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QUANTITATIVE ULTRAMICROANALYSIS

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PREFACE

Ultramicroanalysis is a field of analytical chemistry in which progress has to some extent paralleled the tremendous advances made in other phases of that subject, such as instrumental analysis. The remarkable extent of its utilization by the Manhattan Project and the Atomic Energy Commission in both research and production phases of nucleonics has not been appreciated in detail by the scientific public because of the restrictions of security and the limited release of information. For the same reasons, it must suffice in this volume to state that the contributions made by the use of ultramicrotechniques were unique and virtually indispensable to the development of the atomic bomb and the subsequent nuclear investigations.

In the long range, the biochemist, clinician, and biological research investigator stand to profit most from the use of very small-scale methods, and it is to them primarily that this volume is directed. Not only does the field suffer from a scattered and partially inaccessible literature, but the deviations from the accepted standard methods are such as to generate in the minds of some a hesitancy or even a mistrust of methods designed for so great sensitivity.

Teaching of ultramicrotechniques for a considerable period of years has demonstrated completely that they are grasped at least as easily as are standard microchemical methods and that they often yield better results in the hands of students. It is believed that they offer unique value as laboratory training methods and as technical discipline. In many instances they demonstrate the principles of analysis better than could be accomplished by conventional macro- or even micromethods. With them, the research investigator can often by-pass the difficult sampling problems of biological systems by isolating the small and homogeneous system with which sampling errors are best controlled. By so doing he can also obtain information with a wealth of detail which is unique in biochemical analysis.

This volume is not intended to include every aspect of the

subject nor to exhaust the bibliographical possibilities. Many methods and techniques have been omitted intentionally, not only because of their deficiency but because they did not fit smoothly into the generalized technical scheme which was followed. Errors of omission have undoubtedly occurred, and, however regrettable, it is inevitable in so scattered a literature that not every method should receive detailed scrutiny. It is also true that many of the highly sensitive methods published fall below the standards of accurate analytical requirements which this author feels must be maintained.

I am indebted particularly to the many students and associates whose labors have contributed so greatly to the development and continued interest in this field and without whom this volume could not have been written. Facilities and financing for much of the work reported was generously provided by the Rockefeller Foundation, the American Cancer Society, and the United States Public Health Service, to all of whom a debt of gratitude is due. To many other workers in the field of ultramicrochemistry, particularly Dr. K. Linderstrøm-Lang, Dr. H. Holter, Professor E. J. Conway, and Dr. R. Craig, the author is indebted for voluntary cooperation and much unintentional assistance. Appreciation is due Dr. O. H. Lowry, Dr. P. F. Scholander, Dr. C. B. Anfinsen, and particularly Dr. C. L. Claff for their willing assistance in making available much material included in this volume. Acknowledgment is due to authors, publishers, and editors who have willingly granted permission for reproductions, and special thanks are due the Microchemical Specialties Co. for photographs and access to the latest models of various pieces of equipment described.

PAUL L. KIRK

Berkeley, California
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CHAPTER 1

Introduction

During the first quarter of the present century, the development of microchemical methods and their adoption by chemists, biologists, and others was very extensive. During the following ten years, microtechniques were recognized by a majority of analytical chemists and became an accepted part of their practice. Despite the wide use of various forms of microchemical methods in many fields, there is still no generally accepted definition of the limits which should be termed "micro." The common understanding of the term is certainly that employed in such standard works as Pregl (1), Niederl and Niederl (2), Emich (3), and others (4), in which the sample size is of the order of 1 or a few milligrams. For convenience, this scale of operations will henceforth be termed *milligram analysis,* a term which is relatively unambiguous.

In recent years, the research microchemist has increasingly turned his attention toward a much smaller scale of analysis, and indeed, many and far-reaching applications of smaller-scale analysis have been made. In general, these may be termed *microgram analysis* since the sample size is such as to contain an amount of the material determined of the order of 1 or a few micrograms. Such procedures are generally applied either (*a*) to analysis of very small samples or (*b*) to analysis of traces of material in larger samples. In the former, volumetric procedures have in general been applied; in the latter, the most common methods are colorimetric. These methods are capable of much further reduction in amount of material analyzed than is indicated by the term "microgram analysis" and indeed may in many instances be applied to the analysis of traces of materials contained in very small samples. This volume is intended to summarize the present status of microgram procedures for analyses of all types that have been proved practical and to include the smaller-scale procedures of colorimetry which have been tested. The field is still to be considered as in the developmental

stage, and many practical and desirable methods have not been developed or tested. These omissions should be readily noted from the context.

Magnitudes

Confusion frequently arises in the use of microchemical units, particularly as to their designation as "micro-," "ultramicro-," and "semimicro-." As indicated above, most of this ambiguity and confusion may be eliminated by the terms "milligram analysis" and "microgram analysis." The smaller-scale colorimetric procedures may in certain instances be applied to analysis of amounts of the order of 1 millimicrogram which might well be termed "submicrogram analysis." Table 1 indicates the *approximate* ranges of various scales of analysis and the reductions achieved at each scale.

TABLE 1. SCALE OF MICROMETHODS

	Sample Size	*Reduction*
Macromethods	100 mg.	
Semimicromethods	10–20 mg.	1/5–1/10 from macro
Micromethods (milligram)	1 mg.	1/100 from macro
Ultramicromethods (microgram)	0.001 mg.	1/1000 from micro; 1/100,000 from micro
(Submicrogram methods)	0.000001–0.00001 mg.	1/100,000 to 1/1,000,000 from micro

Semimicromethods are less well defined and developed than the other categories with the exception of the last. No well-defined set of methods has been described between milligram and microgram methods, but the two types of procedures may usually be extended to fill the intervening gap. It is interesting to note that microgram methods may be more readily extended upward than may milligram methods downward.

The *units* (5, 6) commonly employed in microgram and other small-scale analyses may be defined as follows:

$1\ \gamma = 1\ \mu g. = 0.001$ mg. $= 10^{-6}$ g. $= 1$ microgram
$1\ \lambda = 1\ \mu l. = 0.001$ ml. $= 10^{-6}$ l. $= 1$ microliter
$1\ \epsilon = 1\ \mu equiv. = 0.001$ m. equiv. $= 10^{-6}$ equiv. $= 1$ microequivalent

These terms are all analogous to the common designation of microscopic lengths by the use of μ, where

$$1\ \mu = 1\ \mu\text{meter} = 0.001\ \text{mm.} = 10^{-6}\ \text{m.}$$

It is convenient and desirable to make routine use of these symbols in calculation rather than small decimal fractions of the common units for mass, volume, and equivalents. The following simple relationships show that the calculations are of equal simplicity:

$$\text{ml.} \times N = \text{m. equiv.}$$

$$\lambda \times N = \epsilon$$

$$\frac{\epsilon}{\lambda} = \frac{\text{m. equiv.}}{\text{ml.}} = \frac{\text{equiv.}}{\text{l.}} = N$$

$$\frac{\gamma}{\lambda} = \frac{\text{mg.}}{\text{ml.}} = \frac{\text{g.}}{\text{l.}}$$

General Uses of Microgram Analysis

In the field of biology particularly, there are innumerable instances in which the available sample is too small for conventional analysis, even by micromethods. An extreme example is the field of insect physiology, but almost as striking is that of invertebrate biochemistry. Many applications of microgram chemical analysis may be made in both fields, as well as in tissue culture study, protozoology, and other allied sciences. Cytology and cellular chemistry may expect great advances from consistent application of the smallest-scale chemical analysis.

Very important and apt to be underestimated is the possible application to ordinary biochemical studies. Most animal experimentation requires comparatively large amounts of blood, such as may be obtained without slaughter only from dogs or animals of similar size. The equivalent information may be obtained by analysis of tail blood from mice or rats in many instances, without slaughtering the animals to obtain enough blood for ordinary scale analysis.

Another very large field for microgram techniques is in the clinical laboratory where the advantages of analyzing finger or ear blood rather than the much larger amounts usually taken from the arm veins are very evident. The pediatrician is in

position to profit especially from this substitution, because of the well-known difficulties of withdrawing large samples of blood from infants. Probably the most extensive application to the field of biology up to date is in the field of enzymic histochemistry, which has been recently reviewed by Glick (7) and earlier by Kirk (8).

