by Terroine (1919), which shows that the rate of hydrolysis increases as the series is ascended from triacetin to trilaurin, but thereafter it decreases. When examined under comparable conditions it was found that triolein was much more rapidly split than tristearin. Ethyl esters of fatty acids are hydrolysed by pancreatic lipase, and during the Great War studies of the comparative utilisation of such esters of the higher fatty acids and the triglycerides of the same acids were made in order to determine their suitability as substitutes for the latter in food (Müller and Murschauser, 1916). Glycol esters were also examined for the same reason by Franck (1918). As might be expected, since these esters are hydrolysed by pancreatic lipase, they are also assimilated to a considerable extent.

Attempts have been made to determine whether the amount of lipase secreted in the pancreatic juice varies with the diet. Such experiments are fenced about with many difficulties, and it is very improbable that with the technique available at present, a satisfactory answer can be given to these inquiries. Nevertheless, London and Krym (1911) have investigated the lipolytic activity of the chyme collected by means of fistulæ from dogs on different diets, and have found that it is greatest

with a pure flesh diet. On the other hand, Bompiani (1913), using pure pancreatic juice, has not been able to find any difference in its lipase content associated with differences in diet.

In the small intestine digestion and absorption proceed side by side, so that examination of its contents at various levels gives an indication of the rapidity with which these two processes take place. Experiments of this nature have been carried out by Levites (1906), using dogs with permanent fistulæ established at various points in the stomach and small intestine. After a meal consisting of 100 grms. of beef fat and no other food the data given in the following table were obtained. A series of dogs was used, each having one fistula, and out of this the chyme was passed when this situation was reached:—

Site of Fistula.	Absorption, Per Cent.	Per Cent. Free Fatty Acid in Recovered Fat.	[1]Reaction of Contents to Litmus.	Soaps, Grms.
Stomach	Nil.	Trace in 4-6 hours.	Acid.	—
½ cm. below pylorus	,,	,,	,,	—
End of duodenum	3	Just detectable.	,,	—
1 metre below pylorus	11	26	Neutral.	—
Middle of small intestine	36	38	Alkaline.	2·41
1 metre above cæcum	60	42	,,	2·87
End of ileum	96	67	,,	0·45

It will be noted from these results that there is a tendency for hydrolysis to proceed faster than absorption in the lower half of the small intestine.

The Absorption of Fat.—One of the oldest established facts of physiology is that fat is absorbed into the lymphatic vessels (lacteals) of the small intestine. Because of this mode of absorption, experimental inquiry into the processes involved has been rendered easy in so far that in animals the fat laden lymph may be collected from the thoracic duct and its properties and composition determined. In this way much qualitative information has been obtained regarding the phenomena of fat absorption, but so far the quantitative data are very meagre. This is due to the difficulty of establishing a permanent fistula of the thoracic duct in animals, and to the fact that fat absorption gradually ceases or becomes minimal in animals submitted to operative procedures under anæsthetics. Even when a permanent fistula is successfully established, it frequently becomes blocked by coagulation of the lymph or chyle and the flow from it ceases. Most of our knowledge of the processes of fat absorption we owe to Immanuel Munk.

[1] It is not stated by Levites whether the reaction was taken after exposure to the air and consequent loss of carbon dioxide. Such exposure would make the reaction more alkaline.

His work, which was begun in 1879 and carried on for a period of twenty years, served to establish the main facts regarding the passage of fats from the lumen of the intestine to the thoracic duct, and although these facts have been added to and extended by other observers, but little that is new and fundamentally important has been discovered.

Previous to Munk's work it was known that the lymph flowing from the thoracic duct during a fasting period was clear and contained very little fat (about 0·25 per cent.). On the other hand, if the lymph were collected during the digestion of a meal of which fat formed a part, then the lymph was milky and contained neutral fat in varying amounts up to 3 or 4 per cent. It was believed that the hydrolysis of fat proceeded only to a small extent in the small intestine, and that the fatty acids thus produced united with the alkali of the intestinal juices and bile to form soaps. The powerful emulsifying action of the soaps resulted in the rest of the fat being converted into a fine emulsion in which form it was taken up by the intestinal epithelium and passed on to the lacteals, whence it passed into the thoracic duct as chyle. Munk (1880) discovered that the free fatty acids, as well as fats, in the presence of protein would form a fine emulsion if a little soap were added; and thinking that the fatty acids might be absorbed in this form, proceeded to try the effect of administering free fatty acids alone to dogs and collecting the chyle from the thoracic duct. When this was done he was surprised to find that almost the whole of the fat in the chyle was not fatty acid, as he expected, but neutral fat. Only 5 to 10 per cent. of the fat was present as free fatty acid or soap. Thus the first proof was obtained of the resynthesis of fatty acids into neutral fat during absorption from the small intestine. It has been confirmed repeatedly, both in the same and other ways, and is now a well-established fact. The possibility that this synthesis might take place in the lymphatic glands which lie along the course of the lacteals was ruled out by Moore (1903), who showed that the fat of chyle collected from the lacteals before it had traversed any lymphatic glands consisted almost wholly of neutral fat. Moore also showed that the mucous membrane of the intestine at the height of fat absorption contained a mixture of fat and fatty acid, the former preponderating. This established the view that the resynthesis actually took place in the mucous membrane. In 1891 Munk and Rosenstein demonstrated that the synthesis also took place in man. They administered erucic acid to a patient with a lymph fistula from which chyle could be collected. In the chyle only the glyceride, erucin, was found. Munk

took these results to indicate that fat is completely hydrolysed in the small intestine prior to absorption, but part of the fatty acid so formed might be absorbed in a finely emulsified form into the epithelial cells of the villi. Here it would undergo resynthesis to neutral fat. The principal fact which influenced him in arriving at this interpretation was, that if the fatty acids produced by digestion have to be converted into soaps and absorbed as such, then so much alkali would be required for this purpose that the body could not provide it. For instance, a dog can easily absorb 200 grms. of fat in a day. To convert this fat into soaps 39·3 grms. of sodium carbonate would be required, but only one-sixth of this could be furnished by the whole blood. On this account, therefore, Munk believed that a considerable proportion of the fatty acids produced during digestion is absorbed without previous conversion into soaps, and that these fatty acids are taken up in particulate form by the cells of the villi. On the other hand, histological methods had failed to reveal the presence of stainable fat in the free borders of the epithelial cells, although during fat absorption it was easily revealed by fat staining reagents in the body of these cells. This observation was responsible for the alternative view that all fat must be absorbed in solution. Pflüger was a stout supporter of this, and published many papers in support of it during the years 1901 to 1903. His experiments, however, did not provide satisfactory evidence on the point in question (Kingsbury, 1917), and need not be further discussed. The fact that during fat absorption, staining methods fail to reveal the presence of fat in the epithelial cell border does not necessarily mean that fat is not present there. Ample evidence is available to prove that fat may be present in cells in considerable amount and yet be unstainable, possibly because of its high degree of dispersion in the protoplasm (Fischer and Hooker, 1916). In almost any cellular tissue, liver, for example, the usual fat staining methods may fail to show the presence of fat and yet chemical analysis will reveal a considerable amount. Similarly, a solution of oleic acid in egg albumin can be prepared which will not stain with osmic acid. Histological evidence does not help much therefore in a discussion as to the mode of fat absorption. Light on this problem has been sought in other ways. It has been thought that if a solvent for fats or fatty acids could be found in the intestine, then the supposed histological difficulty could be overcome, since, if the fats or their digestion products—the fatty acids—were capable of being brought into solution in the intestine, their passage into the cells of the mucosa would be understandable, and, in this condition, they would not be expected to give the staining reactions of particulate fat or fatty acid.

Old observations by Strecker, Marcet, Latschinoff and Kühne, showed that bile would dissolve fatty acids. Moore and Rockwood (1897) confirmed this, and later, Moore and Parker (1901) found that while solutions of bile salts alone dissolved fatty acids or their sodium, calcium and magnesium salts to a slight extent, if lecithine were present in small amount, then all of these substances were much more soluble. The presence of lecithine in normal bile thus magnifies its solvent action on fatty acids and soaps. Kingsbury (1917) has also shown that, in the presence of bile, the reaction between sodium bicarbonate and fatty acids proceeds much more rapidly than in its absence, and he believes that this makes possible a much greater formation of soaps during the normal periods of digestion than would otherwise be the case. This discovery of the solvent action of bile has therefore given rise to the modern view that fat is hydrolysed to fatty acids and glycerine in the intestine, and that the fatty acids are partly absorbed as soaps and partly as fatty acids dissolved in bile. Obstruction of the common bile duct, whether brought about experimentally or through disease, leads to deficient fat absorption, and supports the view of the importance of bile as a fatty acid solvent, for fatty acids and not neutral fats are chiefly present in the fæces in this disorder. Were it simply a digestive deficiency, neutral fat should be present. But it is not on this evidence alone, for it is insufficient, that the older view of the absorption of fat or fatty acids in a particulate state has been abandoned. The most important facts in support of the modern view have been got by observations on the fate during digestion and absorption of substances which are like fat either in chemical composition or physical properties. The general method of experiment has been to administer such substances by the mouth, and, during the ordinary periods of digestion, to collect chyle from the thoracic duct or otherwise in order to find whether the fat-like material given as food could be detected in it. The fæces have also been examined to determine the degree of absorption of the substance fed. The first experiments of this nature were done by Munk and Rosenstein (1891). To a patient with a lymph fistula, which discharged chyle during fat absorption, cetyl palmitate was given, and the chyle collected during the next 14 hours. On examination, the chyle was found to contain the glyceride tripalmitin, but no cetyl palmitate or cetyl alcohol. If absorption of the cetyl palmitate in particulate form had taken place, then it should have been present in the chyle unchanged. Its absence indicated that it could not be absorbed without previous hydrolysis in the intestine. Further experiments on the same patient with amyl

oleate and with very finely divided charcoal gave results pointing in the same direction. The charcoal was not absorbed, and the amyl oleate was absorbed as triolein.

Similarly, Franck (1898) showed with dogs that, although the ethyl esters of the higher fatty acids were digested and absorbed, only triglycerides appeared in the chyle, and Bloor (1911), using isomannide dilaurate, which is optically active, found no trace of optical activity when the fat of the chyle collected during its absorption was examined, thus proving that no unchanged isomannide ester had been absorbed. Argyris and Franck (1912) have also shown that monoglycerides are converted into triglycerides during absorption. The inference to be drawn from the results obtained with these very varied kinds of esters is that they can only be absorbed after hydrolysis and liberation of the fatty acids they contain, and that the fatty acids are either absorbed as such or after conversion into soaps. Perhaps the most conclusive evidence against the absorption of fat in a particulate form has been supplied by experiments with fat-like substances such as paraffin oil or lanoline, which are capable of conversion into very fine emulsions but which cannot be brought into solution in the intestine. Connstein (1899) fed 20 grms. of lanoline (m.p. 40° to 42°) to a dog, and found that it was almost all excreted in the fæces. Henriques and Hansen (1900) fed a mixture of equal parts of vaseline and lard to rats, a trace of fatty acid being added to cause emulsification. In spite of this, 95 per cent. of the vaseline was recovered from the fæces, and the greater part of the lard was absorbed. Since the fat was absorbed, it must have been dissolved out of the lipoid droplets as soap and absorbed in that way. Bloor (1913) has carried out a series of feeding experiments with mixtures of petroleum jelly or petroleum hydrocarbon oil with either olive oil or cocoanut oil. Examination of the fæces showed that 88 to 100 per cent. of the hydrocarbon given had not been absorbed. In other experiments with dogs in which the same mixtures were fed and the chyle collected from the thoracic duct, no hydrocarbon could be detected in the chyle. This was also the case when the mixtures were fed in an emulsified form. A repetition of Connstein's experiments with lanoline confirmed them, 95 to 100 per cent. of the lanoline being recovered from the fæces. The only positive results indicating the absorption of emulsified hydrocarbons are those of Bradley and Gasser (1911), who stated in a preliminary communication that, on giving to a dog a mixture of olive oil and a petroleum hydrocarbon oil in an emulsified form, the chyle collected during absorption contained triglycerides and petroleum in about the same proportion as in the

mixture fed. No further confirmation of this experiment has been published, so that it is safe to conclude that the absorption of fat or fat-like substances in particulate form has not been proven. Experiments with dyes which are soluble in fat or fatty acids, but not in soap solutions [Mendel and Daniels (1912)] have not thrown any further light on this problem of fat absorption.

It is of interest to inquire why fat must be hydrolysed prior to absorption, and then resynthesised again when it has passed into the epithelial cells of the mucosa. It is obvious that this would prevent the absorption of substances such as petroleum, which are like fats, but unsatisfactory for supplying the body's need of energy, and are, therefore, rejected. This may be the only reason, but it is possible that fat may undergo some modification during absorption, so that the fatty acids resulting from the hydrolysis of the fat in the food are recombined into glycerides, but not exactly in the same way, so that a fat different from that in the food results. There is some evidence that this takes place, and this may, therefore, be a second reason why hydrolysis must precede absorption. Arnschink (1890) noted that the fat in the fæces of dogs fed with mutton fat had a higher m.p. than the food fat, and Munk and Rosenstein (1891) observed that the chyle fat from their patient with a lymph fistula had a lower m.p. than the food fat when mutton fat was present in the food. These observers ascribed the change in the m.p. of the absorbed fat to rejection by the intestinal cells of the fatty acids with a high melting point. Franck (1898), in experiments with dogs, noted a change in m.p. of the chyle fatty acids compared with those of the fat administered. On giving ethyl palmitate the chyle fatty acids melted at 50·5° (palmitic acid, 63°) and had an iodine value of 32·6 corresponding to an oleic acid content of 36 per cent. The food fat contained no oleic acid, so that this must have been added during the resynthesis in the intestine, or have been normally present in fasting chyle. Now the average fat content of the chyle of fasting dogs is 0·25 per cent., so that it is possible to calculate whether the oleic acid found in the chyle fat by Franck represented that which might be expected to be normally present. The 27 c.c. of chyle obtained by Franck in the above experiment contained 0·372 grm. of fat, of which 0·134 grm. was oleic acid (calculated from the iodine value); 27 c.c. of fasting chyle would contain only 0·067 grm. fat. Assuming that the whole of this was triolein, it would not account for the amount of oleic acid present in the chyle in Franck's experiment. We are thus led to the conclusion that some, at least, of the oleic acid was added during the process of resynthesis in the intestine.

Bloor (1912, 1913, 1914) has observed similar changes on feeding isomannide dilaurate, olive oil, ethyl esters of stearic, palmitic and lauric acids and cod-liver oil. The change is not always in the same direction. The saturated fats in general give rise to a chyle fat with a lower melting point and higher iodine value, whereas the unsaturated fats give rise to a chyle fat with a higher melting point and a lower iodine value. For instance, on feeding with olive oil, in six out of seven experiments the chyle fatty acids melted at 27° to 32° (acids fed had m.p. 16°), and had an iodine value ranging from 71·7 to 86·8 (acids fed, 86·1), and on feeding with ethyl palmitate the melting point of the chyle fat was 55° and the iodine value 66·9 as compared with tripalmitin, which melts at 65° and has an iodine value of 0. The mechanism by which these changes are brought about is at present obscure. When fat containing several fatty acids such as oleic, palmitic and stearic acids is present in the food, it may well be that the poorer absorption of the higher saturated fatty acids accounts for the change in the chyle fat, but this explanation does not hold when the ester of a single fatty acid is given. A certain small amount of fatty acid is present in bile either in the form of soaps, neutral fat or lipines, and it may be the absorption of this along with the food fat which modifies the composition of the chyle fat. In one of Bloor's experiments with ethyl laurate 106 c.c. of chyle were collected in 6½ hours. It contained 1·82 grms. of fat with an iodine value of 44. The weight of oleic acid in the chyle fat (calculated from the iodine value), was thus 0·9 grm., whereas that in the same volume of fasting chyle would be 0·26 grm. The excess, 0·64 grm., might possibly have been present in the bile secreted during the 6½ hours, and if the fatty acids of the bile had a higher iodine value than that of oleic acid then less still would be required to account for the change in composition of the chyle fat in this experiment. It is also possible that desaturation of saturated acids, or reduction of unsaturated acids, occurs in the cells of the small intestine, but we have no experimental evidence for this. Whatever the mechanism which accounts for these changes, the evidence does not indicate that from the fats taken in the food an animal always synthesises a fat of definite composition which is distributed to the tissues. The results may, however, be interpreted as suggesting the presence of a process which, during absorption, tends to prevent the taking up of a fat widely different in composition from the average connective tissue fat of the animal. That fat, differing considerably from the normal connective tissue fat of an animal, may be laid down when large amounts of unusual oils or fats are administered is well

known (Lebedev and others, p. 164). In the ordinary diet of animals, modification of fat during absorption is most probably brought about by differences in the degree of absorption of the various fatty acids. This is easily understood from the results of experiments on the absorption of fatty acids of different melting point. It has already been pointed out that Arnschink and Munk noted the difficulty with which tristearin is absorbed, and the former made quantitative comparisons between different fats by estimating the fat in the fæces. Tristearin (m.p. 63°) was absorbed to the extent of 9 to 14 per cent. A mixture of tristearin and almond oil (m.p. 55°) to the extent of 89·6 per cent. and olive oil to the extent of 97·7 per cent. Levites (1907) has also carried out experiments in a dog with an ileocæcal fistula, in which the free fatty acids were administered under good conditions. They were made palatable—an important point in such experiments—by mixing with meal and baking into cakes. The following degrees of absorption were noted: stearic acid 35 per cent., palmitic acid 78 per cent., and oleic acid 98 per cent. Administration of the corresponding sodium salts resulted in much better absorption of the saturated acids; sodium stearate 87 per cent., sodium palmitate 90 per cent., and sodium oleate 100 per cent. Halliburton, Paton, and others (1919) have shown that in man, fatty acids mixed with ordinary fat are well assimilated, and in two experimental periods of 6 days each led to no digestive disturbance. The continued administration of fatty acids along with other food to rats for 2 months had also no deleterious action on either growth or reproduction.

Although experiments of this kind show how complete fat absorption usually is, it has, so far, not been possible to account for the whole of the fat absorbed, by examining the chyle during the period of absorption. No satisfactory experiments on animals have been recorded. Zawilski (1876) has fed dogs with large amounts of fat, and, at different times, after the meal was given, exposed the thoracic duct and collected chyle from it. By uniting the results composite curves have been constructed which show the hourly progress of fat absorption. It begins about the second hour, and shows two maxima: one about the fifth hour, and the other about the tenth or eleventh. After 24 hours, absorption has practically ceased. Attempts which have been made in animals to follow fat absorption during the whole of the absorptive period by collecting chyle from the thoracic duct have been ineffectual. The experiments fail because anæsthetics interfere with fat absorption, and, even when the animal is allowed to recover from the anæsthetic, the collecting cannula easily becomes occluded by

clotting of the chyle. For the same reason, absorption experiments in which isolated loops of gut are used are also unsatisfactory, because the rate of absorption does not nearly approach the normal [von Fürth and Schutz (1907)]. The only quantitative experiments in which the absorption of a meal containing fat has been followed almost to completion are those of Munk and Rosenstein (1891), on a patient with a lymph fistula. This patient, a girl of eighteen, suffered from an elephantiasis of the left leg, in which dilated lymphatic vessels were visible under the skin. In the upper third of the leg below the knee was a small fistulous opening which discharged clear lymph during fasting periods, but, after a meal containing fat, poured forth a milky fluid. This fluid, in appearance and properties, was undistinguishable from chyle. It was on this patient that Munk made most of his classical experiments on fat absorption. Although, on anatomical grounds, it is difficult to understand how the whole of the chyle from the lacteals could be collected from the fistula in the leg, other evidence obtained by Munk pointed strongly to this being the case. At the height of absorption of a meal containing a large portion of cream, the chyle from the fistula contained 3·85 per cent. of fat, and a sample of blood drawn at the same time from the patient contained only 0·169 per cent. of ether soluble material. On another occasion, when the fistula had closed temporarily, the blood during a fasting period contained only 0·171 per cent. of ether extractable material, whereas, when cream was again given, the fat content of the blood rose to 0·417 per cent. These results were taken by Munk to indicate that, when the fistula was open, the whole of the chyle could be collected from it, or, if not, so little was entering the blood that its fat content was not raised above the fasting value. When 41 grms. of "Lipanin" (olive oil containing 6 per cent. of free oleic acid) was administered to the patient, 1134 grms. of chyle containing 25·1 grms. of fat were collected from the fistula during the next 13 hours. Fat absorption was still proceeding when the collection was stopped, so that this experiment showed that at least 60 per cent. of the fat administered had passed into the lacteals. Munk assumed that possibly 2 grms. more fat would have been collected by continuing the experiment till fat absorption ceased, in which case the fat passed into the lymph stream would be 66 per cent. of that taken in the food. Olive oil is very well absorbed, so there is no reason to doubt its almost complete absorption from the intestine in this case. On other occasions other natural fats were administered, but no figure higher than that given by olive oil was reached. It has been concluded from these results that about two-thirds

of the fat absorbed eventually enters the blood stream by way of the thoracic duct, and that the other third is probably absorbed directly into the blood capillaries of the small intestine. The validity of this conclusion is still uncertain because of the lack of absolute evidence that the whole of the chyle was being collected, and that all the fat was absorbed, in Munk's experiments. It has been shown, however, that ligature of the lacteals (Hamburger, 1900), or the thoracic duct (Munk and Friedenthal, 1901), does not prevent but diminishes fat absorption, so that, under these conditions, the path through the lacteals being closed, the absorbed fat must enter the blood directly. Bloor, however, found no increase in the fat in the blood after a meal containing fat, when the thoracic duct had been tied (but see Chap. VII., p. 154). There is no serious objection, therefore, to the assumption that, under normal conditions, a part of the absorbed fat may be passed directly into the blood stream.

The question as to the form in which fatty acids are taken up by the villi of the small intestine is still a vexed one. The evidence against the absorption of neutral fat in a particulate form has already been given. Of modern workers, Croner (1912) is the only one who seriously adheres to the idea of particulate absorption, but opinion is divided as to whether all the fatty acid must be converted into soap before absorption, or whether free fatty acids can be absorbed without such change. It must be remembered that soaps or free fatty acids are both well absorbed, the former better than the latter (Levites, *supra*, p. 134), but those who believe that only soaps can get into the epithelial cells suggest that the alkali of the intestinal juice converts the fatty acids into soaps which are then absorbed, giving rise to neutral fat and so freeing the alkali for the conversion of a further quantity of fatty acid into soap in the lumen of the bowel. There is no satisfactory evidence for or against this view. The reaction of the intestinal contents, determined with the hydrogen electrode with precautions to exclude loss of carbon dioxide (McClendon, Shedlov, and Karpman, 1918), has been found in dogs to lie between pH 5·6 and 6·6 in the ileum, and in the duodenum it is also on the acid side of the neutral point. This is likewise true for duodenal contents in man collected by means of the duodenal tube (McClendon, 1921). These observations do not negative the possible occurrence of soaps in the intestine, for, with alkaline secretions being poured into the intestine from the intestinal glands, local alkaline reactions may occur, especially near the villi where absorption proceeds. It must also be remembered that a fatty acid and its soap, especially in the presence of bile, forms a buffer system in

which soaps may exist, although the reaction may be on the acid side of neutrality. The fact that the absence of bile seriously interferes with fat absorption, coupled with the observation that, under such conditions, the fæces contain large amounts of free fatty acids, suggests that the solvent power of bile for fatty acids is of considerable importance for their absorption, and renders it extremely probable that a part, at any-rate, of the hydrolysed fat is absorbed as fatty acids dissolved in bile. Or it may be that bile so alters the permeability of the cells of the villi that fatty acids in a finely dispersed condition can pass into them. That changes in the diffusibility of fats and fatty acids through collodion membranes may be brought about by bile has recently been shown by Neill (1921).