The widest field of application so far found for microgram chemistry was in connection with the studies of the fundamental chemistry of plutonium, most of which was elucidated before as much as a milligram of the element was in existence. It is to be expected that the new subject of isotope chemistry will give many fruitful applications of these techniques because it is frequently possible to obtain only the most minute amounts of special isotopes without great difficulty. Moreover, since most pure isotopes which become available are the result of formation by neutron or deuteron bombardment in a cyclotron or pile, they are nearly always quite radioactive and should be used in very small quantities if the operator is not to be exposed to the hazards of high levels of radioactivity. Thus, it was necessary to conduct all control analyses in the Hanford plutonium plant on a microgram scale in order to diminish the level of gamma activity to an amount considered safe for the analysts. In this case, it was not deficiency of analytical material which was the determining factor in choosing microgram analytical methods. All control analyses were based directly on the techniques described in this volume.

Types of Method

The analytical principles which have proved useful for microgram analysis are not inherently different from those long employed with the conventional size of sample. These principles are detailed in numerous standard textbooks. The techniques which must be employed are considerably different. If very small amounts of material may be weighed, measured, filtered, ignited, stirred, etc., the more satisfactory analytical methods which have been long employed may usually be adapted to microgram quantities by making the appropriate changes in the technique of each operation. The following outline indicates the present status of various types of procedure.

1. *Gravimetric.* No complete procedure for analysis on a

microgram scale by gravimetric procedures has so far been published. This omission is due to the previous lack of reliable balances that will weigh conveniently to small fractions of a microgram. A number of balances of sufficient sensitivity have been described, but they were all highly specialized, difficult to construct and use, and consequently rather impractical for general analytical application. It is now possible to obtain quartz fiber balances capable of weighing rapidly and reliably to $\pm 0.005\ \gamma$ and probably to $\pm 0.001\ \gamma$ (9), and it is anticipated that gravimetric microgram methods may now be developed as needed.

2. *Titrimetric.* The most accurate and precise procedures so far described belong in this category. Liquid volumes may be reliably dispensed and measured in very small volumes, so that this basic method is one of the most favorable for general application. The limitations of microgram titrimetric procedures are similar to those of larger-scale titrimetric methods, viz., a frequent lack of specificity which may demand the use of separation procedures, and the errors which are characteristic of all titration procedures.

3. *Colorimetric.* Microgram colorimetric methods are numerous (10), though the lower limits of most of them as currently used lie somewhat higher than is typical of the methods described in this volume. They are inherently less accurate than titrimetric methods, and they may or may not be more rapid or specific.

When the colorimetry is performed with the spectrophotometer, the reliability is greatly increased over simpler color measurements, and the errors are correspondingly less, though the precision may not be any greater. In general, the error of spectrophotometric analysis may be readily maintained within limits of about ± 2 to 5%, and under favorable circumstances may be reduced to less than 1%. In the biological field, particularly, these methods are appealing because of the lack of other types of procedure for many of the important organic constituents of the biological system. It is unfortunately true that methods of great practical interest are frequently not well studied and may be of a very low order of dependability. This is equally true of the larger-scale colorimetric methods for these constituents.

4. *Gasometric* (exclusive of gas analysis). Methods based on the measurement of gas volumes are rarely used for microgram

analysis. They are inherently less sensitive than titrimetric methods and much less so than colorimetric. There is no necessary reason for the lack of microgram gasometric methods for numerous constituents, and it is to be expected that certain ones will be developed. In some instances, such methods should be very favorable as compared with other types of procedure.

5. *Physical.* Specialized physical methods for respirometry and for enzyme studies, such as the ingenious Cartesian diver micromanometer of Linderstrøm-Lang (11), have received considerable attention and are widely employed.

Methods for microgram analysis that involve the spectrograph, polarograph, conductometric apparatus, and similar specialized physical equipment show great variations in utility and types of application. In general, they have too low sensitivity to be classed along with the more common microgram procedures. They are much more highly specialized and therefore less available. They rarely show any improvement in accuracy over the commoner types of method. Treatment of these special procedures may be found in other books and will receive little discussion in this volume.

Solutions and Standards

Both titrimetric and colorimetric procedures for analysis depend directly on the use of standards, and the possible accuracy of the method never can be better than the reliability of the standard. The accuracy of microgram analysis is limited by the standard in the same manner and to about the same extent as is that of larger-scale analysis.

Standard solutions for microgram analysis need not be made in as large lots as for larger-scale work, but the stability of a large volume toward evaporation and solution of glass from the container is greater than that of a small one. It is advantageous for this reason to make standard solutions for titrimetric procedures in volumes not less than about 250 ml., and for colorimetric methods in volumes not less than 100 ml. if they are to be stored and down to 10 ml. if they are used directly. If the standard material is weighed and dissolved, it is usually necessary to prepare larger volumes than those indicated because the amount of solid weighed would be too small to obtain an accurate weight. It is advantageous to employ the analytical balance which is

usually available and familiar, and not less than 50 mg. of standard substance should be weighed on this instrument in any single sample.

Titrimetric Analysis. *Standard solutions* are made by either of two methods.

1. Approximate weighing and solution of the weighed material, followed by accurate standardization of the solution against primary standards, or

2. Accurate weighing of the solute and dilution to an accurately known volume.

Method 1 is applicable to all types of solution since it requires no accurate determination of the total volume and allows the use of solutes of unknown composition or purity. Acids and bases are ordinarily made this way, and the method can be adopted for any type of material.

Method 2 applies only when the purity and composition of the solute is approximately as well known as would be necessary for a primary standard. It is suitable for silver nitrate, potassium iodate, alkali halides, carefully prepared and equilibrated sodium thiosulfate, and certain other materials. Unless the material is entirely suitable, method 1 is preferable.

Certain reagents such as potassium permanganate, ferrous ammonium sulfate, and ceric sulfate require special precautions or procedures in preparation of solutions. These will be described in connection with their application in oxidimetry. Direct weighing of the solute and dilution to volume may never be employed with these and other materials of unknown purity or composition.

Purity of solvents must receive special care. Water, acids, and other solvent materials employed in making solutions must usually be purer than is necessary on larger-scale analyses. It is a desirable precaution to redistill all water, since distilled water as it is commonly dispensed from large laboratory stills may be much more impure than is generally appreciated. The amount of water needed is ordinarily small and may readily be provided by a small all-glass distillation outfit. Sulfuric acid, particularly, is frequently too impure to use directly and may also be redistilled from an all-glass still of small capacity. It has been noted that microgram titrations with ceric ion are especially sensitive to small amounts of impurity in the water and sulfuric acid added to the reagent.

Primary standards must meet the general requirements of these materials when used on a larger scale, and the standards need not be better than usual. These general requirements include meeting all or most of the following criteria:

1. Constant composition on standing and during weighing. This includes the composition with respect to water of crystallization.

2. Easily duplicated composition on successive batches or successive crystallizations.

3. Accurate stoichiometric relations, depending on (*a*) absence of measurable impurity and (*b*) absence of side reactions.

4. Sufficiently high equivalent weight to yield satisfactory weighing accuracy.

5. Reasonably wide application to different types of standardizations; e.g., both acidimetry and oxidimetry.

6. Negligible or easily determined titration error.

Standards which meet the greatest number of the above criteria include potassium iodate, potassium biiodate, and sodium oxalate. No universal standard meeting all of them perfectly has so far been found.

Suitable primary standards are indicated in Table 2. The details of obtaining and utilizing these and other definite and pure compounds as primary standards may be obtained from other standard works on quantitative analysis (12–14).

Numerous other primary standard materials might be mentioned, either because of certain intrinsic merits or because of widespread application in analytical laboratories. Those that are omitted have some disadvantage such as difficult availability, difficult manipulation, or lack of general utility. It is not implied that they are necessarily not useful or dependable.