Investigations of fat absorption on animals with intestinal fistulæ have shown that, when the ileo-colic sphincter is reached, absorption is practically complete. They have also furnished no evidence that fat absorption takes place in the stomach. Nevertheless, observations have been recorded which have been taken to indicate that both the stomach and large intestine possess the power of absorbing fat. Greene and Skaer (1914) have described granules in the gastric epithelium which stain like fat. They are present in new-born cats and dogs and in full grown animals, appearing in the latter particularly after food rich in fat. Their presence has been interpreted as indicating that fat absorption may take place in the stomach. On the other hand, Mendel and Baumann (1915), although confirming Greene and Skaer's results, have been unable to find any increase of the fat content of the circulating fluids from the stomach when fat is present in it. Quantitatively, therefore, this phenomenon is of no importance in fat assimilation. In the case of the large intestine, Hamburger (1900) has described experiments which indicate that here, fat absorption may be as vigorous as in the small intestine, and that soaps also are absorbed. This appears very remarkable in comparison with the meagre absorption of other foodstuffs from this part of the alimentary tract, and would merit confirmation by other methods.

The use of the microscope for the investigation of the chemical processes of fat absorption has confirmed in general the results obtained by purely chemical methods. In particular, the work of Noll (1910) and Lamb (1910, 1917) in this field may be mentioned. Noll has compared the microscopical appearances of the intestinal mucosa observed during fat absorption with the results of chemical analysis. Rabbits were used, and at various intervals after the administration of 20 c.c. of olive oil, the animals were killed and examined. A latent period

was observed, during which fat accumulated in the epithelial cells of the villi before it began to be passed into the chyle. The fat thus stored temporarily in the cells was principally neutral fat, and contained only a small amount of free oleic acid. It stained more freely with Flemming's solution (osmic, chromic and acetic acids) than the fat of the chyle, but Noll does not deduce from this that any difference in composition exists between the two. Such differences in staining power may be due to the different degrees of dispersion of the fat in the two situations. He suggests that the temporary storage of the fat in the cells indicates that some change takes place in it before it is passed on to the chyle, but his results do not suffice to indicate what this change is. Similar conclusions have been reached by Lamb, who used a different micro-chemical method. Lamb's work was carried out chiefly on frogs, and the chromation method of Lorrain Smith and Mair (1909-10) was used for staining. By this method only unsaturated fats and fatty acids are stained. Lamb finds that saturated fats or fatty acids, which are unstainable before absorption, stain distinctly when they have been taken up by the epithelial cells and appear as droplets. This, he thinks, may be due to the absorbed substances becoming associated with unsaturated substances already present in the cell, *e.g.*, lipines or cholesterol, or to a desaturation of the saturated fats which have been absorbed. Staining reactions of unsaturated fats or fatty acids were also found to be changed after absorption. These changes were most marked with fatty acids and consisted of a considerable acceleration of the chromation process, so that oleic acid, for example, after absorption stained well after 24 to 48 hours' chromation, whereas outside the cell it did not reach the stainable stage until it had been subjected to the chromation process for 12 days. Lamb, therefore, suggests that the absorbed fatty acid undergoes some change in the cell which facilitates its oxidation by the bichromate solution used for chromation, and that this change is not simply a conversion into triolein. An association with cholesterol is the most probable explanation of the change, since mixtures containing cholesterol are easily stained by the chromation method, and also cholesterol is able to antagonise the irritating effects of free fatty acids [Lamb (1910, 2)].

Disturbances of Fat Digestion and Absorption in Disease.—The failure of the normal process of fat absorption is rendered evident by an increase in the excretion of fat, fatty acids and soaps in the fæces. This change may be due simply to incomplete absorption resulting from diarrhœa, in which case the contents of the bowel pass along too quickly for the normal processes of digestion and absorption to be

exercised completely. If this cause be excluded, then so-called fatty stools are usually due to disturbance in the secretion of pancreatic juice or bile. Experiments on animals and the effects of obstruction to the flow of bile resulting from pathological conditions in man, are in complete agreement in indicating the type of disturbance of assimilation which results from this disorder [Schmidt (1906)]. In general, there is a marked loss of fat in the fæces, the amounts varying from 22 to 78 per cent. of the intake. Schmidt quotes two cases of jaundice with complete obstruction of the common bile duct, in which 83·4 grms. of fat were given daily. One excreted 22·8 to 27·2 per cent., and the other 27·2 per cent. of the fat given, in the fæces. A series of cases examined by Müller (1887), in which the fat intake varied, showed losses of 31 to 78 per cent. The percentage loss varies with the fat intake and with the kind of fat in the food. It is usually greater when the intake is low but the differences are not very great. In normal persons about two-thirds of the fat in the fæces is present as free fatty acids or their soaps, and this proportion is also found in cases of complete stoppage of the bile flow. These results are usually interpreted as indicating that the absence of bile interferes with the absorption of fat and not its digestion. If, however, the absolute values and not percentages are considered, then the amount of unsplit fat in the fæces is considerably increased. Thus, using Schmidt's figures and calculating the amount of neutral fat excreted by normal persons and those with jaundice, the former are found to excrete 1·7 grms. and the latter 7·2 grms. In some of the cases described by Müller the loss is still greater. Absence of bile does, therefore, interfere to some extent with the digestion of fat. This might be expected from what is known of the furtherance of the action of pancreatic lipase by bile.

Arrest of the secretion of pancreatic juice also leads to the passage of fatty stools, but there is not that complete agreement between the evidence obtained by animal experiment and that from a study of disease in man which is met with in the case of biliary obstruction. This is undoubtedly due, in the first place, to the difficulty during life of diagnosing pancreatic disease with certainty, and in the second, to the fact that, when the diagnosis is correctly made, there is always considerable doubt whether there is absolute cessation of pancreatic secretion or merely a diminution in its amount. Experiments on animals in which the pancreatic ducts were ligatured, followed by either complete or partial removal of the gland, or grafting, have always been consistent in showing that the assimilation of fat was markedly interfered with. Simple ligature of the ducts, or better, grafting of the

gland so that the secretion flows on to the surface of the body and not into the bowel, does not lead to such severe loss of fat in the fæces as complete removal of the gland [Lombroso (1904), Fleckseder (1909), Visentini (1911), Jansen (1911)]. In the former case, there is a loss of 60 to 80 per cent. of fat in the fæces, but, in the latter, it is usually greater. McClure, Vincent and Pratt (1916), however, have not been able to confirm this. In animals from which the gland has been completely removed, the administration of pancreas with the food diminishes the severity of the fat loss [Cruickshank (1915)]. These findings are in agreement with other experience of the general nutritive condition of depancreatised animals. The general disturbance of metabolism is more severe than in animals in which grafting of the gland is performed, so that its functions in carbohydrate metabolism may still be exercised. The results of pancreatic disease in man are very variable [Schmidt (1906)], for whilst in some cases, in which the gland has undergone atrophy or its ducts have been occluded, little or no change in the fat content of the fæces has been found, in other cases, a marked loss of fat (20 to 80 per cent.) has occurred. In those cases where fatty stools have been encountered, great variations in the extent of hydrolysis have been found. The amount of neutral fat recorded by different observers has varied from 20 to 90 per cent. of that excreted. To some extent this extreme variation is referable to the nature of the fat given in the food. A natural emulsion such as milk is much better digested than artificially emulsified or unemulsified fat [Abelmann (1890)]. This, in turn, suggests that the hydrolytic agents in cases of complete pancreatic obstruction are gastric lipase and intestinal lipase, which, under suitable conditions, may bring about a considerable splitting of fat [Brugsch (1906)]. It is certain that the experimental evidence at present available does not justify the diagnosis of pancreatic disease by the fat content of the stools alone, for it may be normal with extreme degrees of the disease. It is more probable, as Brugsch has suggested, that a diagnosis of pancreatic disease may be made if the stools are pigmented, and contain an abnormal amount of fat (diarrhœa being excluded), and that pancreatic disease accompanying biliary obstruction may be diagnosed with probability if the fat loss in the fæces is above 50 per cent. In Brugsch's experience, a combined absence of pancreatic juice and bile caused a loss of 80 to 90 per cent. of fat in the fæces.

In the condition known as infantile atrophy or marasmus, a disturbance of fat digestion or absorption has frequently been described. The disease is associated with the passage of soapy stools, and improvement

often follows a lowering of the fat content of the diet. Recent investigations by Hutchinson (1920) of infants suffering from this disorder have shown that the percentage of fat in the fæces present as neutral fat was 8·1 as opposed to 4·8, the value found in healthy infants. The condition is not, therefore, one in which there is a seriously deficient hydrolysis of fat. The degree of absorption of fat in this condition has been found by many observers to vary between wide limits. Hutchinson ascribes this to two causes; firstly, no notice has been taken of the dry weight of the fæces, and secondly, the influence of the fat intake on the output of fat in the stools has not been examined with sufficient care. Both these factors have been shown by him to be very important, for, in a series of investigations on healthy and atrophic infants it was found that the fat output ran parallel to the dry weight of the fæces, and that within wide limits both appeared to be independent of the fat intake. In determining whether fat absorption is defective, it is therefore necessary to take note of this fact. Since in health approximately one-third of the dry fæces consists of fat, Hutchinson has taken this into account in determining the true fat absorption in the series of cases he investigated. When this was done, it was found that the fat absorption was practically normal. It thus appears that the defective absorption described by others has been due to their neglecting to take into account the low fat intake. The approximately constant percentage of fat in the fæces is not peculiar to infants. Hutchinson states that in three adults the percentage was about 30, and in a dog, on widely different diets, the figures 21·8, 17·7, 16·1, 16·9, 19·0 and 14·0 (fat free diet) were found. Drummond (1919) has observed the same phenomenon in rats, but the percentage in these animals was about 6. These observations suggest that the fæcal fat has a function, but it is also possible that fat absorption may take place in the large intestine (Hamburger, 1900), and may vary in extent inversely as the bulk of the fæces. In this case, the fat output would increase or decrease in parallel with the amount of the fæces, and might lead to an approximately constant percentage of fat being present in them.

Another pathological manifestation of fat assimilation has been described by Williams (1907) and others. This consists in the formation in the intestine of definite calculi containing insoluble calcium soaps of the higher saturated fatty acids, along with free fatty acids and calcium phosphate. These concretions are met with in the form of "intestinal sand," calculi in the vermiform appendix, or enteroliths large enough to cause intestinal obstruction. "Intestinal sand" is a fine gritty material like sand in appearance, which separates out from the fæces

when they are disintegrated with water. It has been found in greatest amount in abdominal cases associated with colicky pains, and may have been a factor in the production of the latter. The appendical calculi examined by Williams contained only calcium soaps and free fatty acids, calcium phosphate being practically absent. This was also the case in the large calculi met with in cases of intestinal obstruction. The fatty acids they contain are mostly saturated, and some cholesterol is present. They are unlike the calculi which are sometimes associated with the taking of large amounts of olive oil as a supposed cure for gall stones. These consist essentially of calcium oleate.

CHAPTER VI.

THE FAT IN THE BLOOD DURING ABSORPTION OF FAT.

FAT absorbed from the intestine by lacteals is conveyed by the thoracic duct to veins at the base of the neck, and, therefore, like that portion whatever it may amount to which enters the blood capillaries directly, is conveyed by the blood to the various organs. The difference between the two routes means that fat absorbed by the blood will go through the liver before it reaches other tissues, and that which goes by the thoracic duct will be distributed equally to all parts. The readiness with which the liver in certain circumstances takes up fat may make the lacteal route advantageous when fat is to be laid by in adipose tissue.

The result of absorption of fat must be traced, therefore, by examination of the blood. It has been frequently observed that the serum obtained from the blood of animals recently fed on food containing fat is milky in appearance, and in extreme cases fat may on standing separate as a creamy layer from the serum. Neisser and Braüning (1907) found that human serum was almost always milky 3 to 5 hours after taking 100 grms. of fat; the milkiness was greatest about the sixth hour, and after 12 hours the serum had become clear.

On microscopic examination of human blood, Neumann (1907) found that ultramicroscopical particles were to be seen 2 hours after a meal containing fat, but not after a meal that was free from fat; nor were such particles to be found if the sample of blood were taken before breakfast. Kreidl and Oshima (1907) found ultramicroscopic particles too in the fœtal blood in the guinea-pig, but not till after the fifth week, and then in increasing amounts up to the ninth week. If these particles are composed of fat, it is significant that Imrie and Graham (1920) found that the liver of fœtal guinea-pigs during the later stages of gestation contains increasing and finally very large amounts of fat of high iodine value.

Lattes (1911) has collected data obtained by earlier workers, who

used various more or less unsatisfactory methods. These show an extraordinary variation: in man, for instance, the blood has been found to contain in physiological conditions anything from 0·2 up to 2·0 per cent.; in the dog from 0·1 up to 2 per cent. Lattes himself used the excellent method of Kumagawa and Suto, which gives the weight of fatty acids, from glycerides and other combinations in which they may occur, together with cholesterol or other substances soluble in petroleum ether. His figures for venous blood from eight dogs that had had food containing but little fat for some days and no food for 20 hours before the blood was collected ranged from 0·36 to 0·43 per cent.; for blood from ten dogs fed on milk or other food containing plenty of fat the percentage ranged from 0·46 to 0·77, the blood being collected generally 6 or 7 hours after a meal. These results seem to show clearly enough that the absorption of fat leads to an increase in the amount contained in the blood.

Terroine (1914), however, points out that it is not always safe to argue from comparison of the amount of fat in the blood of one animal in one condition with that found in another animal in another condition, as if the differences found were necessarily due to the difference in the conditions. Any individual dog according to his observations will have very similar amounts of fat in the blood, provided the conditions remain similar, but very different amounts from those found in other animals in those conditions. The variations in the amount of fat found in the blood of any one dog on different occasions are much smaller than those in the amount of fat found in all the dogs of his series compared with one another. So that he speaks of a "lipæmic index" for each individual animal, adhered to in that animal within a variation either way of 15 per cent., whereas the figures obtained from different animals differ more than 50 per cent. on either side of the mean. He, therefore, makes the proof that feeding with fat increases the fat in the blood certain by determining the amount in the same animal before and after a meal containing fat. The increase when moderate amounts of fat are given may be more than 30 per cent. in the sixth hour. But the animal's "lipæmic index" is restored about 4 hours later by the reduction of the amount of fat in the blood to its normal value.

Terroine employed for his determinations the improvement of Kumagawa and Suto's method, which Shimidzu showed to be necessary for blood: the fresh blood being precipitated with alcohol, the precipitate extracted with boiling alcohol and the extract saponified and dealt with as by Kumagawa.

At the same time Terroine also estimated separately the cholesterol in the petroleum ether extract, using the digitonin method of Windaus, so that by deducting the weight of cholesterol from that of the whole petrol extract the weight of the higher fatty acids free from any considerable amount of impurity was obtained. In this way he found that the cholesterol in the blood also increased during the absorption of fat, and in such a way as to keep the ratio of it to the fatty acids practically constant so long as the amount of fat absorbed was not excessive. This ratio, the "lipæmic coefficient," is different in different dogs like the "lipæmic index," but in any one individual dog it varies very little; he gives figures obtained on several occasions from each of four dogs that show a mean variation of 5 or less than 5 per cent. in each animal. Its average value is about one-third, often it is one-fourth, in one dog it was as high as one-half.

Bloor (1914) worked out a nephelometric method for estimating fat (*supra*, p. 62), which makes it possible to work with small amounts of blood and to carry out more estimations. He has found the mixed fatty acids and cholesterol to be doubled somewhere between the fourth and seventh hour after feeding dogs with fat. In one animal the thoracic duct was tied, and no increase in the fat in the blood could then be detected.

He has since developed methods for estimating cholesterol and phospholipines in the same alcohol ether extract of the blood as is used for the estimation of fatty acids and cholesterol together; the cholesterol colorimetrically, the phospholipines by the application to the alcohol ether extract of the blood of Neumann's method for liberating phosphorus from organic compounds and a nephelometric method for estimating phosphorus.

Using these methods on the whole blood (1915) in eleven experiments on dogs, all of which showed definite and some of them large increases in the fatty acids of the blood during the absorption of fat, the variations indicated in cholesterol were small and irregular, sometimes barely appreciable, so that the constancy of the "lipæmic coefficient," found by the gravimetric methods used by Terroine, was not confirmed. (Compare, too, the results obtained by Hiller, Linder, Lundsgaard and van Slyke, 1924.) But the phosphorus present in phospholipines showed an increase which, in a general way, though not exactly, ran parallel with the increase in fatty acids. Separate analyses of the plasma (1916) and of the whole blood, from which the plasma was obtained by a centrifugal separator with graduated tubes, showed that it was not in the plasma, and therefore was in the corpuscles that most of

this new phospholipine occurred. In these experiments again, no definite changes in the quantity of cholesterol were noted to occur regularly in either plasma or corpuscles.

It will be easier to form an idea of what these experiments teach with regard to the absorption of fat by taking an instance from Bloor's experiments, and calculating from the total volume of blood (70 c.c. per kgrm. of the dog's body weight) how much fat could be found in the blood in each form. Thus, in experiment 1, a dog was given 50 c.c. of olive oil, and, after 4 hours, the blood contained 2·5 grms. more fatty acids than at the outset; of this amount 0·7 grm. was accounted for as "lecithine," and 0·5 grm. in other forms in the corpuscles, 0·1 grm. as "lecithine" and 1·2 grms. in other forms in the plasma. In the later experiments of the series, the increase in lecithine was not nearly so pronounced. But Bloor believes the figures furnish "conclusive evidence that the blood cells absorb the fat from the plasma and transform it into lecithine." And he adds that "the results justify the conclusion that most, if not all, of the absorbed fat is so transformed" into lecithine by the blood corpuscles, that is to say. It should be noted, in support of these conclusions, that 8 hours after the fat was given, in all but one of the seven experiments, the amount of "lecithine" in the corpuscles had declined, and was apparently approaching if it had not yet reached, as in one case it had, the figure found before feeding. It is difficult to suppose that the red blood corpuscles had disposed of the additional lecithine that appeared in them by oxidation, and it seems as if they must have given it up to the plasma, and this to the tissues supplied by the blood. But, if this is what happens to the additional lecithine in the corpuscles, there is nothing whatever to show that the same does not happen with the other compounds of fatty acid, in plasma as well as corpuscles, which were double the amount of that inferred to be in the form of lecithine, and nothing to prove conclusively that these other compounds were merely waiting their turn for being converted into lecithine in the corpuscles. Moreover, the deposition of simple glycerides of the fatty acids in adipose tissue, and, in certain conditions, their appearance in the liver in larger or smaller amounts, as well as in other organs, makes it difficult to see how "most or all" of the absorbed fat can be, first of all, converted into lecithine in the blood. This, however, is merely a matter of perspective; the fact that absorption of fat leads to a considerable increase of lecithine in the blood corpuscles is of great interest and importance. If lecithine is to be formed in the blood when fat is absorbed, the amount of phosphoric

acid found in the plasma, and not already in some phospholipine form, is known to be so small, 10 mgrms. in 100 c.c., and that in the corpuscles is so much greater in amount, nearly twenty times (*cf.* Bloor, 1918), that a more extensive formation in the corpuscles than the plasma must be expected, unless the phosphoric acid could be given up by the corpuscles more easily than it appears to be. If, however, the whole of the fatty acids absorbed in the course of this experiment, say 40 grms. from the 50 c.c. of oil, were converted into lecithine in the corpuscles, since 564 grms. of oleic acid require 98 grms. of phosphoric acid for conversion into lecithine, 40 grms. would require about 7 grms.; and the whole blood of this dog, about 560 c.c., to judge from Bloor's valuable figures for the different forms of phosphoric acid in human blood (1918) could not contain much more than a gram of phosphoric acid not already in phospholipine form. A liberal supply of phosphoric acid would have to be available for the corpuscles from some outside source. It would be interesting to know how the amount of phosphoric acid not in phospholipine form, found in the corpuscles at the end of the 10 or 12 hours that the fat absorption takes to be completed, compares with the amount before it began. Bloor's methods should be able to decide whether the corpuscles, in making lecithine from absorbed fat, use up their own stock of inorganic phosphorus, or whether there is some other fund on which they can draw outside the blood stream altogether; it would seem that there must be, for, even for the amount of lecithine which Bloor's figures prove to have been formed, which corresponds to about one-third of the total fatty acids absorbed by and still present in the blood, the amount of phosphoric acid required would be considerable. The average increase of fatty acids at the fourth hour in his seven dogs was in the whole blood, 0·276 grm. in 100 c.c., and that of lecithine 0·118 grm.; this amount of lecithine would contain 30 per cent. of the fatty acids that the blood had taken up and still contained. If this proportion is typical throughout the whole duration of the absorption, much more phosphoric acid would be required than the amount not already in the form of phospholipine, which the blood actually contains. Eckstein (1922) examined the fat of the lymph in dogs during absorption of fat and found that while the total fat in the lymph was increased eight fold, that in the form of phospholipines might be increased only 25 per cent. The fat taken up by the lacteals therefore is not converted into phospholipines.

Bloor and Knudson (1917) further estimated in the blood cholesterol and its esters separately, and Knudson (1917) traced the changes in the

amounts of these compounds at the same time as those in fatty acids and phospholipine phosphorus in both blood and plasma obtained from dogs during the absorption of fat. The results obtained, while they again showed no constant change in the total quantity of cholesterol, its amount sometimes rising and sometimes falling, in either case less than 5 per cent. on the amount for the whole blood, showed that the amount of cholesterol present as esters was considerably increased, particularly in the corpuscles, in which indeed they question whether at other times there is any such combined cholesterol at all. Instead, therefore, of a constant relation between fatty acids and total cholesterol such as Terroine found during fat absorption implying that fresh cholesterol enters the blood together with and in proportion to the amount of fresh fat entering it, this result implies that some of the cholesterol already in the blood combines with the fatty acids as they or their glycerides gain the blood, and the product is found in greater quantity in the blood corpuscles than in the plasma; at the same time another portion of the absorbed fat combines, as we have seen Bloor found before, with phosphoric acid, and the product of this combination also is found particularly in the blood corpuscles: in some of the experiments the amount in the corpuscles of both these combinations of fatty acids has by the eighth hour already returned to the value it had before the fat was given, so as to suggest that in all cases if the period of observation had been somewhat longer the original values would have been obtained. The inference might then be drawn, as in the case of the additional lecithine, that the additional cholesteride found in corpuscles during fat absorption is passed on from the blood to some of the tissue elements that it supplies, ready for another and later stage in the series of uses which fats have to serve: but if so how is the total cholesterol of the blood kept nearly constant throughout the process of absorption of fat as Bloor and Knudson think it is? Their view seems to imply either that cholesterol is absorbed into the blood only, when, and in proportion, as cholesteride is taken up from it by the tissues, or that the cholesteride increase has no physiological significance and is merely the necessary but temporary result of the increase of fatty acids in the blood; as this passes away the cholesterol becoming free again and returning to the plasma. Such facts taken as "evidence that cholesterol esters are one of the intermediate stages of fat metabolism" do not lead far.