Standards for colorimetry have been treated inadequately in most standard books, which is unfortunate in view of the wide employment of colorimetric methods and the accuracy which is often claimed for them. It is self-evident that the analytical values cannot be more accurate than the standards employed for comparison or calibration. It is not uncommon for widely adopted standards to be uncertain by several per cent, and instances of a twofold error have been known to occur because of inadequate standards alone.

Standards for colorimetry may not be treated so systematically

TABLE 2. PREFERRED PRIMARY STANDARDS

Solution	*Standard*	R.E.W.*	*Indicator*	*Remarks*
Acid (HCl, H_2SO_4)	$Na_2B_4O_7 \cdot 10H_2O$	190.61	M.R.†	Requires adjustment of water of hydration
	KIO_3	35.66	M.R.	Special titration, $IO_3^- + 5I^- + 6H^+ = 3I_2 + 3H_2O$, in presence of excess neutral thiosulfate
Base (NaOH, KOH)	$H_2C_2O_4 \cdot 2H_2O$	62.99	P.P.‡	Pure material available commercially
	$KHC_8H_4O_4$	204.06	P.P.	Bureau of Standards material available
	$KH(IO_3)_2$	389.85	M.R.	
Potassium thiosulfate	KIO_3	35.66	I_2-starch	Iodometric
	$K_2Cr_2O_7$	49.012	I_2-starch	Iodometric
Potassium permanganate	$Na_2C_2O_4$	66.98	Reagent	Bureau of Standards material available
	$H_2C_2O_4 \cdot 2H_2O$	62.99	Reagent	
Ceric sulfate	As_2O_3	49.47	Fe-phenanthroline	Requires a catalyst (15)
	$Na_2C_2O_4$	66.98	Fe-phenanthroline	Bureau of Standards material available
	$K_4Fe(CN)_6 \cdot 3H_2O$	422.15	Setopaline C	
Silver nitrate	KCl	74.56	Electrometric or Dichlorofluorescein	

* R.E.W. = rational equivalent weight, defined as the true equivalent weight, reduced by the amount of the correction to weight in vacuum. Thus, it is the nominal weight of 1 equivalent balanced against brass weights.

† M.R. = methyl red.

‡ P.P. = phenolphthalein.

as may volumetric solutions because of the greater diversity of the former and the difficulty of obtaining dependable compounds from which some standards may be made. Solutions against which unknowns are to be compared or instruments calibrated should meet as well as possible the following criteria:

1. A definite, reproducible composition.
2. Availability in pure form.
3. Solubility in water, or in a solvent which is suitable for color development.
4. A reasonably high equivalent weight.
5. Lack of groups or radicals in their structure which will interfere with color development.

Table 3 lists suggested standards for elements and radicals which are commonly determined by colorimetric methods. Alternative standards may be substituted for many of these, and in some cases better compounds may be available. Few of them are ideal in their conformance to the above criteria.

It is important with the standards listed in Table 3 or other similar standards that every effort be made to obtain material that is as pure as possible and always well within the error of the colorimetric method chosen. This is no easy task because common commercial chemicals are frequently more impure than the allowable limits, particularly with respect to water content.

No organic compounds are listed because the standard for determination of an organic constituent must usually be that constituent itself, and the only choice exerted by the analyst is to attempt to obtain material of high purity and reproducibility, which in some instances is very difficult. Physical constant determination, e.g., melting points, boiling points, refractive indexes, etc., should be utilized to check the purity of any doubtful standard when possible. Recrystallization, distillation, or sublimation may often be employed to purify further the commercial product.

The judgment of the analyst is indispensable in determining how far it is necessary to proceed for the particular purpose. It must be noted that many of the analytical data in the literature are highly deficient in this regard.

Operations

Chemical analyses may be considered as a series of operations, each designed to achieve a limited objective such as complete

TABLE 3. STANDARDS FOR COLORIMETRY

Element or Radical	*Standard*
Al	Uneffluoresced potassium alum, $KAl(SO_4)_2 \cdot 12H_2O$
As	As_2O_3, reagent grade, dissolved in NaOH and neutralized carefully
Bi	Pure bismuth metal
Ca	Reprecipitated $CaCO_3$
Cr	Recrystallized $K_2Cr_2O_7$
Co	$CoCl_2 \cdot 6H_2O$
Cu	Electrolytic Cu metal or selected $CuSO_4 \cdot 5H_2O$ crystals showing no effluorescence
Fe	Pure iron wire (for standard) dissolved in 1:3 HNO_3 and boiled to remove oxides of nitrogen
Pb	$Pb(NO_3)_2$ (dry) dissolved in weak HNO_3 solution
Mg	Pure Mg metal dissolved in dilute HCl
Mn	Electrolytic Mn metal in dilute HNO_3 or standard $KMnO_4$ reduced with acid sulfite and with SO_2 boiled out
Hg	$HgCl_2$ (pure)
Mo	Pure MoO_2 in dilute NaOH solution and slightly acidified with HCl after dilution
Ni	Pure Ni metal or uneffluoresced $NiCl_2 \cdot 6H_2O$
K	KCl (pure and dry)
Na	Pure NaCl
Sn	(Stannous) metallic Sn dissolved in concentrated HCl to which is added thioglycolic acid; must be fresh
U	$UO_2(NO_3)_2 \cdot 6H_2O$
Zn	Reagent grade 30 mesh Zn metal dissolved in HCl
Phosphate	Pure, dry KH_2PO_4
Arsenate	As_2O_3, dissolved in NaOH, carefully neutralized, and oxidized exactly with iodine
Silicate	Pure SiO_2, fused with NaOH
Iodine	Resublimed iodine

separation of precipitate, measurement of an exact volume, etc. Assuming a satisfactory chemistry, the accuracy and reproducibility of the analysis will depend on the performance of each of these individual operations with the required accuracy and precision.

Methods for each operation are comparatively standardized for macroanalysis and almost as much so for conventional milligram analysis. In both, the equipment and its corresponding method are designed for the specific operation on its respective scale. Again, if the suitability of the chemical reactions chosen for the microgram analysis is assumed, the development of the method will depend on proper design and manipulation of the equipment so that the operation can be conducted with the neces-

sary expediency, accuracy, and reproducibility. Since the number of necessary operations is limited, many of them being common to a large group of analytical methods, the development of microgram analysis is largely concerned with providing proper types and design of equipment to handle unusually small amounts of material with the same relative dependability as the larger amounts are manipulated in the conventional scale of operations. Also, the limited number of operations allows a considerable degree of standardization to be introduced. If appropriate equipment and methods are available for each operation of a microgram analysis, the method should be practical regardless of other considerations.

The above generalization has been shown by experience to be correct in most instances. Exceptions occur, chiefly due to the following factors which are more important with small amounts than with large.

1. High evaporation loss of solvent from solutions, due to the large surface to volume ratio of small volumes of liquids. This leads to a concentration of solute.

2. High rate of loss of volatile solutes, e.g., iodine, hydrochloric acid, or ammonia, from small volumes of solution. This leads to a lowering of concentration of solute. It may be turned to advantage as in isothermal distillation, or diffusion methods.

3. Surface tension. Manipulation of small volumes of solutions is quite different from that of large volumes in this regard. Surface tension may be a favorable factor in correctly designed apparatus and may lead to much difficulty with improperly designed equipment.

4. Shallow liquid layers, which lead to small light absorption of colored compounds such as indicators. Thus, a greater concentration of indicator is needed with small volumes than with large, leading to a different order of certain errors.

Temperature and pressure changes, absorption, coprecipitation, and similar phenomena have no greater percentage effect on microgram analysis than on other scales of operation.

Errors

Microgram analysis is inherently subject to somewhat different degrees of the common volumetric errors than is milligram

analysis:* The differences are due largely to the effect listed as factor 4, above; i.e., a shallow depth of liquid requires a greater concentration of indicator, in order for the color change to be visible to the eye. An additional reason for the differences in error is the frequently greater concentration of materials present in the microgram methods in which dilutions are often less than in conventional microanalysis.