The increase in cholesteride in the whole blood amounts to about 10 per cent. of the cholesterol, only in one case as much as 15 per cent.

Here again, it may be easier to appreciate the condition of the fat that is absorbed into the blood if figures calculated from some of the experiments described are presented so as to give the actual amount of fat in each of the different forms found in the blood.

In experiments 3 and 5 of the series where cholesterol and its esters were determined separately (Knudson, 1917), the dogs were given 65 and 60 c.c. of olive oil respectively.

In experiment 3 in the whole of the dog's blood (70 c.c. per kgrm.), just before it was fed the fatty acids were distributed as shown in column 1; at the fourth hour these several amounts were increased by the amounts given in column 2—

AMOUNT AND DISTRIBUTION OF FATTY ACIDS.

	Before Feeding.	Increase, 4 hours later.
	Grams.	Grams.
In the corpuscles—		
as "lecithine"	0·645	0·375
as cholesteride	0·024	0·076
in other forms (glycerides, soaps, etc.)	2·221	2·582
In the plasma—		
as "lecithine"	0·712	0·341
as cholesteride	0·243	0·018
in other forms	1·071	0·419

and in experiment 5 of the same series—

	Before Feeding.	Increase, 4 hours later.
	Grams.	Grams.
In the corpuscles—		
as "lecithine"	0·740	0·204
as cholesteride	0·012	0·085
in other forms	1·598	1·071
In the plasma—		
as "lecithine"	0·601	0·046
as cholesteride	0·216	0·027
in other forms	1·013	0·746

With these figures set out in this way it is easier to estimate the force of the inferences drawn in these papers, and at the same time to keep the several facts in due perspective.

In addition to the points already referred to another is brought forward, the constancy of the relation between the amounts of fatty acid and lecithine (Bloor, 1916), and the constancy of the relation between fatty acids and cholesterol esters, and of that between lecithine and cholesterol esters (Knudson, 1917). Such a constant relation between

fatty acids and lecithine was observed before by Mayer and Schaeffer in the corpuscles as part of a systematic study of the quantitative relations in which the different common components of all kinds of cells may be found in the different kinds. A study of such a wide subject as this takes in much more than what we are here immediately concerned with. They obtained evidence, as is well known, of many such constant relations. For the purpose before us, that of tracing the metabolic history of fat in the blood, a constant relation between fatty acids and lecithine might serve as the foundation for important inferences. But the relationship found by Bloor is described by him only as "fairly constant," and a glance at the tabulated figures for the ratio (*loc. cit.*, p. 458) will show what this means: in one case the ratio of fatty acids to lecithine was 1·4 to start with, fell to 1·1 and rose to 1·6 in the corpuscles, a variation of 45 per cent.; and in the whole blood this ratio was at one time 1·5, later 2·3, having been originally 1·8, a range of variation of more than 50 per cent. on the lowest value. This is the most extreme case; variations of 25 per cent. are common, and if the average increase of fatty acids at the fourth hour in all seven experiments be calculated and compared with the average increase of lecithine at the same time in the corpuscles, the figures will be found to be 56 per cent. for the former, 79 per cent. for the latter. This does not seem to be close enough to allow of any very far-reaching inference. And so with the cholesterol esters (Knudson, 1917): the ratio of lecithine to these esters was to start with, in experiment 1, 5·1, and in experiment 4, 5·4; the absorption of fat caused this ratio to rise to 6·4 in the first case and to fall to 4·1 in the other. The constancy of the relations can hardly be pressed to carry any important interpretation.

To sum up then the data with regard to the condition of the fat as it passes through the blood stream from the intestine to the tissues in the absorption of a meal containing it. (1) The amount of fatty acids in different combinations temporarily increases in plasma and corpuscles, rather more perhaps in the latter than in the former. (2) The amount of cholesterol tends to increase up to a certain point but not beyond it, depending perhaps upon the amount available in the intestine from food or bile; the association of cholesterol with the fat appears thus to be rather accidental than essential to the process either of absorption, or of transfer from blood to tissues. (3) The amount of phospholipine increases, mainly in the corpuscles; the newly-formed phospholipine appears to be made from the absorbed fatty acids, the

source of the phosphoric acid and the basic constituents being obscure; it may be reasonably supposed to leave the blood for use in the tissues since the amount in the blood gradually returns to its previous value as the absorption of fat ceases. How much of the absorbed fatty acid is thus dealt with there is no means of saying; the amount may be quite small. (4) The amount of cholesteride in the blood increases, involving some 10 per cent. of the whole amount of cholesterol: the newly-formed ester appears mainly in the corpuscles: whether it is passed on to the tissues as cholesteride, or is merely a temporary combination subsequently reconverted by hydrolysis into free cholesterol and fatty acid, the data cannot decide.

Some experiments have recently been carried out at the Rockefeller Hospital (Hiller, Linder, Lundsgaard, and Van Slyke, 1924) on human subjects, in which the blood was examined before, and 3 and 5 hours after, taking about 60 grms. of butter. In 6 out of 8 experiments done on six normal subjects, the total fat in the plasma rose more or less definitely; in one, it was unchanged; in another, it fell slightly. In six, the cholesterol was estimated in the plasma and in four of these, also in the whole blood; and in three, the lecithine. The figures, as far as they go, do not show any constant relations between either cholesterol or lecithine, and the fatty acids: when the fatty acids in the plasma were increased 80 per cent., the cholesterol in the plasma increased 10 per cent.; when they increased 29 per cent., it increased 13 per cent.: the lecithine was unchanged in plasma, and whole blood alike, when the fatty acids increased in the former 17 and 22 per cent.; and, in the third case, it rose 8 per cent. in the plasma before it rose at all in the whole blood, the fatty acids in the plasma being unchanged. Nor do the experiments done on patients suffering from diseases of the kidneys show constancy in these relationships. But some of the patients had, as has been observed before in these conditions, relatively large amounts of compounds of fatty acids, and also of cholesterol, in their plasma before taking the butter; and, in these patients, the tendency was for the amount of fatty acids to be increased in the plasma by the meal of fat much more than in the normal subjects examined. The suggestion is put forward that both the high initial content and the larger increase during absorption in these conditions are due to some impairment of the facilities for disposing of the fat in the blood.

These experiments are of interest, too, in showing that an active transportation of fat in the blood need not mean an increased amount in the blood at any moment. Blood analyses cannot answer all the

questions in fat metabolism that they are sometimes expected to answer. In some of the experiments on abnormal, as well as on normal, subjects, in which there was no increase of fat in the plasma, the rise of metabolic rate, and the fall of the respiratory quotient, seem to show that, nevertheless, the fat was being effectively conveyed to the tissues.

In the light of these experiments on man, the results previously obtained in animals, which have been referred to, appear not to be capable of general application, and more work must be done before any general laws of fat metabolism can be deduced from what is known of the condition of the fat in the blood after the ingestion of food containing fat.

CHAPTER VII.

THE DISCHARGE OF RESERVE FAT INTO THE BLOOD.

THE fat in the blood has in the last chapter been dealt with in relation to the earlier chapter on the digestion and absorption of fat. But as is well known the large stores of fat that the body contains can be put at the disposal of tissues in need of a source of energy, and for this to be possible must be distributed to them by the blood.

In man and the dog the reserve store of energy in the form of fat constituting 12 per cent. of the body weight amounts to more than 1100 cal. per kgrm. Pflüger found in an exceptionally fat dog more than three times this amount of fat, whereas in seven dogs specially fed up in his laboratory for the determination of the maximum amount of glycogen that may occur in these animals the average for this substance was 20 grms. or 80 cal. per kgrm. The normal average is probably only about half this. Similarly Terroine gives the results of determinations of both fat and glycogen in certain small animals shown in the following figures:—

	Calories per Kgrm. Stored in the Body.	
	As Fat.	As Glycogen.
In the—		
Mouse	61-630	15-32
Bengali Sporoeginthus Melpodus (a small bird) .	176-413	26-36

and points out that Sporoeginthus, as Lapique showed, requires about 42 cals. per kgrm. per hour, and dies if kept without food in at any rate 10 hours, so that it could never live through a night without using up most of its reserve of fat.

The availability of the reserve fat for supplying energy to the body is well known to have been proved by the experiments of Zuntz, Rubner, Atwater, and others: in starvation Zuntz reckons that sometimes 90 per cent. of the energy required for maintenance of body temperature and

the necessary activities is derived from fat. The heart, the organ which with the kidney requires most energy, contains in itself material including all the protein of which it is composed sufficient for something less than 24 hours' work, even if it could all be completely burnt up: of this the glycogen might suffice for 1 hour, the fats for another 6. Even in a starving animal the weight of the heart is maintained, therefore it must for the performance of its work be continually supplied by the blood with food derived in this condition very largely from the reserve of fat in the adipose tissue.

The way in which this reserve is put into circulation and conveyed to the organs where its energy is to be liberated is very little understood. From the consideration of the facts mentioned, a starving man must be able to draw on fat stored in his adipose tissue to the extent of something like 200 grms. a day, or perhaps 200 mgrms. a minute during the waking hours. His blood, or the blood of normal men (Bloor's average from twelve) contains the amount of fatty acids corresponding to 200 mgrms. of fat in almost exactly 50 c.c. or perhaps one-hundreth part of the whole circulating blood. In one minute therefore the transport of fat in such conditions, when by hypothesis 90 per cent. of the energy is derived from fat mobilised from adipose tissue, represents a turnover of something like 1 per cent. of the total amount of fatty acid present in one form or another in the whole blood. It is necessary to visualise this, because otherwise there is a temptation to think that it should be very easy to demonstrate any extensive physiological mobilisation of reserve fat by analysis of the amount of fat in a sample of blood taken from any accessible vein. The temptation may be escaped, moreover, also by the reflection that a physiological mobilisation of fat is presumably brought about by some reaction due or corresponding to the avidity of working cells for fat—the faster the fat is thrown into the blood, the faster it will be removed by hungry cells. If the adjustment is delicate it would be logical to expect the amount of fat in the mixed blood to be unchanged; if it is not, then at some moments the amount of fat must be above, but at others below the normal value, and there is nothing to indicate how the desired figures are to be obtained. It is indeed remarkable how often they have been.

But if only those figures are considered which have been obtained by the best methods and controlled most satisfactorily, it is at once clear that the adjustment of gain and loss of fat in the blood in starvation is not very precise. Lattes (1911) gives the results of analyses of the blood of thirteen normal and eight fasting dogs and the average of

the amounts found is exactly the same in each group. In the case of five of the fasting dogs estimations had been done also before the fast began: in one of these the fasting figure is 30 per cent. lower, in two others 20 and 26 per cent. higher, in the others the differences are very small. He considers that his figures do not prove that the blood contains more fat in starvation. Terroine's experiments are better planned. He had ascertained that while in any one dog the amount of fat in the blood is normally very nearly the same from day to day, different animals may habitually yield figures that are very different. He therefore made several determinations at intervals of a week on each of a series of dogs that were kept without food. In two of them the amount of fat in the blood increased and then fell, in one it fell regularly from the first, two others showed little change and the others different changes at different times. Similar results have been obtained by Daddi (1898), G. Mansfeld (1910) and Polanyi (1911).

It is, therefore, not possible to follow the migration of fat from the reserve depots to the working organs by examination of the blood, as we have seen it to be during the absorption of fat from the intestine. In this latter case the circumstances are different, not necessarily because the rate at which fat is being taken up by the blood is greater, but because the cells of normal organs are presumably not so pressed by hunger as those of a starved animal; their avidity is less, especially when after a normal meal there are, besides fat, other substances conveyed from the intestine by the blood, sugar or nitrogenous or non-nitrogenous derivatives of proteins, that may be more readily made to serve their immediate needs.

In one physiological condition, the later stages of pregnancy, human blood contains, according to Herrmann and Neumann (1912), 30 per cent. more fat than in the intervals of pregnancy. The analyses were done apparently on mixtures of the blood of different subjects in each condition, and so the results may be taken as averages. Such a large difference can hardly, therefore, be explained by the fact that the results were not obtained from the same subject. Kreidl and Oshima (1907) found ultramicroscopic evidence of fat in the guinea-pig's blood during the later weeks of gestation and not before, and as this was the case equally, whether the animal was fed or not, the fat was, in the latter case at any rate, reserve fat put into circulation. In the later stages of pregnancy and early in lactation, Coope and Mottram (1914) found the amount of fat in the liver considerably increased in rabbits. This corresponds with the great increase of fat in the fœtal liver shortly before birth, noted by Imrie and Graham in guinea-pigs. Polimanti

has found large amounts of fat too in the liver of selachian fish during pregnancy. In view of the fact that Marshall (1924) has found evidence of increased pituitary activity in the latter stage of pregnancy, it is particularly interesting that Coope and Chamberlain (1924) find that injection of pituitrin into rabbits causes a considerable increase in the fat of the liver. This suggests that, in pregnancy, the activity of the pituitary may be a physiological stimulus to the mobilisation of fat from the stores in adipose tissue. This is the only evidence that we possess as to a physiological cause of such mobilisation at any time.

If the phenomenon is constant in the human subject it is remarkable, and would seem to imply that the concentration of fat in the blood is raised, either by the tissues not taking up that which is brought from the intestine or by mobilisation of reserves, and that a high concentration is necessary to ensure the fœtus obtaining what it requires. For, incidentally, there is evidence that the fat in the fœtus may come from the maternal blood, in the fact that the fat of the fœtus has been found to contain lauric acid when the mother's food contained cocoanut oil (Hofbauer, 1905). Hermann and Neumann found a similar or rather greater increase of cholesterol esters in the blood, but free cholesterol and phosphorus in combination with fat unchanged. The greater part, therefore, of the additional fatty acid in the blood was presumably in the form of simple glyceride. But there is nothing to indicate whether it was withdrawn from or merely prevented from entering the reserves.

In lactation, it is known that the fat appearing in the milk is brought by the blood to the secreting gland, and not produced by glandular activity. It is known that this may be brought from the alimentary canal, since Engel (1906) found sesame oil in human milk a few hours after the ingestion of very small amounts of this oil, and Bowes detected arachidic acid in the milk of a goat 12 hours after it had received its first dose of arachis oil. But it can also be brought from other parts of the body; a Jersey cow has been found to secrete milk containing 62·9 lbs. of fat in ninety-five days, when its food had contained only 5·7 lbs., but the cow gained 47 lbs. in weight, so the fat in the milk presumably had been made in the body, and not merely brought up from the reserves (Jordan and Jenter, 1897). But Engel has found that a woman kept on a low fat diet may produce an undiminished amount of milk with practically undiminished amount of fat in it. The fat has a higher iodine value than is observed when the food contains fat, and, in this respect, it resembles reserve fat. Colostrum he finds always to have the same iodine value as reserve fat, and is, he thinks,

derived from it rather than directly from the food. The large amount of fat in the blood just before delivery, observed by Herrmann and Neumann, may then be regarded as reserve fat mobilised for the making of colostrum as well as for supplying the fœtus. Caspari (1899) found that iodised fat, when added to the food, passed into the milk and also into the fat reserves; but, as it continued to be secreted in the milk for some time after the animal's food had been changed so as to contain no iodised fat, it must then have been coming from the fat stored in adipose tissue.

But if the fat in the milk is brought up from the stored stock, this mobilisation need not increase the fat in the blood; in order to carry more, the blood need not contain more; it is a question of rate of transfer, not of concentration. Mansfeld found 0·49 per cent. of fat in the blood of a dog six days before delivery, 0·54 two days after the mammary glands had begun to secrete, but 0·31 per cent. nine days later shortly before the glands went dry, and three weeks after they had done so, 0·61 per cent. Lactation does not appear from this to involve a higher concentration of fat in the blood. The data are not sufficient however to settle the point conclusively.

But there are abnormal conditions in which it is known that the fat is put into circulation from the adipose tissue, and which do involve a higher concentration of it in the blood. The clearest cases of this are the conditions that result from phlorrhizin or phosphorus poisoning. Lattes (1911) found the blood to contain more fat than the normal average in twelve out of thirteen dogs treated with phlorrhizin; in ten of them more than the largest amount found in any of his thirteen normal animals, and in two of them twice as much. The drug is administered to fasting animals, and it is universally recognised that the adipose tissue is the source of the fat, and that large amounts are taken up by the liver. Terroine estimated the fat in the blood of four dogs before and at intervals after the administration of the drug; in three the fat in the blood was increased notably, in the fourth it was not; there was less evidence of fat migration also in the organs of this animal. Phlorrhizin then not merely calls out the fat from the storage places, but does so faster than the organs can take it up. In the case of phosphorus, this action is also sometimes marked. Lattes found higher figures than in any of his normal animals in five out of seven dogs poisoned with phosphorus; but the maximum figure was not nearly so high as the highest with phlorrhizin. Mansfeld, in four dogs on the contrary, only found the amount increased once, and,

in that animal later, it had fallen to a lower value than before the phosphorus was given.[1]

Does the action of these substances throw any light on the normal arrangements by which migration of fat is effected? Both these substances tend to cause a large amount of fat to appear in the liver and abnormal amounts also in certain other organs. There is an intense migration of reserve fat; but whereas physiological migrations of this fat are effected generally in such a way that cells which remove it from the blood do so as fast as it is discharged into the blood stream, under the action of these substances this balanced adjustment is out of gear. Where the fault lies, whether it is discharged from the storage cells too fast or whether the cells that should receive it are disabled in some way, it is impossible to decide. Physiological principles may incline us to the idea that normally the mobilisation is effected by some agent or factor arising in cells that are ready to receive the fat, that such cells produce their own phlorrhizin as it were and that cells that are not in the condition to do this cannot receive fat. Administering phlorrhizin in this case results in the discharged fat accumulating in the blood or getting into places where it is not normal for it to be. This seems more reasonable than to suppose that phlorrhizin acts in two totally distinct ways, not only liberating stored fat but at the same time imposing some disability on the cells throughout the body preventing them from dealing with the fat that reaches them.

But it may be of course that phlorrhizin does not act directly on the adipose tissue cells: it may act elsewhere, and its action there may produce agents or factors that distributed by the blood bring about the discharge of stored fat. There is of course another action of phlorrhizin, by which it causes glycosuria; and this may be so intense that everything that can be made into the indispensable sugar is used in this way to the exclusion of almost every other path of chemical change otherwise open to it, a condition in this respect very like that which is apt to supervene in diabetes mellitus in man. And it is well known that in this disease, especially when untreated, the blood may contain almost incredible amounts of fat, so much in fact that the tendency of the fat to separate as a cream spontaneously makes it difficult to take a fair sample of the mixture. In a case such as that described by Imrie (1914) in which what was believed to be a fair sample of the serum contained 12·5 per cent. of fat freed from cholesterol, it is certainly

[1] In the disease of the liver known as acute yellow atrophy, which has certain points of resemblance to phosphorus poisoning, Feigl found the fat in the blood to be much increased up to five or six times the normal amount.

easy to get a sample of the cream containing far more. Neisser and Derlin (1904) found 19·7 per cent. in the blood from a vein, 24·4 per cent. in the blood from the heart after death. The glycosuria that results from removing a dog's pancreas also is accompanied by a lipæmia that is sometimes considerable (Seo, 1910). Is it possible to discover in the glycosuria that is common to these conditions the cause of what is also common to them, the tendency to lipæmia? If the escape of sugar in the urine can be regarded as a cause of "hunger" in the tissues, and "hunger" as a cause of discharge of fat from the reserves, what is it that prevents the fat from entering the hungry cells? This is the cul-de-sac. Can an exit be found by supposing that the same leakage of sugar removes what is necessary to enable the fat to be fixed in the cells in the normal way, that for fat to take part in the life of the cell it must be linked up with phosphoric acid, and that in some way sugar makes this possible? There is no end to the questions that imagination can put, and no beginning has been made as it seems with the answers. When one has been answered, many may become unnecessary. The action of phosphorus hardly even tantalises with the semblance of a clue. Clues there are that have raised hopes and may lead somewhere. The obscure connection of cholesterol with the metabolic fortunes of fat has led to the accumulation of facts which may suddenly illuminate the obscurity, though that has not yet occurred. Adler (1909), who describes three cases of severe lipæmia, found large amounts of cholesterol, 2·6 and 3·6 per cent. of the serum, and speaks of the condition as one of cholesteræmia. Imrie found 1·5 per cent., almost all of it free. Much the fullest study of the fat in the blood in diabetes has been carried out by Bloor (1916), on thirty-six cases in which the blood did not give a clouded serum, and two cases of definite lipæmia in which it did. The former cases, all but two, had more fatty acids in various combinations than he found to be the average, and all but five more than the highest amount that he found in the blood of normal subjects. But his results give no indication of a tendency for the cholesterol to be increased out of proportion to the fatty acids; in the corpuscles there appears to be hardly more than normally. The component that was most affected by the change was the fatty acids present as simple glycerides, though those associated with phosphorus were also present in larger amounts than normal. The change especially in the most marked cases was principally in the plasma, though not confined to it. There is no obvious change in the proportion of the cholesterol which is present in the form of esters of the fatty acids (1916).

In the fact that the cholesterol is increased in amount, as well as

the fat, and not conspicuously out of proportion to it, Bloor sees proof that the fat did not come from the reserves where there is little or no cholesterol, and seeing that in the two cases in which the serum was milky, definitely lipæmic, this condition disappeared when the patients were put on a diet free from fat, he thinks he is justified in stating that the fat had come from the food, and was unassimilated, not mobilised fat. But even that hypothesis does not necessarily make it easy to account for the cholesterol. Terroine found in his experiments with phlorrhizin as close a correspondence between the amount of cholesterol and the amount of fat, and phlorrhizin, beyond question, brings out the fat from the reserves, as the effect is obtained on fasting dogs. Bloor's view may be the right one, but the reasons he gives do not prove it to be. We shall have occasion to return to this point later.

The fact that the most severe lipæmias in diabetes are commonly in neglected cases, in which the acidosis has become severe, has led to the attempt to trace the lipæmia to the acidosis. Mansfeld injected 350 c.c. of N/10 hydrochloric acid into the veins of a dog of 15 kgrms., and found the fat in the blood to be increased nearly 50 per cent. : 500 c.c. of N/4 lactic acid did not, however, change the amount of fat in the blood of a dog weighing 10 kgrms. ; but, in a third dog, of twice the weight, 600 c.c. of N/8 lactic acid increased the fat in the blood 25 per cent. Whatever these experiments are supposed to prove, they prove little with regard to fat mobilisation : there may have been no increase in the rate of discharge of fat when the percentage in the blood rose, and, on the other hand, there may have been when it fell. The amount of fat in the blood depends necessarily on two factors, the rate at which it is added to it, and that at which it leaves it. An observation made by Boycott is interesting. A man died a few hours after drinking, when intoxicated, some spirits of salt. The acid had eroded the stomach, and a portion of the liver was attacked by it. The cells of this part of the liver were killed and fixed, and were found to contain no fat : the whole of the rest of the liver was intensely fatty, containing several times as much fat as is normal for the organ, and the fat was of the connective tissue type, with a low iodine value. The interpretation put forward is that, at the time when the acid reached the stomach, the liver was normal in the matter of fat, as shown by the cells which were fixed by the acid. The invasion of the liver with fat took place in the time subsequent to that, the few hours before death : such rapid invasions are known to occur : this one, it is suggested, was the result of the acid poisoning.

Boggs and Morris (1909) described an interesting form of lipæmia,

brought on in rabbits that had been rendered anæmic by daily removal of 20 to 25 c.c. of blood. It was observed that, when the number of red blood corpuscles had been reduced in this way to something like 2,000,000 per c.mm., the serum was milky, and contained from 2 to 4·5 per cent. of substances soluble in ether, which were extracted by alcohol and ether; the blood, when normal, containing from 0·3 to 0·5 per cent. estimated in this way. Anæmia, of the same degree, induced by pyridine, did not produce this change. It was noted that the fat had a high iodine value, 105 to 134, so that, if it was fat taken up by the blood from connective tissue, it had since undergone some change; but, since emaciation is known to result from a secondary anæmia, and not from the primary forms, it probably had had that origin. In Imrie's case of diabetic lipæmia, where the amount of fat was far larger, the iodine value was also higher than that of human connective tissue fat, though not so high as in these experiments.

Terroine (1919) gives the results of experiments done in 1913-14, in which three or four severe hæmorrhages were repeated at intervals of two or three days in three dogs whose food, bread, rice, and sugar, was practically free from fat and cholesterol. The proportion of both these components to other solids in the blood rose considerably both in corpuscles and plasma; in the corpuscles the cholesterol increased notably more than the fatty acids. But the increases were of the order of 50 per cent., far smaller than those referred to above, observed in the rabbit. Terroine points out that the newly-formed corpuscles must have been richer in fat and still more so in cholesterol than the old. The study of the organs in these dogs at the end of the experiments showed different results in different animals, but one constant change in all three, a strikingly small amount of cholesterol in the suprarenal, one-third; in two of the animals, in fact, less than a quarter of the average amount found in three normal dogs.

In the rabbit again Horiuchi (1920), working with Bloor and using his methods, confirmed the observations of Boggs and Morris, and added new points of interest. Some of the animals were fed on food rich in oil, sunflower seeds, others on carrots that contain but very little. The lipæmia was more intense on the former diet than on the latter, but was severe in this too. There was also an increase in all cases of lecithine and of cholesterol, but a smaller one; there was some increase though a much smaller one in the fat in the corpuscles, but the cholesterol in the corpuscles did not change concurrently. The most important new fact bearing on the question of the mobilisation of fat in this work is that not only are the cells that take up fat from

the blood failing to do so, but the reserves are at the same time giving it up to the blood, both the result of the same experimental abnormality. If the cause was here again essentially hunger, insufficiency of sugar due to loss of blood, and the resulting dilution of it in the vessels (45 c.c. of blood is a large fraction of the blood of rabbit weighing 1675 grms. and 35 c.c. in one of 1190 is more still), this form of lipæmia and those which occur in phlorrhizin poisoning and in diabetes, may possibly be traced to some common cause that both diminishes the power of the tissues to assimilate and at the same time puts the reserve into circulation.

Another point is worth noting, the rabbit fed on carrots had the cholesterol in its plasma doubled at a time when the fat in its blood was much increased by fat that must have come from the reserves. This no less than the earlier results of Terroine referred to above show that the increase of cholesterol together with fat in the blood of diabetes does not prove that the fat in that condition comes from the food.

It has been found by Reicher (1908) that chloroform, and even such an anæsthetic as morphine, increases the amount of fat in the blood of dogs as much as twofold. But this is disputed by Lattes (1911), and Murlin and Riche (1915) state that with chloretone the deeper the narcosis in dogs the less fat is to be found in the blood.

Bloor (1914) gives curves to show a rapid rise, sometimes of more than 50 per cent., in the first hour of ether administration, and in one experiment, in which a dog had been fed with fat abundantly for a week, a rapid rise with chloroform; in this case the dog died in 10 minutes, the same dog previously having stood well and without lipæmia chloroform administration for 3 hours after a week's fast. Alcohol and morphine make little difference at the time, but he observed a somewhat increased amount of fat, generally less than 20 per cent. more than before, on the second or third day after both chloroform and morphine, but not after ether. It is not clear that these results add anything that can be interpreted at present as bearing on the question of the mobilisation of fat.

CHAPTER VIII.

THE TRANSFER OF FAT FROM BLOOD TO ORGANS.

BUT the fat may be called out of the reserves and found in other parts of the body besides the blood. Here again so long as the physiological adjustment of supply and demand is precisely maintained an increase in the rate of supply will not result in accumulation of fat in organs any more than in the blood. And if an unusual amount of fat is found in any organ it may be due either to a failure on the part of the organ to dispose in the normal way of fat brought to it at the normal rate, or else to accelerated importation when there is no corresponding acceleration in the rate at which the organ deals with it. Or of course it may be due to both retarded assimilation and accelerated importation occurring at the same time. It may not be possible to decide which of these explanations should be adopted in any of the conditions to be examined; and it must not be forgotten that when unusual amounts of fat are found in any organ another possibility is that it may have been produced on the spot by synthesis, and yet another, if histological methods alone are used, that the apparent change in the amount of fat may not be real.

Reference has been made above to the doctrine formerly prevalent that increase in the amount of fat in a part implied that the new fat had arisen on the spot by a degeneration of cell substance consisting as ordinarily understood of proteins. This was the meaning for Virchow of "fatty degeneration" whether in the cells of the brain, the liver, the heart, or in any other kind of cell; a fatal disease, to which any living cell was liable, supposed to furnish proof that the chemical transformation of protein into fat was a common phenomenon: the secretion of milk and that of sebum were for Virchow further instances of the same change, in these cases, however, turned to a useful purpose.

The first clear experimental proof that a morbid change previously interpreted in this way was really the result of the migration of fat from adipose tissue was that devised by Lebedev in the case of the fatty liver resulting from the action of phosphorus. He first observed that in a man who had taken phosphorus when he was emaciated by starvation the

liver was not, as in phosphorus poisoning it usually is, fatty. He then made the following experiment: a dog, after being starved till its stock of reserve fat was used up, was fattened with food containing linseed oil. When it was then poisoned with phosphorus, the fat in its liver, far in excess of the normal amount, was found to be different from the fat in the liver of a normally fed dog poisoned with phosphorus, and to resemble the oil with which it had been fattened. The phosphorus had mobilised the reserve fat formed from the linseed oil and this had accumulated in the liver. In this condition other organs, too, may be the seat of accumulations of fat but to a much smaller extent.

This experiment has been repeated and confirmed by Leick and Winkler who used mutton fat for fattening their starved dogs, and distinguished this from the fat generally found in the dog's liver by its lower iodine value. Rosenfeld compared the amount of fat in the liver of dogs that had been first starved and then poisoned with phosphorus with that found in animals that had been normally fed or specially fattened before phosphorus was administered, and confirmed Lebedev's original observation that the liver exhibits the fatty change only in proportion to the amount of fat in reserve available for mobilisation. He also showed that an extremely fatty liver was found in dogs that after five days' starvation received injections of phlorrhizin. Large amounts of fat were found 48 hours after the phlorrhizin treatment began, again only if there was a stock of reserve fat to be put into circulation. He also showed that this accumulation is a rapid process, since it does not begin at once and is not found till more than 30 hours have elapsed; and that it might as rapidly disappear again, since it was no longer to be found if the animal was fed for a couple of days before being killed.

Rapidly induced fatty change in the liver is also known to occur sometimes after chloroform anæsthesia, in the condition known as delayed chloroform poisoning. This is probably not a specifically characteristic action of chloroform, since it has been described as following the use of other anæsthetic drugs, ether and even nitrous oxide (Langmead 1907). It occurs too in acute alcohol poisoning and, as we have seen, apparently sometimes in acute acid poisoning.

In diabetes in man and after removal of the pancreas in dogs the fat in the liver is often excessive in amount, and since in phlorrhizin glycosuria we have evidence that the fat in the liver is brought rapidly from the reserve depots, it is natural to suppose that sudden and rapid mobilisation may occur in these conditions too. There are many other conditions besides these in which the liver may be fatty and probably for the same reason. Because taking human livers as they chance to

be presented at autopsy, if the amount of fat is estimated as fatty acids set free by saponifying and making acid, and the iodine value of these acids is taken, when the results are arranged in order of increasing amounts of fat, they are found to be arranged also in order of diminishing iodine values [Hartley and Mavrogordato (1909), Imrie (1914)]. In normal physiological conditions, when the rate of importation of fat is not greater than the rate at which the liver disposes of fat, the liver like other organs, heart, kidney, spleen, etc., as Mayer and Schaeffer, and Terroine have shown, contains no reserve fat, the fatty acids amount to about 3 per cent. of the fresh weight of the tissue, or a little less, both in man and other mammalian animals, so far as they have been investigated, monkey, dog, cat, rabbit, guinea-pig, rat, pig, sheep, horse, ox, or goat. And then the iodine value of these fatty acids is high as in other organs, generally about 130. But it is not uncommon to find larger amounts of fat than this in the liver, in man and in, at any rate, several of the species of animals enumerated, even when there is nothing otherwise apparently abnormal, and then the fatty acids have an iodine value lower than the physiological normal value in proportion to the amount of additional fat. No exceptions to this rule have so far been observed or recorded. With the maximum amount of fat, 20 to 22 per cent. of the fresh weight, the iodine value approaches very close to that found in the fatty acids from adipose tissue fat of the species, in man 60 to 65. A curve can be drawn (Imrie, 1914) representing the iodine value corresponding to any percentage of fatty acids, on the supposition that in man the liver contains an *élément constant* amounting to about 3 per cent. with the iodine value 135 and that all fat over and above this amount is imported fat with the iodine value 65; for 6 per cent. therefore the corresponding iodine value should be 100, for 9 per cent. 88, and so on. When this is done, and the actual results obtained with individual livers are plotted on the chart, it is astonishing how close the points come to lie to this curve.[1]

As already pointed out, however, such observations do not necessarily mean, in every case, that the rate of importation has been accelerated. The same result would be, sooner or later, obtained, if there was a block on the line of metabolic change in the liver, and it

[1] These calculations are as Mottram has pointed out to us not quite correctly made: if a liver containing 3 per cent. of fat takes up more fat so that, assuming that this is the only change occurring, the fat now amounts to 6 per cent. this probably means that of this amount 3·2 grms. is imported fat and 2·8 grms. fat that was in the liver before. The error for these amounts is small, but with larger amounts of fat it is more considerable. If, however, the correction is made the calculated curve so obtained and the values recorded by Imrie are in even better agreement rather than the reverse.

may be impossible to decide whether the actual cause of the condition is arrested function of this organ, or rapid mobilisation of fat. It is not clear that examination of the blood could remove this uncertainty. In some cases, indeed, there is presumptive probability that the liver may be at fault; in poisoning by phosphorus, for instance, we know that the liver bears the brunt of the attack, and shows very definite signs of suffering seriously in other ways. Something similar is seen, in some cases of chloroform poisoning, acute yellow atrophy, occasionally in cyclic vomiting, and the pernicious vomiting of pregnancy. And even when it does not suffer in other ways, it is possible that a particular function involved in fat metabolism may be singled out, and put out of action by certain agencies or combinations of circumstances. Nevertheless, in cases in which there is reason to think the accumulation of fat in the liver has been rapidly effected, it is almost certain that the mobilisation of fat from the reserve has been accelerated.

In other organs besides the liver, something similar may be found occasionally: but then a quantitative difference is conspicuous. The heart or kidney may occasionally be found to have as much as twice the amount of fat that is normally found; and this fat has then a correspondingly lower iodine value also; but it is commoner than that to find even six or seven times as much fat in the liver as is normal. And this seems to show that the liver plays a part in fat metabolism, which other organs commonly do not at all, or only to a much smaller degree (Imrie, 1914).

The position to which this brings us is then, that at times in certain organs, especially the liver, and to a much less pronounced degree, heart and kidney, evidence is obtainable of a transfer of reserve fat having taken place, and, although it is not generally possible to follow the process, to see when it begins, when it reaches its maximum, and how long it lasts, as can be done in the blood of which a series of samples can be taken at different times, still there is reason for thinking that the lack of physiological adjustment of supply to demand is only temporary, and that, while the fat may gather rapidly, it may, with changed circumstances, be as rapidly disposed of, and the balance restored. In the case of starvation, the normal function of reserve fat is called into play. Mottram (1909) showed that, in starving rabbits, fat was to be found in the liver in distinctly larger amounts than in a normally fed animal, the access of fat recurring at intervals, apparently of a few days, in these animals. Terroine also found, occasionally, but not uniformly and constantly, increased amounts in the liver of dogs kept without food: in the other organs, this was not the case.

In order to account for the special position taken by the liver as a receiving organ for fat which has been transferred from the reserves, we should consider what is known of the subsequent history of mobilised reserve fat. It is certainly oxidised in tissues where energy is set free, and no less certainly, it must be used for repair of the fatty components of the protoplasmic fabric of cells, including the myelin of the nervous system. For oxidation, it is possible that the fatty acids are more readily available if they have unsaturated linkages at which oxidation can take place, and so give rise to lower fatty acids; these we know that the organism can by β-oxidation convert into acetoacetic acid, and thus into carbonic acid and water.

But also, if required for purposes of repair, the fatty acids must in part acquire new unsaturated linkages; for the iodine value of the mixed fatty acids, obtained from cells other than those of adipose tissue, is invariably higher than that of the fat in the reserves or in the food, and, indeed, higher than that of oleic acid, the only unsaturated acid commonly present in any quantity in the food. The iodine value of the fatty acids in the cells of the organs is such as would be given by a mixture of oleic and linoleic acid in about equal parts, but, since saturated acids absorbing no iodine are present together with unsaturated, either linoleic acid must constitute more than half of the total amount of acids, or there must be acids more unsaturated than linoleic present, with three or more double linkages. Now, it is not in accord with what is known of the functions of the liver to suppose that higher fatty acids are, in any great amount, completely oxidised there. The liver is not an organ like the heart or kidney, whose functions involve the transformation of chemical energy on a large scale; and its blood supply is largely venous. If more of the reserve fat is taken up by the liver than by the heart and kidney, it seems that the liver cannot require it for its own use, but must do something with it to render it more suitable for the uses of other cells. Whether the uses to which these cells put it are oxidation for energy or repair of an essential component of their own protoplasm, as we have seen, new unsaturated linkages appear to be required. Is it possible that it is in the liver that these are introduced?

Experiments were done (Leathes and Meyer Wedell, 1909) which seem to show that this must be so. Rats or cats were fed on food containing cod liver oil or the flesh of herrings, into the composition of which acids with a very high iodine value enter. The fatty acids subsequently found in the liver of these animals had an iodine value that was still higher than that of the mixed acids of the oil or fat that

had been eaten. This might be accounted for by supposing that those fatty acids to which the high iodine value of the fats contained in the food was due, acids with three or more double linkages, were taken up by the liver more readily, or, having been taken up, were less readily disposed of than other acids more completely saturated with which the liver commonly has to deal. But there is other evidence which makes the explanation which was originally put forward more probable, namely, that some of the acids had had new unsaturated linkages introduced; the acids in which this occurred being probably those of the stearic and oleic acid series present in the mixture presented by the fish oils, and present also in the mixtures of acids that occur in the fats of the animal's usual food. The other evidence in favour of this explanation is the proof given by Hartley (1909) that the liver of the pig contains an oleic acid different from that in lard, the reserve fat of the same species of animal. In lard the oleic acid is the common acid with the unsaturated pair of carbon atoms exactly in the middle of the chain ($\Delta\,\theta\,\iota$, Δ^{8-9}), as Hartley showed by oxidising it with alkaline permanganate and isolating pelargonic acid. But from the pig's liver the unsaturated acids obtained were an oleic acid that, treated in the same way, gave caproic instead of pelargonic acid, and a dicarboxylic acid with 12 carbon atoms instead of azaleic, which, in the oxidation of lard, though it was not isolated, was presumably formed together with the pelargonic acid; and, in addition to this new oleic acid, a linoleic acid that, on oxidation, gave caproic and acetic acid, the latter derived from malonic acid. In this linoleic acid, therefore, the double linkages were one in the middle of the chain, and the other three places further from the carboxyl group ($\Delta\,\theta\,\iota$ and $\lambda\,\mu$, *i.e.*, Δ^{8-9} and Δ^{11-12}). The hydroxy acids obtained from these two unsaturated acids by oxidising at a low temperature agreed with this view of their constitution. In the pig's liver then two acids were found, neither of which exists in lard, one of which would be formed from the stearic, the other from the oleic acid of the pig's reserve fat by the same change in both cases, the introduction of a new unsaturated union between the sixth and seventh carbon atoms counted from the methyl group as the first. If the same change had been effected in the stearic and oleic acids of the fish oils, by the livers of the cats and rats in the previously mentioned experiments, this would account for the iodine values found in the acids of those animals' livers.

We have thus seen that it is common for the liver to contain fat that has been transferred to it from the fat depots, commoner than it is for other organs, and in far larger amount than other organs; and from

the consideration of all the facts we are led to interpret them provisionally in this way: when fat is called up for use, it is more particularly the liver that, in the first instance, normally deals with it; in the cells of the liver it is prepared for the uses to which it is to be put in the body generally by desaturation of the fatty acids.

Whether the work of the liver stops there, whether the more unsaturated acids are given over to the blood and by it distributed to the other tissues, as simple glycerides perhaps, or whether more complex compounds, the phospholipines, for instance, are prepared in the liver, and it is these substances that the blood conveys from the liver to other organs there are no positive data to decide. It has been shown that in the liver the acids more unsaturated than those found in the fat of adipose tissue are not confined to the phospholipines, but exist also as simple glycerides (Kennaway and Leathes, 1909). But this does not necessarily imply that they leave the liver as simple glycerides. It is possible that they are built up in the liver into compounds containing phosphorus and nitrogen before they pass out into the blood. Experimental methods are not as yet available for determining quantitatively the amount of each of the different kinds of combinations in which fatty acids occur in a tissue or organ. We have to be content for the present with estimations of total fatty acids and of phosphorus in combinations that are soluble in ether. In both lecithine and cephaline there are two fatty acid radicals for 1 atom of phosphorus. Mayer and Schaeffer (1913, 3^{me} Mémoire) estimate the ratio of fatty acids to phosphorus in the organs of normal animals [p. 60], and give the following table of values for this ratio:—

	Liver.	Kidney	Lung.	Pancreas.
Man . . .	36·1	24·7	—	54·8
Dog . . .	21·4	22·1	22·4	27·0
Rabbit . . .	19·6	22·0	25·3	—
Guinea-pig . .	16·0	24·9	18·8	—
Pigeon . . .	32·6	26·6	16·5	—

The human organs examined were those of a man of twenty-three years who was executed: the values for the ratio in the organs of animals were obtained by taking the mean of the amounts of fatty acids from one series of normal animals, and the mean of the amounts of phosphorus from another series. These values are compared with the ratio in lecithine or cephaline, which is about 18 when the acid radicals are taken to contain 18 carbon atoms each. It appears that in most cases there must be some fatty acid present in combinations that are

free from phosphorus, if lecithine and cephaline with two acid radicals each with 18 carbon atoms are the only compounds containing fatty acid and phosphorus that exist in these organs. Assuming this to be evidence for the presence of simple glycerides in organs in which the ratio is higher than 18, it is impossible to say whether these glycerides have come as such from the liver, or from the intestine, or from the adipose tissue. The French physiologists find that there is little or no diminution in the ratio between fatty acids and total solids in the kidney and lung from animals that have died of starvation but received water (Terroine, p. 75). In animals that have died of starvation without receiving water this ratio is found to fall, and at the same time the amount of phosphorus in combinations that are soluble in ether increases in proportion to the total solids, so that the ratio of fatty acids to such phosphorus falls and reaches values lower than that which holds for lecithine and cephaline (Mayer and Schaeffer, 1914).

These results raise a number of questions that await solution. Is the low ratio of fatty acid to phosphorus soluble in ether, which Mayer and Schaeffer found in the organs of starved animals, to be taken as evidence that fatty acids had during starvation been partially burnt out of the phospholipine molecules, and that the oxidative demolition of fatty acid radicals in the cells normally takes place only when and while these acids are under the influence of the phosphoric acid which is built up with them into the same phospholipine molecule, an association that may recall that of phosphoric acid with sugar in alcohol fermentation and in muscle? And is the small amount of simple glyceride that normal organs other than the liver appear often to contain merely a small reserve of fatty acid waiting to take the place of that which is about to burn down in the phospholipine molecules? If the data are sufficient to raise such questions there are certainly none that supply the answers.

The important work of Levene on the phospholipines has done a great deal to clear away the confusion that surrounded these substances. But there must be much to be learnt from the study of the decomposition products of lecithine and cephaline, if that is the nature, as he reasonably says, of all the confusing foreign material which with such skill he succeeded in brushing aside so as to bring out clearly the outline of these substances themselves. The study of those decomposition products is likely to be more difficult still, but it should throw light on the metabolic history of the phospholipines and with them of the fatty acids too. But one fact brought out in Levene's work on the liver lecithine (1921) is that it may contain an unsaturated arachidic

acid, probably one with four pairs of doubly linked carbon atoms, $C_{20}H_{32}O_2$. This would be the same as an acid found by Hartley (1909) in the liver of the pig. Levene does not state the species of animal from which he obtained this liver lecithine. But Hartley's proof of the occurrence of an acid of this formula among the fatty acids in the pig's liver is clear and complete.

Bull (1906) found an acid with 20 carbon atoms of the oleic series in cod-liver oil. Majima and Okeda (1914) give evidence for the existence in Japanese sardine oil of highly unsaturated acids with 20 and 22 carbon atoms in chain. The possibility that these acids with 20 carbon atoms were derived from arachidic acid in the food has not been excluded. If it were, their occurrence in the liver of different species of animals would require explanation, and should be borne in mind in connection with the question of synthesis of fatty acids in the liver.

CHAPTER IX.

THE OXIDATION OF FAT.

FATS yield their store of energy by undergoing oxidation processes, and the extent of these determines the proportion of their total available energy which is utilised. In animals, under normal conditions, the oxidation is complete, and carbon dioxide and water are the end products; but in plants it is much more probable that the oxidation processes stop short of this stage of completeness, and that the intermediate products are utilised for the construction of substances which go to build up plant tissues, such as cellulose, cork, or lignin, which belong to, or are closely associated with the great chemical group of carbohydrates. There thus appears to be a great difference between the oxidation of fat in the animal and plant kingdoms. But it is more apparent than real. There is evidence (Krogh and Lindhard, 1920) that under some conditions in the animal, fat is transformed into carbohydrate. To what extent this is a normal process in animal metabolism is at present very uncertain, but the fact that it may occur and that it appears to be the normal reaction in the plant, shows how closely allied the early stages of oxidation in both animal and plant worlds may be, and that an intimate knowledge of these processes in one kingdom might throw considerable light on their progress in the other.

The Oxidation of Fat in Plants.—In plants, fat is found principally in seeds or fruits, where it is stored in readiness for the period of germination. Investigations of the oxidation of fat in the plant have, therefore, been limited to the changes which this stored fat undergoes during the germination process by which the seedling is produced. The information obtained up to the present is very meagre, and most of it is concerned with the rate at which the fat disappears from the seed and the changes that may be noted in the fat prior to its disappearance. The quantitative results have been obtained chiefly by the ether extraction method, and the substances soluble in ether have been taken to be fat. This procedure is quite unjustified, and although the conclusions drawn from the results are probably broadly true, it is

unfortunate that they have not been obtained by more trustworthy methods.