The *drop error,* defined as the last small portion of added reagent which changes the indicator rather than titrating the sample, is, in general, relatively large in microgram titrations. Because a considerable amount of indicator must change color before the change becomes visible in the thin liquid layer, a correspondingly larger amount of indicator is added, and more reagent is necessary to produce the visible change. When the total material titrated is large in amount, this may constitute a very small fraction of the total titration, but, in adding only a few microliters of reagent in the entire titration, it may be a rather large fraction of the total.

The large drop errors of titration with color indicators are readily corrected by controlling the amount of indicator and using proper blank and control titrations to evaluate the correction. In practice, it is found that the drop-error corrections may be applied so accurately that the residual error is small by comparison with other inherent errors in the method.

The *titration error* is defined by the equation

$$\text{T.E.} = \frac{av}{nV} \times 100\%$$

where a = the sensitivity of the indicator in gram equivalents per liter, i.e., the concentration of the ion in question which must be present in excess to change the color of the indicator.

v = the final volume.

N = the normality of the solution titrated.

V = the initial sample volume.

The titration error is then the error that results when the indicator change is not exactly at the end poind of the reaction but somewhat earlier or later.

* For detailed discussion of titration and drop errors, see Kolthoff and Stenger, *Volumetric Analysis,* Vol. I, Interscience Press, New York, 1942.

If all concentrations are the same in the microgram and the large-scale titration, the titration error is the same. It is less if the concentrations are greater, and vice versa. Since it is customary in microgram volumetric analysis to maintain volumes as small as possible, the dilution of sample and reagent is likely to be less and the titration error also less than that of conventional milligram titrations, and little greater than macrotitrations. Milligram titrations are often performed with solutions more dilute than 0.01 *N*, leading to relatively high titration errors. By measuring minute volumes accurately, the concentration of reagents used for microgram titrations may generally be kept from two to five times as great as this, i.e., 0.02 to 0.05 *N*.

In this connection, it should be mentioned that the attempt to determine microgram quantities of a material with ordinary apparatus and extremely dilute solutions is usually unsuccessful, partially because of the large titration errors and particularly because of the indistinct character of the end points obtained with very dilute solutions. Clinical blood analysis methods especially have frequently been deficient in this respect.

Potentiometric titrations, correctly performed and interpreted, are effective in greatly reducing or eliminating both the drop and titration error. The small electrodes which are necessary and the occasional special complications introduced by applying potentiometric techniques to small volumes have prevented widespread adoption of such methods. There are, however, no insurmountable difficulties in their application, and further study of such methods should yield many useful procedures which will extend the applications of microgram analysis greatly.

Amperometric titrations, also, though they have been seldom applied in microgram analysis, may be expected to show useful applications on this scale and to be relatively free of the errors characteristic of ordinary indicator titrations.

Technical and *equipment errors* of microgram procedures are not greatly different from those of conventional analysis. Somewhat larger errors are to be expected from capillary pipets and burets than from large ones, but the errors may be made practically as small as desired by proper design and use. Volumetric flasks having a content of less than 1 ml. are more difficult to make and employ accurately than are large flasks, and some compromise between accuracy and convenience is necessary. The

limiting accuracy of most microgram methods is of the order of $\pm 0.5\%$ or less, and routine application usually allows all normal results to fall within a range of $\pm 1\%$.

Construction of Equipment

Most microgram (or drop analysis) equipment in general use is commercially available in reliable form and accuracy. However, the analyst who is engaged in research of this type or who encounters unusual problems will profit greatly by the ability to perform simple glass-blowing and mechanical construction. A small, properly equipped glass-blowing bench and a set of hand tools, preferably supplemented by a workshop lathe, are among the most valuable adjuncts of the laboratory. A little ingenuity and simple mechanical and glass-blowing skill permits easy solution of many special problems that arise, and the desirability of acquiring such skills cannot be overemphasized.* The routine microgram analyst will not usually profit to the same extent as the research analyst because standardized equipment is normally employed.

Discussion of certain special burners which allow fabrication with thin-walled capillary tubes, quartz fibers, and similar special materials not commonly encountered by the chemist is given in Chapter 4.

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CHAPTER 2

Volumetric Apparatus and Technique

In this section will be described equipment for the performance of each of the operations involved in a number of volumetric microgram analyses and the techniques of using the various types of equipment. Special apparatus designed for a limited purpose or unusual in design will be described in connection with the method to which it applies.

If maximum emphasis is placed on those designs that have originated in the author's laboratory, it is due not only to greater familiarity with them but also to the fact that in no other laboratory has such equipment been so systematically developed and integrated into different procedures. Moreover, it is at present the equipment which is most readily available from commercial sources.*

SAMPLE MEASUREMENT

Liquid samples for microgram analysis usually range from about 5 to 100 λ. Occasionally it is necessary to measure larger samples, which is a simple operation, or smaller ones, which present greater difficulty. A pipet, constructed from a heavy-walled capillary tube is normally used. This requires a bore small enough so that the uncertainty of meniscus adjustment leads to no large error. The smallest heavy-walled borosilicate glass capillary that is readily obtainable has an 0.5-mm. internal diameter. Since the cross-sectional area of this tubing is about 0.2 $\overline{\text{mm.}}^2$, an error of 0.1 mm. in setting the meniscus corresponds to an error of 0.02 λ. With this size of tubing, this represents the lower limit of probable error of meniscus setting, corresponding to an error of 0.1% when the total volume is 20 λ, and remaining well below 1% with any sample that is likely to be measured with

* Most equipment described in this volume is obtainable from the Microchemical Specialties Co., 1834 University Ave., Berkeley, California.

simple capillary pipets. Smaller capillary tubing of special types such as marine barometer tubing may of course be used to measure smaller samples, but with greater technical difficulty.

The reliability of pipets calibrated for delivered volume and made from capillary tubing is questionable because of capillary and drainage effects. Pregl (1) avoided error from this source by calibrating small pipets with mercury, thus obtaining the total capacity of the pipet rather than its delivered volume. In use, these pipets were rinsed out so as to recover the total capacity. One obvious error was introduced, viz., that due to the difference

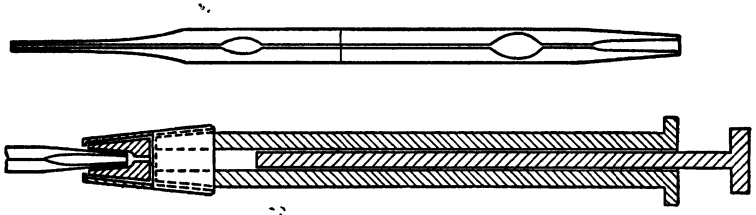


FIG. 1. Capillary transfer pipet and glass syringe control. (¾ actual size.)

in shape of a mercury and a water meniscus. This error may be approximately calculated. If we assume that the mercury presents a hemispherical convex meniscus, and water a hemispherical concave meniscus, the difference in volume resulting is equal to the difference between the volume of a sphere and that of a cylinder having an equal diameter and height. With 0.5-mm. tubing, the volume of the cylinder is about 0.1 λ, that of the sphere approximately 0.06 λ. The error is then the difference, or 0.04 λ. This represents an error of 0.1% when the total volume is 40 λ. It may be applied as a correction for very accurate measurement, but in most instances it is small enough to be disregarded. Obviously, it is smaller, the finer the diameter of the tubing, and the water content of the pipet is always greater than the mercury content by an approximately constant amount.

Pipets utilizing simple design and manipulation based on the considerations above were described by Kirk and Craig (2). They were later modified for easier construction and use and described by Sisco, Cunningham, and Kirk (3), as shown in Fig. 1.

The pipet shown is essentially a capillary tube containing two bulbs, one related to the contained volume and the second serving

as a safety trap to prevent liquid from traveling up into the pipet control. The upper end of the pipet is drawn down to a taper similar to that of the standard blood-dilution pipet used in the clinical laboratory. The pipet is filled and emptied by means of a syringe control which is also shown in Fig. 1. The control con-

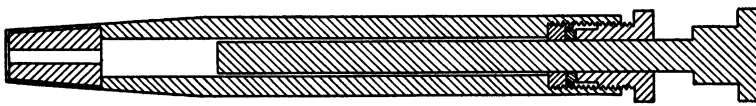


FIG. 2. Metal pipet control. (¾ actual size.)

sists of a glass hypodermic syringe having a special metal band attachment containing a rubber gasket which grips the tapered upper end of the capillary pipet. The control is also useful for blood-dilution pipets as noted above. A similar all-metal control which is virtually indestructible is shown in Fig. 2. Its operation is sometimes more difficult than that of the glass syringe type because of greater difficulty in obtaining tight gaskets. In the newer models this limitation has been effectively overcome.

Operation of Capillary Transfer Pipet

The pipet must be clean and dry. It can be soaked in cleaning solution or detergent solution for cleaning, if necessary. Drying is best accomplished on a vacuum line which sucks air through and sweeps out droplets of liquid as well as evaporates the moisture. Rapid drying may also be obtained with acetone and suction.

The gasket end of a control is fitted rather tightly on the tapered end of the pipet. The combination is grasped in the hand in such a way that the thumb and forefinger rest on the plunger handle. As the plunger is withdrawn, it is rotated slowly between the thumb and forefinger, with a slow, smooth motion that is readily controlled. The sample is drawn either exactly to the mark or very slightly above it. The pipet is withdrawn from the solution being measured, and the tip is wiped. A wad of cotton or cleansing tissue held in the other hand serves admirably for wiping the outside of the pipet tip. Enough liquid may otherwise adhere to the outside to introduce an effective error into the pipet measurement. Wiping is performed with a quick

light stroke that does not disturb the setting of the meniscus. With materials that are not readily removed by wiping, it is permissible to wash the outside of the tip with distilled water or other solvent.

If the meniscus is slightly above the mark, it may be set exactly on the mark by tapping the end of the tip lightly with the finger, which lowers the meniscus very slightly. If radioactive materials are being measured, contamination of the finger may be avoided by means of a microscope slide or cover slip. Similarly, in analysis for sodium or chloride, the sample will be contaminated by perspiration from the finger, and, here also, a slide or slip should be employed. If holding the slide or cover slip in the fingers is also hazardous because of radioactivity or similar reason, the cover slip may be seized by a vacuum arrangement, as shown in Fig. 3. The application and release of vacuum is controlled by means of the orifice on the side of the glass tube. When this is closed, the vacuum holds the slip firmly against the rubber stopper on the end of the tube.

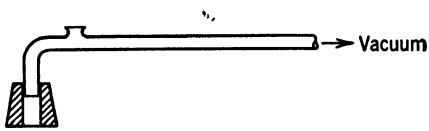


FIG. 3. Cover slip holder operated by vacuum. (½ actual size.)

When the outside of the tip is clean and dry and the meniscus is adjusted exactly, the liquid is transferred by pressing the plunger of the control downward and delivering the expelled liquid into the receiving vessel. A rather slow movement of the plunger is desirable to prevent collection of droplets of liquid in the capillary tube owing to incomplete drainage. The pipet must then be rinsed both on the outside of the tip and on the inside. A drop of water is suspended from the tip of a micro wash bottle, and the entire droplet is sucked into the pipet, thus rinsing both the outside of the tip and the entire inner surface of the pipet when the water is raised to or slightly above the zero mark. If the pipet is very small, it may not be practical to use a droplet suspended in this way, in which case it is often convenient to place a very small droplet on a microscope slide or watch glass and remove it from there. A paraffin block or paraffin covered slide is even more satisfactory because it does not wet. It is always desirable when possible to utilize an entire drop, since this prevents loss to the excess wash liquid of some of the solution

on the exterior of the tip. It is not necessary to use a pipet full of rinse liquid in any case, since the degree of rinsing is practically the same whether the extra volume necessary to fill the pipet completely is employed or not. The rinsings are added to the receiving vessel containing the sample, and thus the entire content of the pipet is recovered. Rinsing should be performed with at least two portions of rinse liquid and more if the character of the material measured, such as a high viscosity, requires it. Experiments have shown that little improvement in accuracy is obtained by a third rinsing when ordinary aqueous solutions are measured.

The ordinary form of capillary pipet has a fairly short stem leading to the tip, and, for the sake of avoiding fragility, this stem is usually made with rather thick walls. For special purposes, such as removal of liquid from a capillary volumetric flask, the pipet tip may require a thinner and longer form. This form may be rather readily made by the analyst, or it may be purchased commercially. Such pipets have the disadvantage of a greater susceptibility to breakage.

Calibration of Pipets

The capillary pipet is calibrated for content in the following manner: A drop of mercury is placed in a porcelain titration dish shown in Fig. 4 or in a small watch glass. With a control attached to the pipet, the tip is inserted into the mercury, the pipet maintained in as nearly a horizontal position as possible. While the pipet is held in one hand, the plunger is withdrawn by the other hand, with a rather quick but short movement. The mercury should flow up the capillary tube and may then be drawn slowly into the pipet so that the meniscus passes the calibration mark at as slow a rate as possible. At the instant the meniscus passes the calibration mark, the pipet is withdrawn from the drop of mercury by being lifted upward, with no effort made to stop the suction at this point. The mercury is expelled into a clean, weighed vessel and weighed. Table 4 gives the conversion factors for converting the weight of mercury balanced against brass weights in air to the true volume of mercury.



FIG. 4. Porcelain titration dish. (Actual size.)

If the pipet is not marked and the position of the mark is to

TABLE 4. CONVERSION FACTORS FOR MERCURY

Temperature, ° C.	*Factor*
20	0.07382
21	0.07383
22	0.07385
23	0.07386
24	0.07388
25	0.07389

be determined, the correct amount of mercury to give the desired volume should be carefully adjusted on the balance. By careful introduction of this mercury into the pipet and adjustment of the bottom meniscus to the tip, the position of the zero mark is located directly. The calibration mark is then placed on the pipet at exactly the right position. This method is used by the manufacturer and is of approximately the same accuracy as the above described method for recalibrating marked pipets.

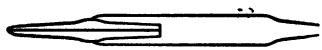


FIG. 5. Self-filling pipet. All glass construction. (½ actual size.)

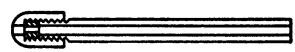


FIG. 6. Metal holder for self-filling pipet tubes. (½ actual size.)

Self-Filling Pipets

Capillary rise of liquid in a tube of small bore has been recognized for a long time (4) as a means of measurement of minute samples. When the capacity of the pipet must be less than about 5 λ, it is advantageous to use a so-called self-filling pipet which is completely filled by capillary rise alone. A practical design for such a pipet is shown in Fig. 5. As a means of avoiding the ring seal in this design, a single holder for a number of capillary pipet tubes may be made as shown in Fig. 6.* When the ring is screwed tight it compresses the rubber gasket on the pipet tube to seal the joint. Another alternative is to seal the short pipet tubes into a larger constricted tube with a little wax or cement.

The self-filling pipet fills automatically when the tip is inserted into the solution to be measured, allowing the capillarity of the solution to draw the meniscus to the top of the fine tube, at which

* This is similar to the pipet holder described by Benedetti-Pichler (24).

point it stops automatically. Pipets of this type are capable of measuring almost indefinitely small volumes and are practical down to at least 0.25 λ. They may not be used for large volumes because the height of column that can be supported by the capillary rise of the solution is distinctly limited and related to the capillary diameter of the pipet through the equation

$$r = \frac{2T}{dgh}$$

where r is the radius, h the capillary rise, T the surface tension taken as 70 dynes per centimeter, d the density, and g the acceleration due to gravity. Thus a tube of 0.7-mm. diameter will support a water column of nearly 4 cm. If the tube were constructed through most of its length with a taper such that the diameter at each point were just small enough to support a column of liquid that high, the total volume of a 4-cm. tube could be made in excess of 0.1 ml. In practice, this is virtually impossible, and, in fact, samples of greater than 10 λ are measured with considerable difficulty, even with a clean pipet.