It has been known for a long time that two stages may be noted during the germination of seeds rich in fat, and this has been confirmed by the more recent investigations of le Clerc du Sablon (1897) and Miller (1910-12). In the early period of germination the total fat of the seedling suffers but little diminution. Thus du Sablon found 50 per cent. of fat in the sweet almond before germination, and 45 per cent. when the root was 2 cms. long; and Miller, studying the sunflower (*Helianthus annuus*), found 56 per cent. of fat in the resting seed and 51·9 per cent. when the cotyledons had reached the surface of the soil. In the next period of the development of the seedling the amount of fat rapidly diminishes. When the rootlet of the sweet almond had attained a length of 20 cms. the seedling contained only 7 per cent. of fat (du Sablon), and in the sunflower a diminution to 13·5 per cent. was observed by Miller when the cotyledons had become completely expanded. In this period of development changes have been observed in the composition of the fat of the seedling. It now contains a considerable proportion of free fatty acids, and the iodine value of the fat is lower than in the original seed. The liberation of free fatty acids from the neutral fat of the seed is undoubtedly brought about by a lipase, as first shown by Green (1890). The isolation of this enzyme from castor oil seed and its properties have been carefully studied by Nicloux (1906) and others. The hydrolysis proceeds gradually during the germination process, and is most marked when the total amount of fat is rapidly diminishing. The following figures obtained by du Sablon (1895) in the germination of linseed illustrate this :—

Length of Root, cms.	Total Oil, Per Cent.	Baryta Required to Neutralise 100 grms. of Oil, c.c.
0·0	37·9	0·3
0·6	36·4	0·3
1·2	30·1	0·9
1·4	27·1	1·1
2·2	22·1	3·9
3·2	17·5	4·1
3·5	14·1	8·7
4·0	11·0	11·4
4·2	9·3	17·7

The isolation or identification of the glycerine, which must be liberated in the hydrolytic process at the same time as the fatty acids, has not yet been achieved. This would indicate that it is used up almost as rapidly as it is liberated.

The diminution of the iodine value of the fat during germination has been observed in many different species and appears to be a constant phenomenon [Schmidt (1891), von Fürth (1903), Miller (1910-12), Ivanov, 1912, i.), and others]. According to the observations of Schmidt, the diminution is most marked in the free fatty acids and the change is greater in the hypocotyl and root than in the cotyledons. The diminution may be considerable. In the case of linseed the iodine value sinks from 173·4 to 93·4 in 8 days, and in the poppy, from 140·2 to 71·6 (Ivanov). Two explanations have been advanced to account for this. Ivanov believes that the more unsaturated acids are used up first and consequently the iodine value falls as germination proceeds. In favour of this view may be quoted the fact that the fat disappears most rapidly from seeds containing highly unsaturated oils, although perhaps this has not been widely or deeply enough investigated, and also the fact that it is in the free fatty acids that the diminution is most marked. Miller (1912), on the other hand, thinks that oxidation of the unsaturated acids with the production of hydroxy acids or saturated acids with a lower molecular weight is the cause of the change. There is no evidence, however, that a change of this type takes place to a sufficient extent to account for the marked fall in the iodine value. Thus von Fürth (1903) compared the acetyl value of the fat from the seed of *Helianthus annuus* with that from the seedling and found values of 87·5 and 50·5 respectively. This evidence is against hydroxylation as a cause of the decrease in iodine value. Miller (1910-12) also working with sunflower seedlings found a slight decrease in the acetyl value of the ether extract of the cotyledons, but a very marked increase in that of the ether extract of the hypocotyl and root. From this he infers that hydroxylation is important as a stage in the breakdown of fatty acids and that this, together with the production of fatty acids of lower molecular weight (as evidenced by an increase in the saponification value), indicates the reason for the lowering of the iodine value. It is, however, unsatisfactory that these data were got from an ether extract of the dried seedlings which might therefore have contained many other things besides fats and products derived from them. On account of this, deductions of value concerning the stages of degradation of the fatty acid molecule during germination cannot be safely made from Miller's results. It is of great interest that Miller found in the hypocotyl and root a considerable increase in the amount of water soluble acids in the ether extract as germination progressed, and it seems likely that a careful investigation of the nature of these acids would throw light on the stages of fatty acid breakdown.

Although the steps by which fat is oxidised when once it has undergone hydrolysis in the germinating seed are very obscure, it is quite certain that the main end products of the process are known. These are carbohydrates. This conclusion has been established by the work of many observers, Peters (1861), Green and Jackson (1905), Maquenne (1898), du Sablon (1897), Miller (1910), and Ivanov (1912). The following figures of Ivanov indicate the extent of the accumulation of total carbohydrate, as estimated by reduction after hydrolysis with 2 per cent. sulphuric acid, during the process of germination. They show clearly the large increase in carbohydrate that accompanies the decrease in fat :—

	In Seed, Per Cent.	In 4 Days' Seedling, Per Cent.	In 8 Days' Seedling, Per Cent.
Linseed—			
Fat	33·6	26·4	16·0
Carbohydrate . .	4·5	6·7	17·6
Hempseed—			
Fat	31·3	17·8	11·3
Carbohydrate . .	2·8	7·9	10·2
Poppyseed—			
Fat	47·0	38·5	36·3
Carbohydrate . .	1·2	6·8	17·4

If the rate of increase of different sugars in the seedling is followed it is found that the appearance of cane sugar precedes that of reducing sugars [du Sablon (1894-5-7), Green and Jackson (1905)]. This is true of all the oil rich seeds so far investigated and has led to the view that this sugar is the one formed primarily from the fat which disappears during germination. Later, reducing sugars appear in increasing amounts and this is due, according to Miller, to the development of the characteristic activities of the leaf by the cotyledons.

In conclusion, therefore, it may be stated, that so far as the available evidence goes, there is little or nothing to show by what series of chemical reactions the fat of seeds is broken down during the process of germination nor how carbohydrates are synthesised from the oxidation products.

The Oxidation of Fat in Animals.—Animals use fat primarily as a source of energy. This does not mean that fat is only of importance to the animal as a source of energy, for, as pointed out in the following chapter, fatty acids in one form of combination or another are indispensable constituents of animals and when the quantity in the body is reduced by inanition below a certain minimal figure, death ensues. It

is, however, true to state that the greater part of the fat which is taken daily as food is utilised for the liberation of energy as heat and work. Were this fat simply stored, animals would gain in weight very rapidly and in a short space of time resemble bladders of lard. This self-evident truth, that the bulk of the fat taken by an animal is oxidised and not stored up is supported by evidence gained from a study of the gaseous exchange on different diets. For the complete oxidation of fat to carbon dioxide and water it is possible to calculate the amount of oxygen required per gramme molecule of fat. For triolein we find the following:—

$$(C_{17}H_{33}COO)_3C_3H_5 + 80O_2 = 57CO_2 + 52H_2O.$$

From this it is seen that in oxidising 1 gramme molecule of triolein 80 gramme molecules of oxygen are required and 57 gramme molecules of carbon dioxide are produced. The relative volumes of carbon dioxide and oxygen involved are therefore as 57 to 80 or 0·71 : 1. This ratio is termed the respiratory quotient, (R.Q.). If fat were being exclusively oxidised in the body the ratio of the volume of carbon dioxide given off in the expired air to the volume of oxygen absorbed in the lungs would be 0·71 : 1. By similar reasoning it can be shown that if carbohydrates alone are being oxidised the ratio would be 1 : 1. In order to determine these ratios experimentally the amount of protein undergoing oxidation must be known and that can, on certain assumptions, be determined by estimating the nitrogen excretion in the urine. By calculation, the oxygen used in the oxidation of the protein can thus be obtained and subtracted from the total amount of oxygen used by the animal. Similarly, the carbon dioxide produced by the oxidation of the protein can be subtracted from the whole of the carbon dioxide produced and the "non-protein" respiratory quotient determined. From the value of the quotient, lying as it does, between 0·71 and 1·0, the ratio of the amounts of carbohydrate and fat undergoing oxidation may be determined. On a diet rich in fat and poor in carbohydrate the value of the R.Q. approaches 0·71, and conversely, on a diet rich in carbohydrate and poor in fat it approaches 1·0. This is in conformity with general experience that under normal conditions the healthy animal uses as a source of energy, day by day, the whole of the fat in the food. It is only when the amount taken exceeds the daily requirements that it is stored. When the R.Q. reaches a value outside the limits of 0·71 and 1·0, it is apparent that when the value is below 0·71, oxygen is being used in the body in oxidation processes which do not give rise directly to carbon dioxide. This must be so since it is known that free

oxygen cannot be stored in the body to any marked extent. Were this not true it would not be possible to make the statement just given. If the R.Q. has a value higher than 1·0, then, if the washing out of carbon dioxide from its store in the tissues by excessive respiration be excluded, it may be assumed that oxygen is being taken up by partly oxidised substances, rendering them so unstable that they give off carbon dioxide in greater amount than corresponds to the oxygen taken up. An example of this type of change, which may occur under certain conditions in the body, is the oxidation of β-hydroxybutyric acid to acetoacetic acid, and the hydrolysis of the latter to give acetone and carbon dioxide—

$$2CH_3 . CH(OH) . CH_2 . COOH + O_2 = 2CH_3 . CO . CH_2 . COOH,$$

$$2CH_3 . CO . CH_2 . COOH + 2H_2O = 2CH_3 . CO . CH_3 + 2H_2O + 2CO_2.$$

The carbon dioxide-oxygen ratio of this reaction is 2·0. The series of reactions involved in the formation of fat from carbohydrates will also give a higher value for this ratio than 1·0.

If the empirical formulæ of glucose ($C_6H_{12}O_6$) and stearic acid ($C_{18}H_{36}O_2$) be considered, it will be seen that the carbon and hydrogen bear the same ratio to each other in these substances, but the glucose contains relatively much more oxygen. The intermediate stages of the series of reactions by which fatty acids are made from carbohydrates are not known with certainty, but it is very probable that, in some of them, carbon dioxide is liberated, and this may be taken to be produced by the utilisation of the excess of oxygen in the glucose. Such a series of reactions taken by themselves would give a R.Q. that is infinitely great. And, on the other hand, if the fatty acid be converted into glucose, which contains relatively much more oxygen, a large amount of oxygen would have to be taken up without the concurrent discharge of carbon dioxide. Such a process taken by itself would have a R.Q. of zero. A partial oxidation process, such as the oxidation of butyric acid to acetoacetic acid, which is known to take place in the body, would also have a R.Q. of zero—

$$\underset{\text{Butyric acid.}}{CH_3 . CH_2 . CH_2 . COOH} + O_2 = \underset{\text{Aceto acetic acid.}}{CH_3 . CO . CH_2 . COOH} + H_2O.$$

These examples are given in order to emphasise that the respiratory quotient is only of value in indicating the proportions of fat and carbohydrate which are being oxidised when it is certain that the oxidation

is proceeding *to completion* in the man or animal under experiment, and that values of the R.Q. outside the limits, 0·7 and 1·0, merely indicate the type of reaction taking place, and do not identify it necessarily as being the conversion of fat into carbohydrate, or vice versa. When we consider, however, that satisfactory proof exists, that carbohydrates are converted into fat in the body, it is reasonable to assume that, when the R.Q. is higher than 1·0, then this reaction is the main one causing the high quotient. The reverse proposition, that when the quotient is lower than 0·7, fat is being converted into carbohydrate, cannot be so easily accepted. This reaction has not, as yet, been fully proved to take place in the body, though it undoubtedly does in plants. The best evidence in its favour is the low R.Q. which has been observed in hibernating animals, but this may be due to other reactions of oxygen with fats, whereby substances richer in oxygen are produced, which are not carbohydrate. The subject is an important one, for the unlocking of the secrets of the processes of oxidation of fat in hibernating animals would reveal the nature of the reactions by which the potential energy of the fats is made available for the body.

It is in the muscles that the greater part of energy transformation takes place, and attempts have therefore been made to determine how muscle, in doing work and evolving heat, makes use of fat. Leathes (1906) found no evidence of fat consumption in muscles which had been tetanised for several hours by stimulation of the sciatic nerve. Winfield (1915), using surviving frog's muscle, found no change in fat content after simple rigor or prolonged stimulation under anærobic conditions. The exhaustive process of stimulation under anærobic conditions followed by recovery in oxygen, several times repeated, also produced no change in fat content. It is now generally accepted that the cycle of processes associated with the contraction of muscle (contraction—relaxation—recovery) is the same in the muscles of the intact animal as in isolated muscle, so that the experiments of Leathes and Winfield indicate that muscles do not make use of fat directly, in the contraction process. On the other hand, Lafon (1913) has found by a comparison of the amount of fat in the arterial and venous blood of the *levator labii superioris* of the horse and ass, that more fat was removed from the blood when the muscle was working than when at rest. He also states that a similar change may be demonstrated in a dog's hind limb when the muscles are excited electrically. Krogh and Lindhard (1920), in a series of careful experiments, have recently compared the efficiency of fat and carbohydrate for the supply of energy to the muscles during work. They find that when fat is utilised to supply the energy

then 4·6 calories represent the expenditure per calorie technical work, and that when carbohydrate is catabolised to supply the energy, 4·1 calories are expended. There is thus a waste of energy of about 11 per cent. when fat is used by muscle. They also find that on a fat rich diet, producing a low R.Q., the quotient rises on transition from rest to work. On a carbohydrate rich diet with a high R.Q. the reverse is found. When the R.Q. lies between 0·8 and 0·9, there is little or no change on transition from rest to work. Krogh and Lindhard conclude provisionally from these results that when the R.Q. is below 0·8 some fat is being converted into carbohydrate and when it is above 0·9 carbohydrate is being converted into fat. They also conclude that under ordinary conditions the amounts of carbohydrate and fat consumed by muscles during rest and work is a function of the relative available quantities of the two substances. It is a consequence of these views that neither fats alone nor carbohydrates alone are suitable for the energy requirements of the body but that the utilisation of either of these substances requires the concurrent catabolism of the other. That the oxidation of fat only proceeds to completion when carbohydrates are being oxidised at the same time is generally held to be true, and the evidence for this will be discussed later, but the hypothesis that the oxidation of carbohydrates only takes place completely when fats are being catabolised is a new one, and so far there appears to be no evidence in its favour other than that supplied by Krogh and Lindhard. The current conception of the working of muscle as a machine certainly provides no explanation of the hypothesis provisionally put forward by Krogh and Lindhard. The work of Fletcher and Hopkins, Hill, Embden, Meyerhof, and others, has combined to produce a fairly clear picture of the processes in a muscle, which result in the transformation of the stored energy of carbohydrate into heat and external work. It is briefly as follows: Three phases of the process are distinguished. Firstly, the phase of contraction, in which some precursor, apparently carbohydrate, or closely related substance, is decomposed, and gives rise to lactic acid. In this phase the condition of tension is set up. Secondly, the phase of relaxation, in which the lactic acid is neutralised, or its action on the muscle fibre annulled, by combination with some substance or substances in the muscle fibre. In this phase the condition of tension disappears. Thirdly, the phase of recovery, in which the lactic acid liberated in the first phase, disappears. This is the phase in which oxidation processes predominate, and, as a result, a part of the lactic acid disappears entirely, with the evolution of an equivalent amount of carbon dioxide, while the rest of the acid is reconverted into

the precursor. The R.Q. of this phase is unity, and, therefore, some substance is oxidised, which has the general formula, $C_x(H_2O)_y$. Whether this substance is lactic acid, or carbohydrate, is uncertain, but it cannot be fat, because the R.Q. of this stage is not 0·7, and the experiments of Winfield show that fat does not disappear from muscle that is subjected to alternate stimulation and recovery. It is, therefore, necessary to seek for some other situation in the body in which the oxidation of fat takes place. Furthermore, it also appears necessary, if the current explanation of the action of the muscular machine is accepted, that the oxidation of fat must give rise to substances which are either carbohydrates, or closely allied to them. It may be said at once that what is known at present of the oxidative degradation of fat in the body supplies no inkling of the means by which fat is made use of in muscle. The products, which have been recognised as coming from fat as a result of oxidation, are not related to the carbohydrates, nor do they fit in any way into the currently accepted scheme of muscular contraction.

The following general methods have been used in seeking information as to the nature of the structural changes which fatty acids undergo on oxidation in the body: (1) lower fatty acids have been administered, and intermediate products of their oxidation have been sought for in the urine; (2) phenyl derivatives of the lower fatty acids have been given to animals, and the products of oxidation isolated from the urine; (3) fatty acids have been administered to diabetic animals and man, and their influence on the excretion of glucose and the "acetone bodies" determined; (4) the surviving liver has been perfused with blood to which salts of the lower fatty acids have been added; (5) fats or fatty acids, differing in composition from the normal fat of animals, have been given in the food, and the organs of the animal have been examined subsequently for their presence in the original or some altered form.

Schotten (1882), and others, have administered sodium salts of the normal fatty acids from formic to caproic to dogs by the mouth, and have found no oxidation products in the urine. Formic and acetic acids appear to be less readily oxidised than the higher members of this group. More recently, Blum and Woringer (1920), have detected small amounts of lævo-lactic acid in the urine of dogs that had received subcutaneous injections of considerable amounts of sodium propionate. Intravenous injection of the same salt in rabbits yielded a little pyruvic acid in the urine. Blum and Woringer conclude from these results that propionic acid undergoes oxidation at the α carbon atom, pyruvic acid being produced, and that this then undergoes asymmetric reduction

to give lævo-lactic acid, a process that pyruvic acid is known to undergo in the body—

$$CH_3 . CH_2 . COOH \rightarrow CH_3 . CO . COOH \rightarrow CH_3 . CH(OH) . COOH.$$

Lactic acid may, however, be found in the urine following the administration of other simple acids such as butyric, β-hydroxybutyric and malonic [Knoop and Jost (1923)], so that its formation from propionic acid directly by α-oxidation is open to doubt.

The scarcity of data regarding the intermediate stages of the oxidation of fatty acids was relieved when Knoop (1904), published his classical investigations on the fate in the body of phenyl derivatives of the lower fatty acids. The series of acids investigated by Knoop, and the products which they yielded in the urine when their sodium salts were administered to dogs, are given in the following table :—

Acid Administered.	Formula.	Acid found in Urine (Combined with Glycine).
Benzoic . . .	$C_6H_5 . COOH$	$C_6H_5 . COOH$
Phenylacetic . .	$C_6H_5 . CH_2 . COOH$	$C_6H_5 . CH_2 . COOH$
Phenylpropionic .	$C_6H_5 . CH_2 . CH_2 . COOH$	$C_6H_5 . COOH$
Phenylbutyric .	$C_6H_5 . CH_2 . CH_2 . CH_2 . COOH$	$C_6H_5 . CH_2 . COOH$
Phenylvaleric . .	$C_6H_5 . CH_2 . CH_2 . CH_2 . CH_2 . COOH$	$C_6H_5 . COOH$

These results led Knoop to conclude that the oxidation of the fatty acid side chain of these aromatic acids resulted in the removal of two carbon atoms at each stage with the production of a fatty acid side chain containing two carbon atoms less than the original acid, and when benzoic or phenylacetic acid were eventually produced they were excreted in the urine, since they are very resistant to oxidation in the body. The changes undergone by phenylvaleric acid may be pictured thus—

$$C_6H_5 . CH_2 . CH_2 . CH_2 . CH_2 . COOH \rightarrow C_6H_5 . \mathbf{C}H_2 . CH_2 . COOH \rightarrow C_6H_5 . COOH.$$

The carbon atom which is oxidised to give the COOH group is indicated by heavier type. The formation of phenylpropionic acid as an intermediate product between phenylvaleric acid and benzoic acid in the oxidation of the former was proved by Dakin (1909). Knoop's results led to the formulation of the theory of β-oxidation. According to this theory the fatty acid side chains of the phenyl fatty acids undergo oxidation at the β-carbon atom, the two end carbon atoms being removed and an acid formed containing a side chain,

shorter by two carbon atoms. It was pointed out by Knoop that this process was probably applicable to the oxidation of the higher fatty acids normally found in the body. If this were so, then a degradation of these fatty acids step by step, would result in the formation of a series of acids, each containing two carbon atoms less than its precursor and finally, since the naturally occurring fatty acids contain an even number of carbon atoms, butyric and eventually acetic acid would be produced. Important evidence for the formation of butyric acid is furnished by the fact that two derivatives of this acid, namely β-hydroxybutyric acid and acetoacetic acid are excreted in the urine in severe cases of diabetes when a considerable amount of fat is being oxidised and there is marked inability of the body to utilise carbohydrates for the liberation of energy, [Minkowski (1884), Geelmuyden (1897), Magnus Levy (1899), and others]. It has been pointed out, however, by Hurtley (1916), who has made a careful analysis of the available data, including those from his own valuable investigations of diabetic patients, that there is no absolutely direct proof that the β-hydroxybutyric acid and acetoacetic acid, together with the acetone which arises from the hydrolysis of the latter, which are found normally in such cases, are derived from fat. There is, however a considerable amount of indirect evidence. Normal, healthy individuals excrete a small amount of acetone in the urine, usually less than 100 mgrms. in 24 hours. The amount increases when large amounts of fat are taken and butter fat appears to cause the greatest increase. The increases are, however, not great, the absolute amount of acetone excreted represents only a minute fraction of the fat given and in some cases no increase has been obtained [Geelmuyden (*loc. cit.*), Hagenburg (1900), Schumann Leclercq (1901)]. Other evidence in favour of the origin of "acetone bodies" from fat comes from a quantitative study of the excretion of these substances in severe diabetes. This was first carried out by Magnus Levy (1901), and the results have been extended and confirmed by others. The amount of carbon in the "acetone bodies" excreted over a given period is sometimes greater than that of the carbon in the protein catabolised during the same time, so that the whole of these substances cannot arise from protein. But it is well established that in severe cases of diabetes such as Magnus Levy investigated, a considerable amount of the protein which is catabolised gives rise to the formation of glucose, so that only a part is left for "acetone body" formation. The evidence for the participation of fat in the formation of these substances is therefore strengthened. Some collateral evidence on this point has recently been published by Kahn (1923). Kahn

prepared a triglyceride of margaric acid which contains an odd number of carbon atoms and, therefore, should not give rise to the formation of acetone bodies if it underwent successive β-oxidation. It is absorbed to the extent of about 95 per cent. On giving this to a fasting normal man or to several diabetics with ketosis the "acetone bodies" disappeared from the urine. Sevringhaus (1924) has, however, not been able to find any diminution of "acetone body" excretion when glyceryl margarate in amounts up to 100 grms. daily was taken by a normal person who was excreting acetone bodies owing to a deficiency of carbohydrate in the diet. The results of further experiments on this interesting and important question will be awaited with interest. The inorganic sulphates of the urine give an indication of the extent of protein catabolism, and Satta (1904), has shown that there is no constant relationship between the excretion of sulphates and acetone in the urine in acetonuria. In addition, in starvation, normal individuals excrete "acetone bodies" and this increase in acetone is accompanied by a decrease in the urinary nitrogen excretion.

Evidence of quite a different kind in favour of Knoop's hypothesis has been furnished by Dakin (1908-9), who showed that when phenylpropionic acid was administered to animals the urine contained besides hippuric acid, a series of substances analogous to the "acetone bodies." These were, β-phenyl-β-hydroxypropionic acid, benzoylacetic acid and acetophenone. In addition to these substances cinnamic acid was also detected—

$C_6H_5.CH_2.CH_2.COOH \rightarrow C_6H_5.CH(OH).CH_2.COOH$,
β-phenyl-β-hydroxypropionic acid.

$C_6H_5.CO.CH_2.COOH$, Benzoylacetic acid. $C_6H_5.CH{:}CH.COOH$, Cinnamic acid. $C_6H_5.CO.CH_3$, Acetophenone.

$C_6H_5.COOH$.
Benzoic acid.

The acetophenone, no doubt, arose from the decomposition of benzoylacetic acid just as acetone arises from acetoacetic acid, but the production of β-phenyl-β-hydroxypropionic acid and benzoylacetic acid, which may be considered as derived from β-phenylpropionic acid by oxidation at the β-carbon atom, suggests that the "acetone bodies" met with in diabetes, and related conditions arise from the β-oxidation of butyric acid. Dakin also showed that β-oxidation of fatty acids took place if their ammonium salts were warmed with hydrogen peroxide. Thus ammonium butyrate, if digested at 37°, with hydrogen peroxide, gave rise to acetone and acetoacetic acid, as well as other products.

Further evidence of the oxidation of fatty acids in the body, with the production of "acetone bodies," has been put forward by Embden and his co-workers (1906, 1908), on the basis of liver perfusion experiments. When the liver of a freshly-killed dog is perfused with defibrinated blood, a small production of acetone and acetoacetic acid is observed. The latter has not been identified as such, but its presence is inferred from the fact that some substance is present in the blood after perfusion, which yields acetone on heating. When different normal fatty acids were neutralised, and added to the blood with which the liver was perfused, it was found that those acids, containing an even number of carbon atoms, increased the formation of acetoacetic acid, whereas those with an odd number did not. Acids from butyric to decoic were investigated. The following table makes this relation clear:—

Acid Added to Blood.	Number of Carbon Atoms.	Acetone Formed per Litre of Blood, in mgs.
No addition . . .	—	12-27
Butyric acid . . .	4	128
Valeric acid . . .	5	20
Hexoic acid . . .	6	100
Heptoic acid . . .	7	12
Octoic acid . . .	8	60
Nonoic acid . . .	9	19
Decoic acid . . .	10	58

This difference in behaviour of the acids, with an even and uneven number of carbon atoms, supports Knoop's hypothesis in a striking fashion, for an acid, with an even number of carbon atoms by a series of successive β-oxidations, would eventually yield butyric acid, which, in turn, would give acetoacetic acid, whereas acids, with an uneven number of carbon atoms, would yield propionic acid, and this has been shown not to yield acetoacetic acid in liver perfusion experiments. On the other hand, perfusion experiments of this kind are open to criticism. Fully oxygenated blood must be used, whereas the liver is normally supplied with a mixture of oxygenated and venous blood via the hepatic artery and portal vein respectively. The yield of acetone is less than 20 per cent. of the theoretical, reckoned on the basis of the amount of acid added to the perfusion fluid. Acetoacetic acid has not been definitely identified as the parent substance of the acetone, though this seems very probable. The acetone has been estimated by the iodoform method, but has only been identified definitely in one instance—the experiments with butyric acid—and it is possible that other iodoform yielding substances, besides acetone, have been estimated as acetone.

On the whole, the experiments of Knoop, Dakin, and the Embden school may be taken to indicate that the lower fatty acids and their phenyl derivatives undergo catabolism at least in part by β-oxidation in the body, and the excretion of acetone bodies when the metabolism of carbohydrates is in abeyance lends support to the view that the higher fatty acids also undergo this change.

The mechanism of β-oxidation is not yet clear. The chief evidence which indicates the nature of the changes that are involved has been obtained by studying the fate of phenyl and furyl derivatives of propionic acid when administered to animals. It has already been mentioned that on giving β-phenylpropionic acid subcutaneously to dogs or cats, Dakin was able to isolate the following substances from the urine: β-phenyl-β-hydroxypropionic acid, benzoylacetic acid, cinnamic acid, acetophenone and benzoic acid. In vitro, benzoylacetic acid easily loses carbon dioxide and is converted into acetophenone, so that it is very likely that the latter arises from the former in the body by simple hydrolysis—

$$C_6H_5 . CO . CH_2 . COOH + H_2O = C_6H_5 . CO . CH_3 + H_2O + CO_2.$$

Also benzoylacetic acid on reduction gives rise to β-phenyl-β-hydroxypropionic acid. The latter on oxidation is reconverted into benzoylacetic acid, or by the loss of water it may give rise to cinnamic acid. Cinnamic acid, by the addition of the elements of water, may be converted into β-phenyl-β-hydroxypropionic acid—

$$C_6H_5 . CO . CH_2 . COOH \underset{\text{oxidation}}{\overset{\text{reduction}}{\rightleftharpoons}} C_6H_5 . CH(OH) . CH_2 . COOH.$$

$$C_6H_5 . CH(OH) . CH_2 . COOH \underset{+ H_2O}{\overset{- H_2O}{\rightleftharpoons}} C_6H_5 . CH : CH . COOH.$$

From a consideration of these reactions, which may be caused to take place *in vitro*, it is apparent that any one of the three acids, benzoylacetic, β-hydroxy-β-phenylpropionic or cinnamic may be the primary oxidation product and the other two secondary. There is still some doubt as to which is the primary product of oxidation of β-phenylpropionic acid in the body. On administering any one of these acids to animals, the other two are produced and excreted in the urine, and all three are oxidised in part to benzoic acid. That the β-hydroxy acid is the first product of oxidation is rendered improbable by several facts. Dakin (1922) has shown that it is much less readily

oxidised in the body than phenylpropionic acid. This would tend to make it accumulate if it were produced by direct oxidation of the latter, and considerable amounts of it should be excreted in the urine. This is not found to be the case. It has been shown by Blum (1910), Dakin and Wakeman (1910), Friedmann and Maase (1910), and Neubauer (1910), that acetoacetic acid is easily reduced asymmetrically by fresh liver tissue to give β-hydroxybutyric acid, and Friedmann (1910) and Dakin (1911) have shown that benzoylacetic acid undergoes the same kind of change, yielding lævo β-hydroxy-β-phenylpropionic acid. The lævo acid is also the one found in the urine when phenylpropionic or benzoylacetic acid is given to animals, and this renders it probable that in part, at any rate, the hydroxy acid is produced by reduction of the keto acid. These results do not definitely exclude the formation of the β-hydroxy acid as the primary oxidation product, but they render it very unlikely. Whether benzoylacetic acid or cinnamic acid is first produced by oxidation of phenylpropionic acid is much more difficult to decide. The oxidation of fatty acids in vitro has so far not been shown to yield α-β unsaturated acids, but Dakin (1908) has shown that β-ketonic acids are formed when hydrogen peroxide is the oxidising agent. This does not exclude the possibility of the formation of α-β unsaturated acids by oxidation in the body, but it renders it more probable that the β-keto acid is the first product. The formation of unsaturated acids by loss of water from β-hydroxy acids is a well-known chemical reaction, and because of its common occurrence in vitro has generally been considered as a satisfactory explanation of the formation of cinnamic acid from phenylpropionic acid—

$$C_6H_5 . CH_2 . CH_2 . COOH \rightarrow C_6H_5 . CO . CH_2 . COOH$$
$$\rightarrow C_6H_5 . CH(OH) . CH_2 . COOH \rightarrow C_6H_5 . CH : CH . COOH.$$

But it is not possible to decide on this mode of formation as against that by direct oxidation of phenylpropionic acid until more quantitative evidence is forthcoming regarding the relative ease of oxidation of these various acids in the body. Such data are difficult to get because it may always be argued legitimately that the rate of oxidation of a substance produced in the tissues in small amounts may be very different from that which results when larger amounts are injected subcutaneously or given by the mouth. Bearing this in mind and giving it due weight when interpreting such experiments, the available quantitative evidence appears to favour the idea of a direct production of cinnamic acid from phenylpropionic acid, and not by intermediate stages from benzoylacetic acid and hydroxyphenylpropionic acid. Dakin (1908) has shown that

the last-named acid is much less readily oxidised than phenylpropionic acid, and experiments of Friedmann (1910), with benzoylacetic acid, indicate that much less benzoic acid is formed from this acid than from phenylpropionic acid when doses of a similar magnitude are given. The formation of cinnamic acid from phenylpropionic acid is not the only instance of its kind in the body. Sasaki (1910), on giving furfurpropionic acid to animals found furfuracrylic acid in the urine—

$$C_4OH_3 . CH_2 . CH_2 . COOH \rightarrow C_4OH_3 . CH : CH . COOH.$$

Einbeck (1913, 1914, 1922) has shown that isolated muscle will convert succinic acid into fumaric acid and the latter then takes up water to form malic acid—

$$COOH . CH_2 . CH_2 . COOH \rightarrow COOH . CH : CH . COOH$$
$$+ H_2O$$
$$\dashrightarrow COOH . CH(OH) . CH_2 . COOH.$$

Further, Leathes and Meyer-Wedell (1909) on administering unsaturated oils to certain animals, found, in the liver, fats with a higher iodine value than that of the oil given, and therefore formed from it probably by desaturation. The idea therefore, that unsaturated acids may arise directly in the body by the oxidation of saturated acids is supported by biochemical evidence, but still lacks confirmation by its demonstration *in vitro*. Dakin (1923) has made an important investigation on the ease of oxidation in the liver, as measured by acetone and acetoacetic acid formation in the perfused organ, of caproic acid, β-keto-caproic acid, β-hydroxy-caproic acid and α, β-hexenic acid. The amount of acetone obtained in these experiments, done under strictly comparable conditions, was such as to indicate that these four acids were all equally oxidised, and suggested that the α-β unsaturated, β-keto and β-hydroxy acids are readily interconvertible. The available evidence as to the mechanism of β-oxidation in its early stages is therefore not yet sufficient to give a decision as to the relative importance of β-ketonic, or α-β unsaturated acids, as intermediate stages in its progress. Whatever the intermediate stages of β-oxidation may be, the final result is the removal of the two end carbon atoms of the acid attacked whereby an acid of smaller molecular weight is produced. Attempts to discover the mechanism of this process have so far been fruitless. It is natural to think, in this connection, of the well-known acid hydrolysis of acetoacetic acid, in which, under the influence of strong alkalies, two molecules of acetic acid are produced—

$$CH_3 . CO . CH_2 . COOH + H_2O = 2CH_3 . COOH.$$

But no evidence has been obtained that this reaction takes place in the body. Although fresh liver tissue is able to cause the disappearance of acetoacetic acid when the two are brought together, this is not due to acid hydrolysis but to reduction of the acetoacetic acid to β-hydroxybutyric acid. It seems more probable that the ultimate steps by which acetoacetic acid is decomposed are oxidation processes. Acetoacetic acid is readily oxidised by hydrogen peroxide (Dakin, 1922, p. 43), and Shaffer (1921) has shown that, in this reaction, the presence of glucose greatly accelerates the disappearance of the keto acid. The further study of the nature of this reaction promises to be of much importance, for its elucidation may provide an explanation of the mode of removal of the two end carbon atoms in the final stages of β-oxidation. It is also an *in vitro* analogy to the well-known inhibiting effect of carbohydrates on the formation of the "acetone bodies" in diabetes and allied conditions.

The effect of carbohydrates in preventing the formation of "acetone bodies" was first pointed out by Hirschfeld (1895), and it is now a well-established fact. The excretion of the "acetone bodies" in the urine only occurs in conditions of carbohydrate starvation and is independent of the method by which the starvation is brought about. It may be due to disease such as diabetes, to the influence of drugs such as phlorrhizin or to voluntary abstention from carbohydrate food. When the acetonuria has made itself manifest as a result of simple starvation the administration of carbohydrate causes its prompt disappearance. To describe the formation of "acetone bodies" the word *ketogenesis* has been coined and the name *antiketogenesis* is therefore used to designate the effect of carbohydrates in inhibiting their formation. Since the "acetone bodies" are undoubtedly produced to a large extent by the incomplete oxidation of fat it follows that fats are only completely oxidised when carbohydrates are being metabolised at the same time. This antiketogenic action of carbohydrate has received several explanations, but none of them are entirely satisfactory because of the difficulties of direct proof. The simplest explanation is to regard ketogenesis as a manifestation of over production of a substance, *e.g.*, acetoacetic acid, under conditions in which it cannot be oxidised as rapidly as it is produced, much as lactic acid is formed in severe muscular exercise more rapidly than it can be oxidised and therefore increases in amount in the blood and may escape in the urine. Deprivation of carbohydrate increases the body's need of fat and this flooding of the metabolic mill with fat may conceivably result in its incomplete oxidation and the production of "acetone bodies." It is possible that

repeated fasting would produce some adaptation of the body with regard to its power to oxidise fat and this is in accord with the observations of Folin and Denis (1915) on two obese patients who were subjected to repeated fasts. The above view of ketogenesis suggests that the antiketogenic effect of carbohydrates is due to their fat sparing action and is simply the result of introducing into the body a substance that is oxidised in preference to fat. To determine whether this represents the true state of affairs it would be necessary to equate the amount of carbohydrate which just suffices to inhibit "acetone body" formation to the amount of fat which had been catabolised in the production of the "acetone bodies" which have disappeared. In other words it would be essential to know how many molecules of any one of the "acetone bodies," *e.g.*, acetoacetic acid, are produced from each molecule of fat catabolised, and of that we are still in ignorance. Other attempts to explain antiketogenesis invoke hypothetical chemical reactions between glucose or substances closely related to it chemically, and one or other of the "acetone bodies." Geelmuyden (1904) has proposed a combination of "acetone bodies" and carbohydrate before the oxidation of the former can proceed, and he suggests that the compound formed by "acetone bodies" with carbohydrate is possibly a glycuronate. No experimental evidence has been brought forward of such a conjugation and the uncertain position of glycuronic acid as a normal intermediary metabolite of glucose renders it unlikely. Woodyatt (1910, 1916) suggests that antiketogenesis is due to a reaction of glucose, glyceric aldehyde, or some simple sugar, with acetoacetic acid, which is of the nature of a coupled reaction, the sugar being oxidised and the acetoacetic acid simultaneously reduced. There is no experimental evidence for this view nor for that of Ringer (1914), who ascribes antiketogenesis to a hypothetical combination between glucose and β-hydroxybutyric acid in order that the latter may be oxidised. Underlying these various speculations is the fundamental idea that antiketogenesis is due to a chemical reaction between glucose or its metabolites and one or other of the "acetone bodies." It implies that all the fat that undergoes catabolism can only do so completely when carbohydrates are being catabolised at the same time, and that the interaction between the two takes place when the fats have reached the stage of degradation represented by acetoacetic or hydroxybutyric acid. The reaction, whatever it may be, should be capable of expression by a chemical equation in which one or more molecules of glucose react with one or more molecules of acetoacetic or hydroxybutyric acid. As a step towards determining whether this view is

true, Shaffer (1921, 1922) has recently attempted to calculate the molecular quantities of carbohydrate and "acetone bodies" involved, by a study of the composition of the food mixture, which is being metabolised in the body in cases of acetonuria. The cases studied were of two types: (*a*) those of mild acetonuria, produced in normal people, by a restricted diet, or by starvation, and (*b*) some cases of the severest form of diabetes, in which the acetonuria was very marked. The method used by Shaffer is briefly as follows: The food mixture metabolised by the body is considered to contain substances which give rise to acetone bodies as necessary intermediates in their metabolism (*ketogenic substances*), and substances which, in the process of their metabolism, oppose the formation of acetone bodies, or are essential in promoting their oxidation (*antiketogenic* or *ketolytic substances*). The ketogenic portion consists of (1) the fatty acids derived from neutral fat, which are assumed to form 1 molecule of acetoacetic acid per molecule of fatty acid, and (2) the leucine, tyrosine, and phenylalanine of the protein metabolised, which are also supposed to form equimolecular quantities of acetoacetic acid. To calculate the amount of these, an analysis of ox muscle protein by Osborne has been used, but the ketogenic value obtained by this method was subsequently raised by 50 per cent. (Shaffer, 1922). The antiketogenic portion consists of (1) carbohydrates, (2) a portion of the protein metabolised, *i.e.*, that part which, in severe diabetes, is converted into glucose, and (3) the glycerol of fat. It was assumed, in the first instance, by Shaffer (1921-22), that 1 molecule of acetoacetic acid reacted with 1 molecule of glucose, or some closely related antiketogenic substance, but further investigation has shown that better agreement between the theoretical and practical results is obtained, when it is assumed that 2 molecules of acetoacetic acid enter into reaction with 1 molecule of glucose (Shaffer, 1922). The food mixture undergoing metabolism in the body is determined from the urinary nitrogen excretion together with the results of direct or indirect calorimetry. From these, the amounts of ketogenic and antiketogenic substance undergoing metabolism are calculated, and expressed in gramme molecular quantities. If the ratio of ketogenic to antiketogenic molecules is greater than 2, then acetone bodies begin to be excreted in the urine. If the ratio is less than this, then acetonuria does not occur, or is very slight. Calculations made by Shaffer from data on diabetic and other cases, which have been published from time to time, bear this out, and it is obvious that these deductions have an important bearing on the dieting of diabetic patients. But that aspect of the matter cannot be further dealt with here. What is more important for the point

at present under discussion, namely, the rôle or mechanism of anti-ketogenesis as it bears on the oxidation of fat, is to determine whether calculations of the ketogenic ratio support the idea of a quantitative reaction between definite molecular quantities of acetoacetic acid and glucose. If the ketogenic ratio exceeds 2, then the excess of ketogenic substance undergoing metabolism gives rise to "acetone body" excretion. Further, it should be possible to predict the amount of these "acetone bodies" when the food mixture undergoing metabolism is known. So far, the available cases suitable for the calculation of the ketogenic excess have generally given results which approximate to those predicted when Shaffer's method of calculation is used. A summary of the figures from some of the cases is given in the following table:—

Nature of Case.	Period Used for Calculation of Ketogenic Excess.	Total Acetone Bodies as Hydroxybutyric Acid. Grms.	
		Expected.	Found.
Fasting . . .	27 days	133	162
,, . . .	1 day	20·8	24·6
,, . . .	1 ,,	28·3	17·3
,, . . .	1 ,,	29·8	20·1
,, . . .	5 days	88	72
,, . . .	4 ,,	8·3	17 (daily average)
Severe diabetes . .	4 ,,	369	247
,, ,, . .	5 ,,	193	193
,, ,, . .	9 ,,	130	95
,, ,, . .	8 ,,	510	634

The agreement is as good as can be expected in view of the uncertainty of the figures for the total metabolism in some of the cases, and also because of the nature of the assumptions that have to be made. The chief difficulties in the way of accepting Shaffer's conclusions in support of the idea of a quantitative reaction between "acetone bodies" and glucose lie in the fact that, to get agreement even as good as that shown in the above table, assumptions have to be made for which, as yet, there is insufficient evidence. The basis for the calculation of the ketogenic value of the protein metabolised is unsatisfactory. An analysis of muscle protein was first used to determine the amounts of those amino acids which yield acetone in liver perfusion experiments, and the value thus obtained was increased by 50 per cent. because the calculated results agreed better with the experimental ones when this higher figure was used. It is possible to justify this procedure to some extent, for it is well known that the methods for the estimation of

certain amino acids in proteins are deficient, and it is likely that the published analytical results are too low. Another difficulty in these calculations is the determination of the amount of fat which is metabolised during the experimental period. This has to be carried out indirectly from the figures available for the total metabolism of the subject, and, in many cases, the basal metabolism only has been measured, and arbitrary additions have been made to cover increased metabolism resulting from muscular movement during the day. It has to be assumed also that acetone bodies cannot be oxidised to any extent in the absence of carbohydrate. And, finally, it is assumed, though there is absolutely no proof of the fact, that each molecule of fatty acid metabolised gives rise to a molecule of acetoacetic acid. At present, therefore, owing to the nature of the assumptions that have to be made, the results of Shaffer cannot be finally accepted as indicating that the only path for the catabolism of fatty acids is *via* acetoacetic acid, or that a quantitative reaction between this acid and glucose or some closely related substance has been definitely proven.

There is another source of evidence by which the ability of the theory of β-oxidation to explain the facts of fat catabolism may be tested. This is the series of experiments carried out by Knoop and Dakin on the oxidation in the body of phenyl derivatives of the simpler fatty acids. These have already been referred to. The acids investigated give as end products of their oxidation either benzoic or phenylacetic acid. Now both these acids when administered to an animal are practically unattacked, and are excreted quantitatively in the urine in combination with glycine. It may be assumed, therefore, that if either of these acids arise in the body as end products of the oxidation of higher acids of the same series, then for each molecule of the higher acid, 1 molecule of benzoic or phenylacetic acid, as the case may be, should be produced. One gramme molecule of phenylpropionic acid, for instance, should give rise to 1 gramme molecule of benzoic acid, and 1 of phenylbutyric to 1 of phenylacetic acid. The extent to which these expectations are realised may, therefore, be used as a measure of the completeness of β-oxidation. It is unfortunate that the experimental data available which can serve as evidence for the discussion of this question are very scanty. In many cases the experiments have been carried out with only the qualitative end in view, but some of them are capable of a roughly quantitative interpretation. For instance, Dakin (1908), administered 5 grms. of phenylbutyric acid to a dog, and obtained a little over 2 grms. of phenaceturic acid together with a very small amount of phenylhydroxybutyric acid. If β-oxidation

were the only process occurring, then 5·9 grms. of phenaceturic acid should have been obtained. The yield was thus not much over 50 per cent. Similarly, Knoop obtained 0·5 grm. of hippuric acid from 1·5 grm. of phenylvaleric acid; a yield of 33 per cent. Again, from 2 grms. of cinnamic acid, 1·2 grm. of hippuric acid was produced; a yield of 50 per cent.; and 0·65 grm. of phenaceturic acid was obtained from 2 grms. of phenyl isocrotonic acid; a yield of 27 per cent. So far as these examples go they do not support the idea of exclusive β-oxidation.

The liver perfusion experiments of the Embden school in which the acetone forming capacity of the salts of a series of the lower fatty acids was demonstrated are not capable of quantitative interpretation for the purposes of the present discussion. The amount of acid actually attacked was not determined so that it is impossible to calculate the percentage of acetone that it yielded. It is, however, of interest that in no case was the yield of acetone greater than 20 per cent. of the theoretical, assuming that all the acid used in the experiment was attacked and that successive β-oxidation had taken place.

It may be concluded therefore that, so far as present evidence shows, the theory of successive β-oxidation is insufficient to explain completely the catabolism of fatty acids. Each molecule of fatty acid undergoing catabolism has not been proved to give rise to 1 molecule of acetoacetic acid, as the theory would require, but to a smaller amount. The natural inference is that some other oxidation process proceeds as well. The only other catabolic process which fatty acids have been shown to undergo, and which may be considered as an oxidation, is that first demonstrated by Leathes and Meyer-Wedell (1909). This is the formation of unsaturated fatty acids from the higher saturated acids or the increase in unsaturation of unsaturated acids. The experiments which led to the discovery of this change were carried out by administering to rats, oils of known iodine value. Some hours later the animals were killed, the fatty acids isolated from the liver and their iodine value determined. It was found in many cases that the iodine value of the liver fatty acids was higher than that of the fatty acids in the oil fed and still higher than that of the acids in the livers from control animals. It was deduced from these results that it is a function of the liver to render saturated acids unsaturated or to increase the degree of unsaturation of unsaturated acids. The possibility that the observed increase in iodine value is due to the shifting of double linkages in the fatty acid chain in such a manner as to render them more liable to react with iodine cannot be excluded, but it is rendered improbable by other evidence in favour of desaturation.