The self-filling pipet is not readily emptied, the operation usually being performed with a pipet control (Fig. 1) or by application of mouth pressure through a rubber tube. With the very small volumes which are sometimes measured by such pipets, the entire content may well adhere to the outside of the tip instead of being transferred to the receiving vessel. For this reason it is desirable when possible to deliver the sample into a drop or more of water or solution which may also be used for preliminary rinsing of the pipet.

Calibration of Self-Filling Pipets. Self-filling pipets are inherently more difficult to calibrate than capillary transfer pipets because the tubing is smaller and therefore difficult to fill with mercury. Also, the adjustment of volume depends on changing the length of tube rather than changing the position of the mark. Since 1 λ of water weighs 1 mg., which is in the range of the microchemical balance, the pipet may be calibrated by filling the weighed measuring tube with pure water and weighing it. As the volume is diminished, the accuracy of this method decreases. If pure methylene iodide (density = 3.325 g. per milliliter) is used, the weighing error can be considerably reduced, allowing reasonably accurate calibration of pipets as small as

0.25 λ. The volatility of both methylene iodine and water makes it desirable to weigh the pipet in a small, closed container or to fit the ends with caps. In order to minimize the total load on the balance, the pipet capillary should be calibrated before it is mounted in a supporting stem. Mercury may be used to calibrate self-filling pipets, but the technique of filling them with this liquid is quite difficult.

An alternative method of calibrating self-filling pipets which is sufficiently accurate for many purposes and is less susceptible to technical difficulties is to measure with the pipet a series of samples of a concentrated reagent which is readily and accurately determined by titration. A very satisfactory solution for this purpose is strong potassium chloride solution which may be titrated potentiometrically with silver nitrate to a high degree of accuracy and precision. Various other solutions may be substituted, but few can be determined with as great accuracy. Thus, strong mineral acid solutions may be used and titrated with standard caustic solution, or a standard ferrous ammonium sulfate solution may be employed which can be titrated quite accurately with ceric sulfate, with *o*-phenanthroline ferrous complex as indicator. Calibration by titration is inherently less accurate than weight calibration because all the errors inherent in the buret, the end point, and the normality of the titrating solutions are included in the pipet calibration. Even with very careful technique, these may easily amount to several tenths of a per cent. However, if the accuracy of the manufacturer's calibration is questioned, this procedure is capable of determining reasonably well whether the pipet meets the requisite standards of accuracy.

It should be noted that, because of the principle of operation, self-filling pipets are inherently less subject to filling errors than are any other type, i.e., the precision is very high.

Self-Adjusting Pipets

It is evident that, if a self-filling pipet contained a bulb, it would no longer fill automatically, but, if suction were applied to draw the meniscus past the bulb and the diameter of the upper tip were small enough, a considerable volume of solution would be measured with the precision of the self-filling pipet. The design of such a pipet tube is shown in Fig. 7. Similar pipets have been described by Mika (4), Benedetti-Pichler (24), and

more recently by Anderson (38). It may be held in a stem by sealing as shown in Fig. 5, clamping in the device shown in Fig. 6 or by cementing. Pipets of this type are practical with volumes up to 50 to 75 λ.

Some care is required to operate pipets of this type. Suction must be applied just to the necessary extent to fill the bulb and then must be immediately released. Otherwise, liquid might be drawn through the capillary to form a droplet at the upper tip. When such pipets are properly employed, they are as rapid as the capillary transfer pipet and are definitely more reproducible. They have the definite advantage over other recorded applications of this principle in that their application is very flexible, readily adapting itself to all types of solution measurement problems.



FIG. 7. Self-adjusting pipet tube. (½ actual size.)

Linderstrøm-Lang and Holter (5) described two types of pipet operating on the principle of the self-filling or self-adjusting pipet. These were subsequently widely used in studies of enzyme activity and distribution in biological materials (6–8). The first served to measure materials which were restricted in quantity such as the original sample being analyzed. It is shown in Fig. 8. The measuring tube is drawn to a capillary tip which is slightly bent so as to contact the side of the test-tube titration vessel. The sample is drawn into the pipet with gentle suction until it is slightly above the mark, after which the meniscus is allowed to drop slowly with both vessel outlets closed so as to operate against a slight vacuum. As the mark is reached, the vessel containing the solution is quickly withdrawn by being lowered on a support operated by a rack and pinion. Capillary action is sufficient to maintain the measured sample in the tube until it is gently expelled by pressure into the receiving vessel. Such pipets measure samples of 5 to 10 λ with an error of less than 3% (5).

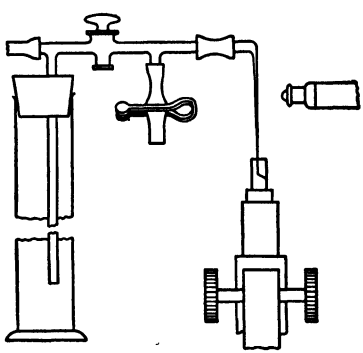


FIG. 8. Sampling pipet of Linderstrøm-Lang and Holter.

The second type of pipet (5) was for measuring reagents (ca. 30 λ) in plentiful supply (Fig. 9). It represents more closely the true self-adjusting pipet and varies from that previously described (Fig. 7) chiefly in its method of filling, which is performed by forcing in liquid from the outer reservoir which encloses the top of the pipet. After the level of liquid in the reservoir is lowered to free the upper pipet tip, a pressure of 50 mm. of water is applied to force the contents of the tube slowly

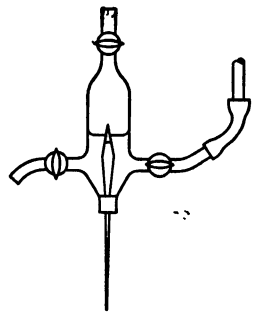


FIG. 9. Reagent pipet of Linderstrøm-Lang and Holter.

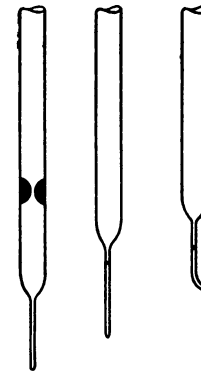


FIG. 10. Levy-type pipets.

into the receiver. The error of a 30-λ pipet was found to be less than 0.1%, thus demonstrating the great reproducibility of pipets operating on this principle.

Levy (9) introduced a modification of the self-adjusting principle of pipet operation which has been widely adopted. The design of such pipets is shown in Fig. 10. This type of pipet, which Levy termed semiautomatic, is attached to an air pump and pressure regulator as shown in Fig. 8. The latter is adjusted so that the capillary forces at the tip and at the constriction just balance the pressure when the tip is free. When the tip contacts a liquid surface, the pressure is then sufficient to discharge the contents without altering the adjustment. This allows rapid operation, but the adjustment requires some time and patience. The pipet is filled just a little above the constriction, either by capillary forces (small pipet) or by suction (larger pipet). With the latter, the solution is allowed to flow down until the hydro-

static pressure is just balanced by the capillarity of the liquid at the constriction. Since the length of such a pipet is critical with respect to the size of the constriction, considerable care is necessary in constructing the pipet and possibly in allowing for variations of surface tension of the solutions being measured. When the Levy pipet is constructed for the more minute volumes, its construction becomes very critical. It is, however, capable of measuring extremely small volumes, even as little as 0.05 λ,* with satisfactory accuracy. Although this is probably the smallest volume ever measured and transferred quantitatively, it is apparent that the inherent accuracy of the Levy pipet and of the straight capillary self-filling pipet for minute volumes is about the same. The most serious problem with both is calibration when the volumes are too low for convenient transfer and weighing.

TABLE 5. CONVERSION OF APPARENT WEIGHT OF WATER TO TRUE VOLUME

Temperature, ° C.	*Factor*
20	1.0028
21	1.0030
22	1.0033
23	1.0035
24	1.0037
25	1.0040
26	1.0043

Borsook and Dubnoff (10) mounted the large-type Levy pipets in multiple with a common manifold to apply suction and pressure and with a separate stopcock in the connection to each pipet tube so that any pipet could be operated without disturbing the others. The advantage of this procedure lies only in that the constant changing of pipet tubes is avoided, since in each instance only one pipet is used at any one time. An objection to the designs of Linderstrøm-Lang and Holter and of Levy is their rigid and inflexible mounting and mode of application, which make it necessary to move all containers to and with respect to the pipet rather than the usual method of moving the pipet to the container as needed.