Hartley (1907-9), found that the fatty acids of pig's liver contain an oleic acid in which the position of the double linkage is very different from that in the oleic acid present normally in pig's connective tissue fat. Since fat is mobilised to the liver prior to oxidation it is reasonable to assume that this new oleic acid is produced in the liver from stearic acid. Experiments by Raper (1913), in which cocoanut oil was administered to animals in various ways and the liver subsequently treated for the recovery of the volatile fatty acids from the cocoanut oil found there, showed that these fatty acids had a greater power of taking up iodine than the volatile fatty acids of the original oil.

The exact significance of the process of desaturation is not known. It is true that the unsaturated fatty acids are more liable to oxidative attack because of their double linkages than the saturated acids, but there is no proof that this accepted fact of the chemical laboratory applies equally to that of the living organism where oxidations are brought about by very different methods. It may be accepted provisionally, however, that the unsaturated acids are probably liable to attack in the body at the positions of the double linkages and that a rupture of the fatty acid chain at these points precedes the oxidation of the fragments so produced. The insufficiency of the theory of successive β-oxidation as an explanation of the catabolism of fatty acids may be due to this. Some of the fragments produced from unsaturated acids by disruption at the double linkages may have an uneven number of carbon atoms, and Ringer (1912-13) has shown in the case of the lower members that such acids, if they belong to the acetic series, do not give rise to acetoacetic acid in diabetic animals but rather to increased glucose excretion. For instance, oleic acid has the unsaturated linkage in such a position that if oxidation occurred there, pelargonic and azelaic acids would be produced. These both contain 9 carbon atoms and by β-oxidation would not be expected to yield acetoacetic acid. Mottram (1924), however, has been unable to show that substitution of butter by cod liver oil, which is more unsaturated, in the diet of diabetic patients, causes any diminution in the excretion of "acetone bodies." The experiments were only of short duration.

The only intermediate products of the oxidation of fatty acids in the body which have been discovered so far, if we except the unsaturated acids referred to above, are the "acetone bodies." This fact has often given rise to the query: Why, if the theory of successive β-oxidation applies to a considerable part of fat catabolism, is it not possible to detect higher β-keto acids, or β-hydroxy acids, or even higher

ketones and acids such as butyric, caproic, caprylic, etc., in the body in conditions such as diabetes when a large amount of fat is being catabolised? If it be remembered, however, that when 100 grms. of fat a day are being oxidised in the body the rate of oxidation works out at less than a tenth of a gramme per minute, it is obvious that higher keto acids (or the hydroxy acids derived from them) would not accumulate unless they were more difficult to oxidise than their parent fatty acids. There is no evidence that this is so except that a somewhat analogous case,—the relative ease of oxidation in the body of benzoylacetic acid and phenylpropionic acid—has been studied by Friedmann (1910). The experiments show that the keto acid is less readily oxidised. It would be incorrect to apply this result without reservation to the higher fatty acids, and the subject must therefore await further investigation before a decision on this point can be reached.

A suggestion put forward by Hurtley (1916) must be mentioned. It is, that oxidation takes place not only at the β-carbon atom but concurrently at several carbon atoms with the production of compounds containing the "multiple ketene" group of Collie (1907). This change may be illustrated as follows:—

$$- CH_2 . CH_2 . CH_2 . CH_2 . COOH \rightarrow - CO . CH_2 . CO . CH_2 . COOH.$$

There is no proof of this, but the instability of such compounds would explain the non-detection of intermediate compounds other than the acetone bodies in the oxidation of fat.

The Utilisation of Fat by Muscle.—One of the main objects in studying the mode of oxidation of fat in the body is to determine how the organism makes use of fat as a source of energy either for the production of heat or the performance of external work. Since heat is continually being produced in the body even when no external work is done the process of β-oxidation together with the subsequent changes, still unknown, by which two carbon atoms at a time are removed, are sufficient to explain how heat may be produced from fat. These are oxidation processes in which heat is evolved and eventually lead to the formation of carbon dioxide and water, at which stage the maximum possible heat evolution has taken place. There is no difficulty, therefore, for the physiologist in accepting the present day conception of the oxidation processes which fat undergoes as true, in so far as it shows how fats may liberate their energy in the form of heat. That they may do so may be inferred from the experiments of Murlin and Riche (1915), who showed that injection of an emulsion of lard into a vein in dogs

caused a rise of heat production and fall of respiratory quotient. Difficulties are met with, however, when the use of fat as a source of energy for the performance of external work is considered. The muscles are the organs by which external work is done, and, as mentioned earlier in this chapter, there is no experimental evidence which indicates that muscles can use directly, the fat which they contain, although it is accepted that they can use their store of glycogen.

It is perhaps necessary to inquire in the first instance what evidence exists that muscles use fat as fuel for the contraction process. It is by experiments on the intact animal alone that the view has been established that muscles can use fat as a source of energy for the performance of mechanical work. The older experiments of Zuntz and his school were devised with the object of determining the value of fat for muscular work as compared with carbohydrate. Zuntz (1911), as a result of these investigations, concluded that fat and carbohydrate have an isodynamic value for the carrying out of muscular work. In these experiments the value of the respiratory quotient was taken as the criterion that fat was being utilised and the relative amounts of fat and carbohydrate undergoing combustion were calculated from it. Benedict and Cathcart (1913) have also made a study of the influence of diet on metabolism during muscular work, and it may be concluded from their results that for moderate work, at least, fat and carbohydrate are equally well utilised. The most recent experiments are those of Krogh and Lindhard which have already been referred to. They show that fat is utilised by muscle for the production of mechanical work, but in the transformation of the potential energy of the fat there is a waste of energy of 11 per cent. as compared with carbohydrate. These experiments then are decisive in showing that in the intact animal fat may be used to supply the energy for the muscular machine.

Experiments carried out during the last decade and initiated by the classical researches of Fletcher and Hopkins on lactic acid production in muscle, have been concerned almost entirely with the phenomena of contraction in isolated amphibian muscle. It is a disadvantage for this discussion that this is so, for it is uncertain to what extent the conclusions arrived at may be held to apply equally to mammalian muscle. If they do apply, then there appears to be no place for fat, or for those oxidation products of fat, which are known to occur in the body, in the muscular machine. The prevailing conception of the chemical and thermal changes, which have arisen as a result of this work, is that lactic acid, which is produced from carbohydrate, is formed during the contraction of the muscle, and that the acid is

neutralised during the relaxation process. In the final stage, in which oxidation processes take place, the lactic acid disappears and some carbohydrate reappears. The carbohydrate corresponds to about two-thirds to three-fourths of the lactic acid, which disappears. The gaseous exchange during this stage shows that the R.Q. is 1·0, so that fat is not oxidised to supply the energy for the synthetic process. A study of the heat changes by Hartree and Hill (1922) has shown that they are such as to suggest that the energy liberated in the oxidative stage is partly utilised in rebuilding lactic acid into carbohydrate, and part appears as heat. They conclude that, under favourable conditions, about five-sixths of the lactic acid which is formed during the contraction is rebuilt into carbohydrate (or the lactic acid precursor), and that the remaining one-sixth is oxidised. It is also possible that the whole of the lactic acid is rebuilt into carbohydrate, and that the energy for this process is got from the oxidation of some other substance, presumably carbohydrate, but the available experimental evidence is not sufficient to give a decision on this as opposed to the view that lactic acid is the substance oxidised. In this cycle of changes it is evident that carbohydrate alone can supply the muscle with its necessary fuel, and it is logical to infer from it either that fat can only be used in muscle by previous conversion into carbohydrate, lactic acid or some intermediate substance between the two, or that by some entirely unknown process, fat may be utilised independently of carbohydrate. There are two serious obstacles to overcome before the former view can be accepted. The first is, that the process of successive β-oxidations of a fatty acid molecule is inadequate to explain how carbohydrate or lactic acid or any intermediate substance containing 3 carbon atoms beween the two (such as methyl glyoxal, for instance) can be formed from fat. The second is, that in any oxidation process by which carbohydrate could be formed from fat a waste of energy would occur, and that this would be greater than that actually found by Krogh and Lindhard in their experiments on the sources of energy of the muscular machine. If it be assumed that the whole of the carbon of a molecule of fatty acid, *e.g.*, stearic acid, be converted into glucose as indicated by the equation—

$$C_{18}H_{36}O_2 + 8O_2 = 3C_6H_{12}O_6,$$

then 284 grms. of fatty acid would produce 540 grms. of glucose. Or for each gramme of fatty acid with a calorific value of 9·3, 1·9 grms. of glucose with a calorific value of 1·9 × 3·7, *i.e.* 7·0 would be formed. This represents a loss of energy of 22 per cent.,

whereas actual experiment by Krogh and Lindhard showed a loss of only 11 per cent. This difference in the theoretical and experimental values cannot at present be reconciled. It is possible that an investigation of the contraction process in mammalian muscle, analogous to the studies of Fletcher and Hopkins, Hill, Meyerhof, and others, on amphibian muscle will indicate more exactly how it makes use of fat; but the energy relations of the process as understood at present, and the correspondence they bear to the known chemical changes in which carbohydrates alone appear to be involved, make it difficult to escape from the conclusion that fat must first be transformed into carbohydrate or some closely related substance before it can be used in the contraction process.

A study of the metabolism of the heart has been made by Evans (1914), using the heart-lung preparation. The results indicate that fat is extensively used by cardiac muscle, this being inferred from the values of the respiratory quotient. From the nature of these experiments in which the heart is practically isolated, it would appear that the oxidation processes in heart muscle are such that fat may be made use of directly. They are thus different from those occurring in isolated amphibian muscle, which does not appear to be able to utilise fat directly as a source of energy for the contraction process.

CHAPTER X.

THE PART PLAYED BY FATS IN THE LIFE OF THE CELL.

THE most commonly recognised use of fats in the body is as a reserve fund of energy. In dogs in a fair state of nutrition the store of fat amounts to about 12 per cent. of the body weight, though much larger amounts than that can occur in exceptional cases. On this basis a man who has in his body 120 grms. of fat for every kilogram of his weight would have a fund of energy equivalent to 1100 calories per kgrm., or enough to provide him with 30 calories per kgrm. daily for five or six weeks, if the fat were all available for oxidation. The fact that most of the fat is deposited in adipose tissue not in solution but as fat itself allows large amounts of energy to be put by in a small volume with a very great economy of weight. All other substances capable of supplying energy require the carriage in the body of many times their weight of water. The utilisation of fat as a source of energy in muscle has been discussed above, p. 178 and p. 195.

But the substances of which an organism is composed may serve in other ways than merely as fuel. Carbohydrates themselves may form an integral and essential part of the fabric of the living machine, without which the organism could not be held together; in plants especially this is the case, but also in large classes of animals in which, for instance, a chitinous exo-skeleton is relied upon for maintaining the organic cohesion of the body. Carbohydrate groups are similarly built into the chemical structure of cartilage where they cannot be regarded merely as a reserve store of energy in the sense in which glycogen is in the cells of the liver or the muscles. Fats and compounds of higher fatty acids, in virtue of their insolubility in water as well as their general chemical inertness, are similarly capable of being put to many uses in the organisation of plants and animals. As the bee uses wax to build the cells of the honeycomb to store a concentrated solution of hygroscopic sugars safe from dilution by the moisture of its surroundings, and thus, at the same time, from invasion by lower forms of living organisms, so too, waxes and fats secreted in the superficial parts of plants protect the underlying cells from loss or access of moisture, and

also from the solvent action of the common enzymes. No less significant are the fats and waxes in the bodies of certain bacteria; in the tubercle bacillus, for instance, the peculiar fatty substances in which the cell body is enclosed or with which it is permeated have a very low iodine value, and are remarkably resistant to saponification (Bulloch and Macleod). They are probably justly looked upon as conferring on this organism much of its vitality and special powers of resistance. It is said that the bacillus of leprosy contains 35 per cent. of fats and cholesterol.

In animal cells, it is true, proteins account for much the greater part of the cell substance; and, in animals generally, the fabric, the working parts and framework of the organism, may appear to be built upon a basis of protein material. There has, indeed, been in the past a tendency to look upon "protoplasm," living matter, as exclusively of a protein nature. Protein, with the growth of more exact chemical ideas as to its composition, came to be almost the equivalent for the earlier "protoplasm," and fats and carbohydrates have been regarded merely as so much unorganised matter serving the living protein only as fuel. But there are facts which point to some at any rate of the compounds of higher fatty acids as being indispensably built into the most intimate organisation of the living cell, and forming part of the machine no less than the proteins themselves. Hoppe Seyler, in 1866, first pointed out the fact that cholesterol and lecithine are both of them to be found wherever the phenomena of life are to be observed, and taught that they stood in a very close relation to the life of protoplasm. In the same year, L. Hermann pointed out that the aliphatic narcotics, alcohol, ether, chloroform, all of them liberated hæmoglobin from the red blood corpuscles. He had recently shown the presence of lecithine in the corpuscles, and he traced both the narcotic and the hæmolytic action of these substances to their power of dissolving lecithine, cholesterol, and fatty substances. This idea was taken up more than thirty years later by Hans Meyer (1899) and Overton (1901). The large group of narcotics, called by Schmiedeberg the alcohol group, includes, besides alcohol, ether and chloroform, many other substances chemically very different from one another. Their common pharmacological properties could not depend upon any common feature of chemical constitution, and must therefore depend upon a common physical property. Meyer formulated the following theses:—

1. All substances that have the power of dissolving in fats or similar compounds, although they may be chemically indifferent, must act upon living protoplasm as narcotics whenever they have access to it.

2. Cells will be liable to this action in proportion as the fats *enter into their chemical structure and are the seat of their functions*, which is notably the case with nerve cells.

3. This action must depend upon the mechanical affinity of the substances to fat on the one hand, and to the other components of the cells, principally water, on the other, that is to say, upon the partition coefficient of the substances between fats and water.

Meyer measured the narcotic action of a number of these substances by determining the minimum concentrations which at certain temperatures caused complete narcosis of tadpoles. He determined also the coefficients of partition as between olive oil and water for the same substances at the same temperatures. And he pointed out that the narcotic action of the substances increases with the amount of them taken up by oil from water. The figures given from his paper in the following table show this:—

	Temperature.	Coefficient of Partition. Per Cent.	Minimum Concentration for Complete Narcosis. M.
Alcohol	3°	0·026	0·33
,,	36°	0·047	0·14
Chloral	3°	0·053	0·02
Monoacetin	36°	0·066	0·014
,,	3°	0·093	0·011
Acetone	3°	0·140	0·33
,,	36°	0·235	0·14
Chloral	36°	0·230	0·004
Benzamide	36°	0·437	0·005
,,	3°	0·672	0·002
Salicylamide	36°	1·400	0·0017
,,	3°	2·223	0·0008

The one irregularity that this series of figures shows, the case of acetone may, as he says, be accounted for by the fact that lecithine and such substances as lecithine behave with this solvent quite differently from oil; oil dissolves in acetone and lecithine does not. And there is evidence to show that the fatty acids which "enter into the chemical structure" of living cells do so in the form of phospholipines as much as or more than in that of simple glycerides.

According to these results, and the views based on them and on others of this nature by Meyer, the narcosis, the suspension of vital activity, which these substances induce is due to the physical changes which they bring about in the relation between fats and the other constituents of the living matter, a relation so intimate and essential that any interference is incompatible with the performance of the vital functions.

Now anyone who makes systematic estimations of the compounds of fatty acids in the organs of large numbers of animals must be impressed with two facts: firstly, that all the organs contain fat in comparatively large amount; that whereas the carbohydrates rarely exceed or even reach 1 per cent., except in the liver, the fatty acids almost always exceed 2 per cent. of the fresh tissue and very often exceed 3 per cent.; they constitute generally 10 to 12 per cent. of the dry matter. Even in the blood serum the liberated fatty acids amount to three or four times the quantity of sugar present. And seeing that in most cells these fatty acids occur largely as phospholipines the actual compounds of these acids that exist in the cells must form a considerably larger proportion of the solid matter than do the fatty acids separated from them. The other fact that cannot fail to impress itself is that no considerable variation occurs in the amount of fatty acids obtainable from any organ with the one exception of the liver: in the spleen the amount is always low, about 2 per cent.; in the voluntary muscles when free from interstitial adipose tissue it is low, except that in those muscles like the diaphragm which consists largely of red fibres it is decidedly higher, especially when for comparison muscles consisting mainly of pale fibres are taken: in the heart it is higher than in the diaphragm (Leathes, 1904; *cf.* too Embden and Lawaczek, 1923). For each type of tissue there seems to be a characteristic composition from which the departures are small in comparison with the widely different amounts that are characteristic for other organs. Observations of this nature are the foundation on which are based the views of André Mayer, developed in a long series of studies, that the compounds of fatty acids are an inseparable essential constituent of the protoplasmic system in every cell. In a cytological study, to begin with, of the mitochondria or granules of Altmann, which can be stained by fuchsin in the cells, especially of the liver, Mayer accumulates evidence for regarding these granules as composed of material of a fatty nature, probably of phospholipines, in which unsaturated fatty acids are important factors. They are completely removed from sections by solvents for these substances such as alcohol and xylol; they are fixed by acetone solutions containing a little calcium chloride, and also by many oxidising reagents; and it is only after oxidation, best by chromic and osmic acid mixtures, that they stain with fuchsin: in this respect they then behave like the hydroxy acids obtained by the oxidation of unsaturated fatty acids. The substances extracted by alcohol from the tissue behave similarly (A. Mayer, F. Rathery, and G. Schaeffer, 1914).

But a systematic series of analyses, carried out side by side with this

histological study, established the fact that the compounds of fatty acids, estimated in terms of higher fatty acids by the method of Kumagawa, in most organs were a constant quantity; in the muscles the amount varies and certain variations occur in the liver in the animals examined, otherwise the constancy for any organ is remarkable. When an animal, a dog, a rabbit, or pigeon, for instance, is starved to death it is commonly said that all the fat in the body is used up; this is not true of the fat in the kidney, lungs, spleen, and heart, not even of the liver or muscles. As was pointed out in a previous reference to this work, there is, he finds, no *élément variable* in the amount of fatty acids obtained from the first four in this list of organs; the *élément constant* accounts for the whole of the amount in them, the greater part of that usually found in the liver, and a definite not inconsiderable part in the muscles. In the cells of most organs, therefore, he argues, the higher fatty acids are present in combinations which are inseparable from the life of the cell. The fatty acids are there not as a reserve of oxidisable fuel for the evolution of energy but as an essential part of the living substance. The combinations in which the fatty acids occur appear to be to a large extent compounds containing phosphorus. Lecithine and cephaline yield about eighteen times as much fatty acid as phosphorus; in the kidney, lungs, and as a rule, too, in the liver, the proportion of the total fatty acids to the phosphorus in combinations which are soluble in ether is but little greater than in these phospholipines, so that it would appear that three-quarters or more of the fatty acids present in these organs are in this form and only a small part, one-quarter or less, in the form of simple glycerides. Indeed after complete starvation the proportion of phosphorus associated with fatty acids that he found was even higher than that which is required by the constitution of lecithine and cephaline as ordinarily accepted; as if in the cells' necessity certain of the molecules of these substances had been compelled to sacrifice one of the two fatty acid radicals they contain for oxidation, or perhaps their fatty acid chains had suffered oxidative shortening; and a picture is presented as it were of the emaciated figure of phospholipines perishing at their post in the starving cell an immovable part of its protoplasm. There is at present no evidence for the physiological occurrence of either of these intramolecular changes in phospholipines; though if they occurred they might account for some of the difficulties recognised by everyone who has worked on these substances, in obtaining pure preparations. In addition to lecithine and cephaline, which the work of Maclean, and more recently Levene, has set on sure foundations, a

number of other phospholipines have been described, the existence of which as chemical entities is questionable and which, with the exception of sphingomyeline, are dismissed by Levene as mixtures of lecithine and cephaline with the products of their own breakdown. In some of these the proportion of fatty acid to phosphorus is less than in lecithine and cephaline as found by Mayer in the organs of completely starved animals, in "cuorine," for instance, 14·5 instead of 18.

André Mayer and his fellow-workers then have done much to substantiate the conception of cell protoplasm that Hoppe Seyler, and Hermann, indicated many years ago. Protoplasm is not merely an old word for a solution of proteins; nor are the phenomena of life manifested merely in a colloidal solution of proteins in which certain electrolytes and certain reserve food-stuffs play merely subsidiary parts. Protoplasm is a complex equilibrated system in which, side by side, with colloidal solutions of proteins, fatty components together with cholesterol, though quantitatively less in amount, play a rôle which it is impossible to say is less essential than theirs.

André Mayer, moreover, thinks that this system has for each type of cell a composition characteristic for that type, and that the proportion in which the components occur in the protoplasm determines its behaviour. His estimations of water, total solids, total fatty acids, phosphorus associated with fatty acids and cholesterol in the different organs, are an attempt to define the characteristics of the protoplasm of these organs. If only he could include determinations of the amino acid groupings in their proteins, their chemical pattern and molecular structure, his attempt would bring us nearer the truth: but, with these, he is, in these studies, not concerned. The relation which he finds, however, between the "lipocytic coefficient," and the amount of water held by the living cells of each organ, is an interesting illustration of his thesis. The lipocytic coefficient, the proportion that is between cholesterol and fatty acids, is higher in the corpuscles than the serum, in the serum than in the lung, and, after the lung has decreasing values in the kidney, the liver and the muscles (Terroine and Weill, 1913). He finds that the amount of water held by these tissues in proportion to their solids, and also the amount of water they take up when immersed in pure water, varies inversely with the amount of fatty acids, and directly with the amount of cholesterol they contain. In the muscles, which contain a variable amount of reserve fat that is not built up into the protoplasmic fabric, differing in this respect from the other organs, the amount of water taken up varies directly with the cholesterol, and inversely with the amount of phosphorus associated with fatty acids.

When the proportion of cholesterol to fatty acid changes in starvation, the amount of water taken up by the tissue changes in the same sense. The red blood corpuscles of different species are hæmolysed by different degrees of dilution of the serum. Those most sensitive to lowered osmotic pressure are those that have the highest lipocytic coefficient, that contain less fatty acids, or more cholesterol, those, that is, that imbibe water most rapidly. The assumption of water by a protoplasmic system is always, according to Mayer's generalisation, limited by its fatty component, and the limiting action of this is checked and counteracted by the cholesterol associated with it.

A concrete definition of the physical relationship in the structural organisation of cells between fatty substances, on the one hand, and other constituents, on the other, is at present not possible. Attempts, however, to form some ideas on this relationship are frequently made. They may be grouped for review under two heads; those that deal generally with the structure of protoplasm, and those that are concerned with the properties, more particularly of cell membranes.

With regard to the structure of protoplasm, Bayliss has given reasons for having to regard protoplasm as liquid; the free Brownian movement of particles in living cells which stops when the cells die; the rhythmically reversed streaming movement of ingested spores in mycetozoa, described by Arthur Lister, and the fact also described by him, that the plasmodial syncytium can be made to pass through cotton wool, so as to filter off the ingested spores; and many others. In liquid protoplasm, however, fatty material is present, and in cells of most organs of higher animals, as we have seen, in relatively considerable and constant amount.

In some cells this fat is conspicuous: a single large spherical mass may occupy the greater part of the body of a connective tissue corpuscle, or sometimes of a liver cell, as if it were a parasitic organism. Its nature is obvious by its optical properties, and its behaviour with solvents or suitable stains. In other cells there may be several smaller droplets, the composition of which can be determined in the same way; and, in others, the smallest microscopic granules may be distributed throughout the cell substance, which also, by staining properties and solubility, may be recognised as of a fatty nature. The evidence given by A. Mayer for regarding Altmann's granules as being fatty material has been referred to above (see p. 202). There is evidence, too, that particles of fat in the blood plasma, at any rate, may be of ultramicroscopic size (see p. 143); it is probable that such particles occur also in cells. There may be every gradation of aggregation and dispersion

compatible with cellular dimensions and the resolving power of available optical technique.

It is well known too that the normal cells of some organs, when stained by methods that in the case of other tissues or cells successfully bring into view droplets or granules of fat, may give no evidence whatever of their containing anything of the kind, although on chemical analysis it may be possible to show that fat is present forming indeed when estimated as fatty acid seldom less than 2 per cent., generally 3 per cent. or more of the cell substance, 8 to 12 per cent. of the dry weight, although invisible. The normal heart muscle in guinea-pigs and other animals treated with a saturated solution of Scharlach R. in 70 per cent. alcohol shows no sign of the presence of fat in the cells. But within 24 hours of the injection of diphtheria toxin these cells treated in the same way may be densely studded with deeply stained droplets and granules (Dudgeon, 1906). Chemical analysis shows however that the normal heart also may contain as much fat as the deeply stained poisoned cells, or even more, though none of it is revealed by the staining. It is clear that whatever the explanation may be the fat in the cells may be microscopically invisible without necessarily being in smaller amount than when it is microscopically conspicuous; and one explanation at least is that the degree of dispersion of fat in protoplasm may not be limited by microscopic visibility. The conversion of invisible fat in cells into fat microscopically conspicuous, the unmasking or phanerosis as it has been called, then becomes merely an alteration in the degree of dispersion, or an aggregation of fat into a less highly dispersed state.

The changes that are obvious in the myelin of degenerating nerve are in part probably to be explained in this way; and the methods such as that of Herxheimer for staining fat in cells that cannot be stained by other methods, depending on the addition of 2 per cent. of sodium hydrate to the usual solution of Scharlach R. (Bell, H. Bullard) may succeed because of the change in dispersion effected by the alkali; just as the action of the diphtheria toxin is to convert fat that does not stain into fat that does by disturbing that balance of forces which is necessary to permit the high degree of dispersion of fat which is normal for heart muscle.

So far then as these facts and observations go the protoplasm of cells appears as an emulsion of fatty material in an aqueous continuous phase, and there is no obvious reason why the fatty material should maintain in any type of cell a constant proportion or why it should form any integral part of the structure of the cell at all. Nor has it been common to suppose that it does.

Leaving now for the moment this question of the distribution of the fat within the cell, we have to consider the ideas that have been current with regard to the relation of fatty substances to cell membranes.

A unicellular organism that floats in water retains its contents, products of the digestion of its food, salts and others, from diffusing into the surrounding water. A red blood corpuscle retains its hæmoglobin and allows none of it to escape in the plasma, in which this substance is easily soluble; it also retains certain salts in very different concentrations from those in which they occur in the plasma. If the plasma is diluted so as to alter the molecular concentration outside the corpuscle, water passes into the latter, but its contents do not pass out until so much water has passed in that the corpuscle appears to burst like an over-distended sac and its contents escape. Both these types of cell we say are bounded by a membrane which is impermeable to substances contained in the cell. But it is not impermeable to all substances alike; for if the plasma be diluted not with water but with a solution of urea or of ammonium chloride isotonic with the plasma, although there is no difference of osmotic pressure set up on the two sides of the surface of the corpuscle the urea and ammonium chloride, unlike sodium chloride, can pass into the corpuscle and will tend to attain the same concentration within it as without; but in doing so, in order to preserve osmotic equilibrium, they will be accompanied by water, so that the result is the same in the end as if the plasma had been diluted with pure water. We have, therefore, to say that the membrane bounding the corpuscle is specifically permeable to some and specifically impermeable to other molecules or ions. Moreover, there is evidence that these properties in many types of cells are subject to variation; on excitation, under the action of anæsthetics or of certain disturbances of a balanced proportion of oppositely endowed ions, Ca and Na, for instance, changes in the permeability of the surface layer may momentarily, or it may be lastingly, take place. The high resistance to the passage of electric currents exhibited by the corpuscles or by tissues composed of living cells, although these cells contain electrolytes, is another illustration of the restrictions imposed, in the resting condition of the cells, on the movements of ions within them.

Such phenomena imply retaining membranes composed of some material which is not dispersed in the liquid protoplasm but invests it in a coherent continuous layer; the membrane cannot be seen, it must be a mere surface film, but penetrable by water though not by the substances found in the protoplasm.

The material out of which these retaining films are formed has been supposed to be of a fatty nature. Hans Meyer as we have seen argued from the action of anæsthetics which dissolve fats that cells were liable to this action "in proportion as fats enter into their chemical structure and are the seat of their functions": but all cells are acted on by these substances. Others have attempted to define the way in which fats "enter into the chemical structure" of the cell, and impressed by the picture of blood corpuscles swelling in hypotonic solutions till they rupture or at least release their hæmoglobin, and by the fact that ether, chloroform and other fat solvents such as solutions of bile salts or soaps also abolish the power of retaining hæmoglobin, have fancied the fatty material as forming a pellicle investing the exterior of the cell. Overton attempted even to define the permeability of cells as limited to substances that are soluble in the fatty pellicle, such as urea and not sugar, lower fatty acids not the amino acids, ammonium not sodium salts. Much of his argument rested on the behaviour of dyes, only those being able to penetrate the cell which are soluble in the lipoid membrane. The access of water to the interior of the cell had to be accounted for by the fact that lecithine which swells in water occurs in all cells and would probably be among the fatty materials in this membrane.

But the behaviour of dyes was not exactly as he pictured it (Loewe, 1912); and in many other respects the simple idea of a fatty pellicle was inadequate to account for the capricious permeability of cells and for the functional variations that this permeability exhibits. The substances which through insolubility in fat should be characteristically excluded from cells, and in some circumstances are, sugar, amino acids and certain salts, in other circumstances undoubtedly must gain entrance to them. So that this conception of the relation of fatty substances to cell structure is by itself inadequate, in spite of the saving virtue of the undefinable term "lipoid," that leaves it open to assign or not to assign to the membrane, at will, any of the specific properties, chemical or physical, that belong to fats.

Clowes has made a suggestion which might help to make good some of these deficiencies, that the surface layer of cells is a system in two phases, one fatty the other an aqueous solution of proteins. At one time the fatty phase is the continuous phase and encloses drops of watery fluid, at another the phases are inverted, the aqueous solution is continuous and encloses drops of oil. Such phase inversion can be obtained if an emulsion of oil by sodium soap in water is treated with a calcium salt, the emulsion then becoming one of water in oil; and by altering the balance between sodium and calcium first in one direction

then the other this inversion of phases can be repeated many times. If such a system exists in the cell membrane, when the aqueous phase is continuous it is suggested, substances dissolved in water inside or outside the cell could pass through ; when the oil is the continuous phase only substances soluble in it could pass. The resting condition of the cell would correspond to that state of the system in which the oil is the external phase : only, in the resting condition, the cell can take up and give out water. The attractive feature of the suggestion is that cell permeability in some well-known experiments is increased by unbalanced sodium ions, the normal impermeability being restored by balancing the sodium with calcium ions. But Seifriz (1923) has shown that, while phase inversion can be obtained in emulsions produced by many emulsifying agents, in all cases, including those in which sodium hydrate may produce a change, the sodium ion as supplied by sodium chloride fails to do so ; while it is the physiological action of sodium as chloride that gave the suggestion made by Clowes much of its interest for biologists (see, however, Clowes, 1918).

Bayliss, admitting that the possibility of phase inversion should be borne in mind, pointed out that the surface of liquid protoplasm will necessarily contain in a concentration greater than that in the interior of the system any substances that lower free energy, and therefore, in addition to fats and cholesterol, proteins ; and also that in the case of cells in higher animals, some contribution to the components of the surface layer will come from the lymph or blood plasma surrounding the cells. Moreover, proteins, he suggested, may behave as egg albumin is known to do, which forms a solid film at any free surface of its solutions that is exposed to air ; and, if so, they may contribute to the tenacity of the membrane.

Two remarks may be made with regard to this conception of the formation of cell membranes. In the first place, where two substances that lower the surface tension are present in a solution that one will be in possession of the surface which lowers it to the greater extent, and the other will be uniformly distributed through the system. Ramsden has shown that the aggregation of egg albumin at the surface of its solutions so as to form a solid coagulated pellicle does not occur if soap or bile salts are present in the solution at the same time, since these substances lower the surface tension more than the albumin does. So it is questionable whether the great complexity which Bayliss recognised that the surface membrane must have can be explained by the crowding together in it of everything in the protoplasm that lowers surface tension. Secondly, it would be wrong to represent the

account given by Bayliss of the mode of production of cell membranes as one that satisfied him. While it is clear that surface concentration must play a part in determining the composition of the surface, and that particularly it fits the fact that injuries to protoplasm surfaces are sometimes instantly repaired, after puncture, extrusion of part of the protoplasm or the entry of a foreign body in phagocytosis, it is equally clear that a chance agglomeration in the surface of all those constituents of protoplasm that reduce surface energy, among which substances of a fatty nature, cholesterol and proteins are specially mentioned, leaves undefined the relations in which those substances stand to each other in the constitution of a membrane with the extraordinary properties of selective semi-permeability, alternating with moments of permeability, on which so many physiological functions of the cell and of every kind of cell ultimately rests.

Surface concentration taken by itself, as Bayliss would have been the first to admit, cannot account for the physiology of cell surfaces.

Whether, therefore, we consider the distribution of fats in the protoplasm of cells or in their surface layers, it is only too clear that we have to confess our ignorance. Fats and water which will not mix are, nevertheless, intimately associated in the structure of the living fabric, and there are many indications that the association is one that is essential to its very nature. But, till more has been learnt of the relationships that fats, and more especially the phospholipines, are able to establish with the aqueous solutions of the cell, even this, though we may feel it to be true, is perhaps too much to say.

New facts are being learnt from the important work of Adam on the structure of thin films of fatty acids and similar substances on aqueous phases. Fats and higher fatty acids differ from paraffin waxes at one point only, where the carboxyl or ester grouping confers on that end of the molecule the power of entering into relations with water which no part of the paraffin molecule possesses. In stearic acid, the 17 other carbon atoms are purely paraffin, with all the propensities and disabilities which that implies. The paraffin chains cling together and withdraw from contact with water; the carboxyl or ester grouping clings to water, but it is unable to overcome the paraffinity of so long a paraffin chain, and the molecule as a whole is completely insoluble. Moreover, such substances, when all their molecules are attached at one end to water, exhibit properties that are entirely different from those that characterise them otherwise; palmitic acid in this case is liquid at the room temperature, and passes at a temperature still 30° below its melting point into a condition resembling the gaseous

state, with the molecules however still anchored to the surface of the water-like captive balloons. Lecithine also at temperatures as low as 5° C., far below the body temperature, is in such films expanded like a gas; while cholesterol, which is liquid up to 40° C., tends to bring both expanded fatty acid and lecithine molecules closer together without however packing them as close as in the liquid state (Leathes, 1923).

We do not know that these new properties of compounds of fatty acids and cholesterol do not contribute to their behaviour in the surface of protoplasm, nor do we know that similar formations are incapable of arising within it, double films with the paraffin chains of one bound to those of the other and the polar groups of each facing outwards, so as to constitute a boundary between two portions of an aqueous phase. And what additional characteristics cephaline, sphingomyeline and galactolipines, all known to be widely distributed in animal cells, may confer on such formations, and what relations with the specific groupings in the proteins peculiar to each type of cell any of these molecules may be able to establish, it is, for the present, also impossible to say. But the facts already known show that chemical characteristics determine the position that different kinds of molecules assume in relation to each other, even when no chemical reaction in the ordinary sense takes place. And it would be in harmony with these facts to picture in the surface of protoplasm, instead of a formless random agglomeration of anything and everything that lowers surface tension, an orderly structural relationship between different types of molecules, a pattern capable of modification, offering interstices now of one form and size, now of another, and flanked at one time by chemical groupings with affinities for one type of ion or molecule, at another for another. In such an ordered juxtaposition of molecules in the surface an important place must be reserved for compounds of fatty acids. For Hans Meyer and Overton argued from unquestionable premisses, though the form in which their conclusion was worded was open to objection.

Measurements have been made by Adam's method of the area occupied by a molecule of lecithine in a monomolecular film which show that the amount of lecithine given by Bloor for the red blood corpuscles in man is on calculation at least as much as would be necessary to invest the corpuscle with a bimolecular film of this substance alone, and together with the cholesterol which the corpuscle contains at least half as much again. In other types of animal cells for which figures exist compounds of fatty acids, including lecithine, form from four to six or more times as large a proportion of the cell

substance as they do in the red blood corpuscle, so that in all cases there would be much more of these substances than would be required for a bimolecular external film. Either, therefore, a much greater thickness is required for their surface film, or these substances are not confined to the surface, and must be for the most part distributed through the cell protoplasm. It is difficult to see why the former should be the case, and in the latter case difficult, as pointed out before, to see why, if these substances are merely dispersed through the protoplasm, any particular amount of them should be characteristic for each type of cell. It thus becomes easier to suppose that they are not distributed at random through the protoplasm, but here, too, on a plan, forming the continuous phase, a multilocular spongework of bimolecular films in which cohesion largely depends upon the paraffin chains of fatty acids with their polar groups facing the enclosed aqueous phase on each side.

Such a conception gives the compounds of fatty acids and the cholesterol associated with them a part in the structure of protoplasm of a kind for which there is much to be said. The fatty membranes would then permeate the cell contents, in far more intimate relation with them than that of a cell envelope. The appearance of fatty granules might be the thickening of the nodes in the meshwork with a tendency to phase inversion, completed when actual globules are found dispersed through the protoplasm.

Another line of study which is being followed and may have bearing on the formation of such films in the substance of cells is that of the movements executed by lecithine in contact with water, and the influence exerted upon them of various substances organic and inorganic present in solution in the water. A streak of lecithine on a glass slide under a drop of water projects from its surface budding protrusions in a way which has long been known but remarkably little studied. This is not the place for a full description of what can be observed, but the apparent nature of the forces at work is relevant in this connection. The essence of the phenomenon seems to be similar to the spreading of fats and fatty acids, so as to form a monomolecular film on a water surface. Paraffins will not spread in this way. It is the polar carboxyl group in a fatty acid that causes it to spread, the glyceryl ester group in a fat. In lecithine this grouping is complicated by additional components, phosphoric acid and choline, and for this larger polar group with new polar points, that necessarily confer upon it properties that a simple glyceride cannot have, there are only two instead of three paraffin chains. Additional relationships

with an aqueous phase thus become possible. Lecithine spreads on water as oleic acid or triolein when applied to it in benzene solution, but lecithine in substance behaves quite differently from a simple oil in contact with water. The more complex polar group appears to be drawn more strongly to the water; as many molecules as possible crowd up to the water surface; the surface presented by the lecithine to the water grows, and the amazing myelin forms and movements are the result. Film formation takes place in all directions not merely along a flat surface. At the same time water, by passing in between the molecules, but without abolishing the cohesion of the paraffin chains, is imbibed into the budding protrusions causing sometimes apparently further polarisation patterns among the deeper molecules within them. It is impossible to believe that these phenomena have no relation to the distribution of fatty material in the cell substance, though it is impossible with the scanty information available to say more than this. Indeed, the only justification for alluding to these phenomena is that the difficulties presented by the physiology of fats must remain insoluble until new lines of investigation have led to the establishment of new facts.

Clearer ideas as to the physical relation between insoluble fats and the aqueous protoplasmic systems of the living organism, and of the part played by cholesterol in this relationship, are indispensably necessary before we can answer some of the commonest questions in physiological inquiry. The unreflecting catechumen can confidently say how it is that fat is absorbed in the intestine, but very little reflection is necessary to shake that confidence. He should be introduced to the microanatomical studies of E. R. and R. L. Clark on the reaction of lymphatic endothelium to droplets of oil, or of oleic acid injected into the tissues in the tail of the tadpole; he should learn how the leucocytes and lymphatic endothelium cells are actively drawn along invisible paths through the tissue fluids to where these fluids surround the passive drop of insoluble fatty material. If he knows that these fluids, serum at any rate, may contain in the case of the eel, 26 grms. of fatty substances in the litre, including 6 grms. of cholesterol, all of which is invisible in a clear fluid, he may be led to think that molecular affinities lay down the invisible paths along which molecular tractions can work, lines of force of a kind of which little or nothing can as yet be learnt from books; let us hope that he will learn to look to observation and reasoned experimentation for the answer to the questions that must occur to him, though they are not found in the catechisms Compounds of fatty acids, with their attendant cholesterol, which form

something less than 1 per cent. of the red blood corpuscles, and often more than 10 per cent. of the liver, are present in the nervous system to the extent of 30 per cent. What have the catechisms to say about this? All secretory processes must remain a mystery till we understand how the contents of the cell are normally confined within it, and even within it, are restricted in their movements and spheres of activity. Whether this lack of homogeneity in the systems within cells will become intelligible when the distribution in them of insoluble fatty substances is understood, is a question that it is, perhaps, unprofitable to ask. The tendency these substances have to range themselves in aqueous solutions, with their paraffin chains adhering to each other in the thinnest layers, possibly two molecules thick, can only be hinted at, and this provokes the question whether such layers would be an obstacle to free movement of any molecules or ions, and if so, which, questions which we are still further from being able to answer. The brilliant work of the last twenty years on the physiology of muscle has furnished so much food for thought that this subject, at least, should enjoy immunity from untimely speculation, on, for instance, the ultimate nature of muscular contraction. And yet much has come to light that points to the changes in tension being set up in surfaces of changing permeability, and it is difficult to repress altogether the inquiry, where are these surfaces, what is their chemical nature, and how are they composed? This inquiry cannot become a practical and profitable one until we have some clearer understanding of the positions taken up by the double faced fat molecules in the ordered juxtaposition of the immiscible components of protoplasm.

Such information, too, is bound to throw light upon the property which fatty acids and their soaps have, of causing hæmolysis. This property is much more marked in oleic acid and other unsaturated acids than in the saturated. McPhedran (1913) found that the amount of sodium palmitate required to give complete hæmolysis in a given amount of blood was more than twelve times as great as that of sodium oleate: and, from time to time, it has been surmised that this or that form of anæmia was due to the action of unsaturated acids introduced into the body in some abnormal way, or liberated abnormally from combinations that occur in it; although no clear evidence that this is the case, it may be parenthetically observed, has ever been obtained. Why the oleate should be so much more active than palmitate or stearate is entirely unexplained; but McPhedran's experiments showed that this activity did not depend on the unsaturated group as such, since this group might be saturated with halogens, and the compound

be as active as the oleate; while, on the other hand, the dihydroxy stearic acid obtained by oxidising oleic acid was much less active even than stearic. In Adam's experiments, it may be noted, saturated bromo acids approach in their behaviour that of unsaturated rather than saturated fatty acids. It is, perhaps, worth noting, too, that the amount of oleate required to cause complete hæmolysis, so far as the calculation is possible, is approximately that which would give a monomolecular film on the surface of the red blood corpuscles.

The fact pointed out by Neuberg that olive oil containing no free fatty acid is not hæmolytic makes inadequate the simple explanation that the fatty acids act as solvents for the lipoid membrane in Overton's sense. More relevant, probably, are observations on the influence of fatty acids on the permeability of cells: Loeb found in his experiments on artificial parthenogenesis that the longer the non-polar carbon chain in a fatty acid, the more certainly did cell penetration take place; nonylic acid, for instance, acted ten times as strongly as octylic, and octylic, at least, ten times as strongly as butyric. The conditions limited the experiments to soluble acids. Loeb holds that both in fertilisation and in artificial parthenogenesis the first change leading to activation of the egg is a cytolytic change in its surface layer. This can be effected, not only by fatty acids, but also by bile salts and saponin, and generally by those agents which are effective in causing hæmolysis.

Another phenomenon in which hæmolysis is in some way due to fatty compounds is the action of snake venom. Flexner and Noguchi (1902), found that the breakdown of red blood corpuscles by cobra venom did not occur if the corpuscles were first washed free from serum; but then, after treatment with the venom, the addition of serum, even of another species, caused hæmolysis. Calmette showed that this was the case even if the serum was heated. Kyes then found that washed corpuscles treated with venom could be broken up by something extracted from serum by alcohol and ether which he identified as lecithine, and he thought that a combination of venom with lecithine was the active agent (1908). Wilstätter and Lüdecke (1905), on the other hand, found that the active substance was a derivative of lecithine, which contained only one fatty acid radical and that radical a saturated one, an unsaturated fatty acid having been split off. The subject is too confused and contentious to be dealt with in detail here (see Landsteiner, 1910). But Levene and his colleagues have recently reawakened interest in it by isolating monostearyl and monopalmityl lecithine and monostearyl cephaline from the products of

the action of cobra venom on egg yolk, and Noguchi showed that these substances, lysolecithine and lysocephaline as they are called, have strong hæmolytic properties. It is remarkable that these compounds which retain only the saturated and not the unsaturated acid chains should have hæmolytic properties resembling those of unsaturated rather than saturated acids. It suggests that this property in the case of lysolecithine depends upon the large size of the polar group, carried by a single fatty acid chain tending to diminish the cohesive force between the paraffin chains, just as the presence of unsaturated linkages or of halogen atoms (Adam, 1923) in such a chain does in a fatty acid. But how it is that cell membranes are weakened by contact with such compounds it is impossible at present even to speculate with any profit. The weakening probably consists in greater permeability, but with that we are brought to a stop.

The influence of cholesterol in counteracting the hæmolytic action of soaps (Meyerstein, 1912) is an indication, such as there are many, that to understand the part played by the fatty acids and their compounds it is necessary to understand that played by cholesterol. If hæmolytic soaps and fatty acids act by increasing the permeability of the containing layers in the corpuscles (L. Bayliss, 1924), in counteracting this cholesterol must tend to preserve their semi-permeability. It is worth noting in this connection the remarkable action of cholesterol in diminishing the area of a monomolecular expanded film of fatty acid or lecithine (Leathes, 1923). It is possible that this property of cholesterol, which has not been explained, is related to its action in cell membranes. It is well known that some substances which cause hæmolysis are substances, such as saponin, which engage cholesterol. If cholesterol causes greater cohesion of the molecules in fatty films and so diminishes their permeability, we should expect the presence of saponin by diverting this cholesterol to increase their permeability.

These few remarks on hæmolysis refer only to those aspects of the subject in which the importance of fatty components of the cell are most conspicuous—there is perhaps no subject on which more labour has been expended during the last twenty-five years than has been devoted to the elucidation of hæmolysis. The specific hæmolysins are formed and exert their action in a manner typical of the origin and action of all substances that confer immunity. The eyes of none that work on immunity can be kept away for long from the apparently simple case of the red blood corpuscle. If it is in the red blood corpuscle that the secret of immunity is locked up the key will be found when the manner in which fatty substances enter into their

chemical structure is understood. Moreover, if the existence and properties of cell membranes, exterior and interior, depend upon the paraffinity of fatty acid chains, the significance of these chains in the problems of hæmolysis and of immunity itself is but an instance of the general biological significance of fats in the structure of cells. For behind the capricious semipermeability of cells lie the mysteries of physiological absorption and secretion, of excitation in muscles and in nerves, and of the division of cells and their nuclei.

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