Calibration of self-adjusting pipets is accomplished most easily by weighing the pipet both empty and filled with water or

* H. Holter, private communication.

methylene iodide, as described for self-filling pipets. Table 5 shows the conversion factors for transforming the weight of water in air against brass weights to the true volume, as calculated from data given by the International Critical Tables.

MICROGRAM TITRATION EQUIPMENT

Burets

When small liquid volumes are to be titrated for constituents present in ordinary dilutions, the titrating liquid must be added either in an extremely dilute solution or in a very small volume. As discussed in Chapter 1, titration with very dilute solutions leads to increased errors and to difficulties in locating end points. The use of precisely measured small volumes is simple and is definitely to be preferred, since the concentrations can be main-

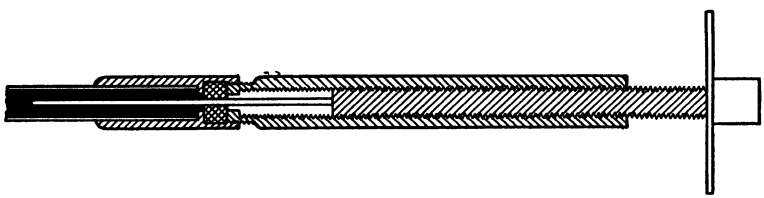


FIG. 11. Modified Rehberg-type buret control.

tained at normal values and the corresponding errors can be diminished. Burets for adding precisely measured small volumes to a sample are almost invariably made from capillary tubing in which a small volume occupies a considerable tube length, thus allowing small differences in volume to be read with minimum uncertainty. Most capillary burets have capacities of 0.1 ml. or less, and it is uncommon for them to be used with solutions more dilute than 0.01 *N*.

The flow of liquid in a fine capillary tube cannot be satisfactorily controlled with a stopcock as is customary with conventional burets. Several principles for controlling the flow with great delicacy have been described and employed in the design of capillary burets. The best known and most widely applied of these is the Brandt-Rehberg principle (11), in which the agent is drawn into or forced from the capillary by mercury, the movement of which is controlled by a screw-operated plunger inserted through gaskets into a closed mercury reservoir, as shown in Fig. 11. An early application of this principle to smaller burets

than that of Rehberg was made by Linderstrøm-Lang and Holter (5). Their buret consisted of an upright capillary tube attached at the bottom to a Rehberg-type control, with the upper end bent over and down so that the delivery tip could be dipped into the solution being titrated. Numerous modifications of the Rehberg-type buret have been described (12–17).

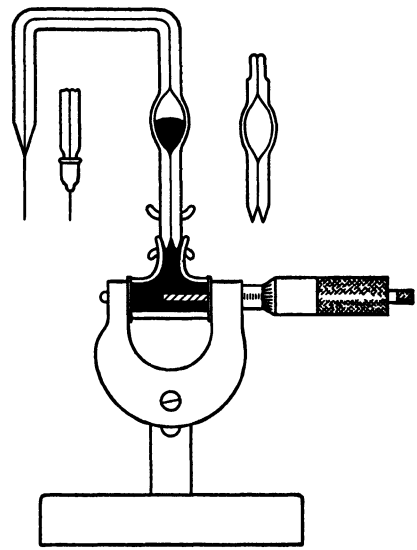


FIG. 12. Micrometer buret of Scholander.

Three general objections to the vertical buret operating on the Rehberg principle are recognized. (*a*) The pressure of mercury in the tube is rather high, causing frequent leakage around the gaskets of the screw plunger. (*b*) The solution is in continuous contact with mercury, causing in some instances corrosion of the mercury and contamination of the solution or deposition of insoluble mercury compounds in the capillary, tending to dirty or plug it. (*c*) The buret is bulky and inflexible, necessitating that all other titration equipment be arranged and adjusted to it. Certain modifications have been made to overcome one or more of these difficulties, but only a few effectively overcome all.

A principle similar to that of Rehberg was described by Widmark and Ørskov (18). Mercury was forced into and out of a capillary by means of a plunger, but the plunger was actuated by a micrometer screw, and the volume delivered was read from the screw rather than from the meniscus position. Such burets are less bulky, and the pressures generated may be much lower than in the conventional Rehberg-type buret. They may be susceptible to thermometer effects, however, which introduce volume errors not present when the meniscus is read. With soft gaskets such as rubber, some error from creeping of the

gasket is also expected in this type of buret. Düsing (19) and more recently Scholander (20) have described similar burets which have proved very useful. The design of the Scholander buret is shown in Fig. 12.

Most difficulties inherent in the Rehberg-type buret have been effectively eliminated in the design of Kirk (12), the latest modification of which was described by Sisco, Cunningham, and Kirk (3). It is shown in Fig. 13. This buret consists of a capillary of about 0.5-mm. internal diameter, mounted on a rotating metal back which is supported on a swivel clamp which also allows vertical adjustment on a ring stand. The mercury contained in the buret cannot contact the titrating liquid, being held only in the lower extension of the tube, while the titrating solution is entirely contained in the calibrated region of the tube. Liquid is drawn into the buret by retraction of the plunger, because of the negative pressure created by the falling column of mercury, and it is expelled from the tube when this small negative pressure is released by forcing the mercury upward. The over-all height of mercury in the tube is always small, making the problem of leakage past the gasket negligible. This design has been adapted to volumes of 25 λ to as much as 1 ml. by changes in the length and internal diameter of the capillary and the diameter of the plunger.

The mercury control is made of steel. It contains a plunger which is usually 2 mm. in diameter, operating through a gasket of hard wood or polytetrafluoroethylene (Teflon). A large wheel operates the screw plunger to give slow, carefully controlled delivery during titration, a smaller nob serving for rapid filling and emptying of the buret. In addition to the adjustment allowed by the swivel clamp, a further axial adjustment is usually installed which permits movement along the axis of the calibrated tube. The analyst can then insert the tip of the buret into the tubes or other apertures in fixed apparatus without danger of breakage. The buret requires virtually no cleaning if it is thoroughly rinsed with water after each use and stored full of water. There is no aperture for entrance of dust or laboratory fumes except the fine capillary tip, which, because of its location and direction, does not tend to collect dirt.

Before the buret is filled, the water is expelled and the tube rinsed with the titrating solution. Filling is performed by draw-

ing the solution through the tip by rotating the plunger nob rapidly in a counterclockwise direction. The liquid meniscus is

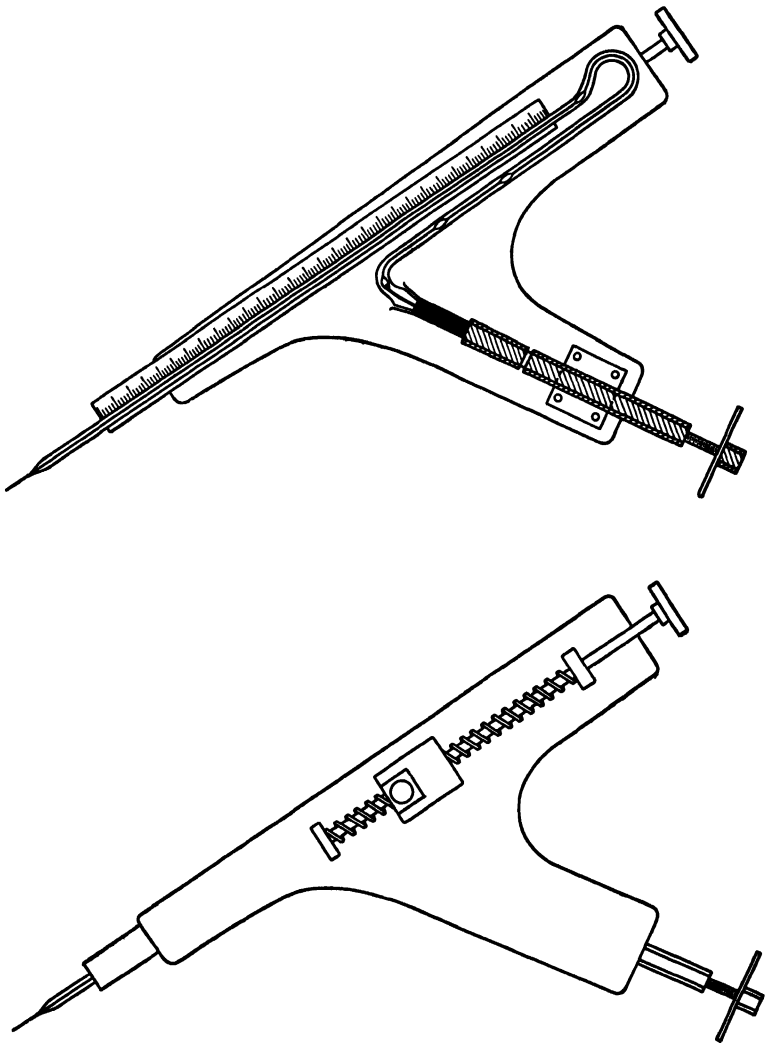


FIG. 13. Universal-type capillary buret, front and rear views.

set exactly on zero, the solution container withdrawn, and the outside of the tip wiped dry with a cotton wad or absorbent paper.

The tip of the buret is inserted into the solution to be titrated, and the large wheel of the control is rotated clockwise at a slow and uniform rate. Complete emptying of the buret should not occupy less than 5 minutes in order to standardize and minimize drainage errors. Rapid delivery during titration inevitably gives rise to a greater holdup of liquid and greater chance of significant error. This effect is entirely comparable to the necessity of slow delivery from any other type of buret or pipet, but the delivery rate must bear a definite relation to the size of the bore if the delivery error is to be controlled. Conway (21) has shown that, "The coefficient of variation for the single delivery from a straight tube which is given by the expression $0.022/\sqrt{dt}$ is in this whole question of glass error the most significant for micro work, for by altering the meniscus descent in an inverse relation to the tube diameter the delivery error remains unchanged." In the above expression, d = tube diameter and t = the mean time in seconds for the meniscus to fall 1 cm. This author also points out that drainage error is avoided when the delivery tube is horizontal or when a device such as that of Rehberg is employed. In practice, the rate specified has been shown to give reproducible delivery when the internal diameter of the capillary is about 0.5 mm.

Another widely used principle for control of flow from capillary burets is that of Schwarz (22). As originally described, the Schwarz buret consists of a vertically mounted tube, bent as shown in Fig. 14. The delivery end is drawn to a very fine tip, and the opposite end is attached to a suction tube as shown. The entire buret is mounted in such a way that the tip can be easily raised or lowered into the solution being titrated by sliding on two parallel upright supports. Stopcocks, mercury controls, and other devices are eliminated by the action of the fine tip in which surface tension and capillarity of the titrating fluid serve to stop the flow. The capillarity of the tip is sufficient to support a considerable negative hydrostatic pressure produced during titration. If the tip is not immersed, the surface tension of the first expelled droplet prevents the flow of liquid against a reasonable positive pressure such as that applied by mouth during delivery. When immersed in the sample, the tip serves merely to prevent rapid flow, but a gentle pressure or suction gives rise to a slow liquid movement in either direction.

The Schwarz buret is operated by being pressed down until the tip is immersed in the solution being titrated, after which a little pressure from the mouth causes a slow delivery of titrating liquid into the sample. This flow is stopped merely by release of the downward pressure on the buret, which is then forced upward by the spring so as to remove the tip from the sample. Surface tension immediately comes into play at the unimmersed tip to stop the flow, after which mouth pressure is released.

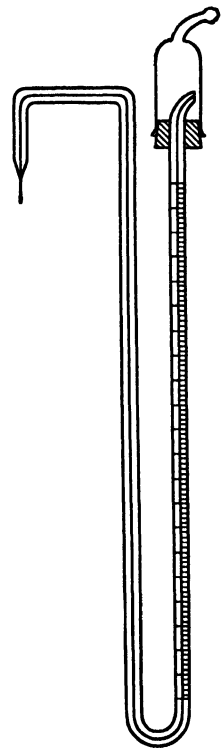


FIG. 14. Tip-controlled buret, Schwarz type.

As originally described, the parallel supports of the Schwarz buret were difficult to mount accurately enough to allow free and smooth motion in the vertical plane. This difficulty was overcome in the author's laboratory by bending of the delivery tube outward toward the operator and mounting of the buret on a hinge so that pressure on the bottom of the mount drops the delivery tip into the sample. A spring retracts the buret to the vertical position. Other designs in which control is achieved by the Schwarz principle have been described by Heatley (23) who adjusted the operating pressure by means of a mercury leveling bulb, by Benedetti-Pichler (24) who used a comparatively involved pressure reservoir and manometer arrangement, and by Linderstrøm-Lang and Holter (25) who employed a Rehberg control and manometer to maintain a proper operating pressure.

The major objection to the Schwarz type of control is based on the fact that, if the capillary tip is fine enough to balance a considerable hydrostatic pressure (allowing a long buret tube), the rate of filling and emptying is extremely slow. If the tip is made large enough to allow a reasonable speed of flow, the length of tube in which the liquid pressure can be balanced is inconveniently short. This objection is overcome only with pressure-regulating devices or with a horizontal tube. The main advan-

tage of the Schwarz design is its ease of construction which allows any amateur glass blower the advantages of such a device.

Probably the most satisfactory design for operation with this principle is a horizontal type in which a small, constant hydrostatic pressure is maintained automatically and the tip is used

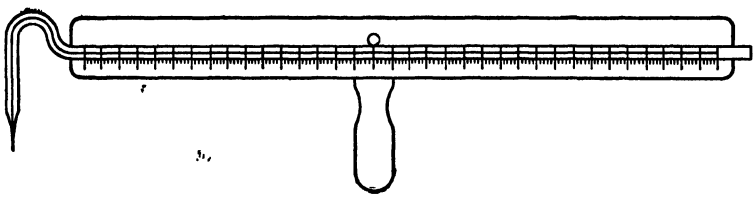


FIG. 15. Tip-controlled horizontal self-filling buret.

for control. The advantages of such a design are (*a*) tube lengths may be longer, allowing greater volumes and higher sensitivity; (*b*) a constant pressure allows uniform delivery rate from all portions of the tube; and (*c*) the drainage error from horizontal tubes is highly reproducible. Although several variations of this design are possible, that shown in Fig. 15 has been found quite satisfactory in the author's laboratory. The buret is mounted on a swivel at or just above the center of gravity. It is filled automatically by being tilted slightly in a clockwise direction which raises the tip and makes the buret a sort of capillary siphon. When the titrating liquid touches the tip, the buret ordinarily fills without application of any suction. When the buret is full, the flow stops automatically as the liquid reaches the end of the capillary at the 0 mark. The buret is then rotated until it is horizontal. When the tip touches the liquid being titrated, a slow uniform rate of flow is established, which is stopped instantaneously when the contact with liquid is broken. This buret may be modified for filling from a reservoir, as shown in Fig. 16, by means of a

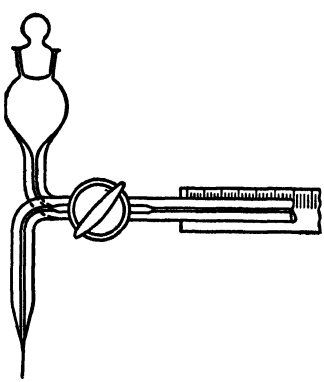


FIG. 16. Horizontal tip-controlled reservoir buret.

capillary three-way stopcock which fills the buret without tilting it and which starts or stops the flow. It is not useful for controlling the rate of flow, which must be done by adjustment of the size of the tip orifice. This principle has been applied to larger burets by Hybinette and Benedetti-Pichler (24) and by Reiman (39).

One further principle has been successfully applied to control of the flow from capillary burets, viz., that of Conway (21). The

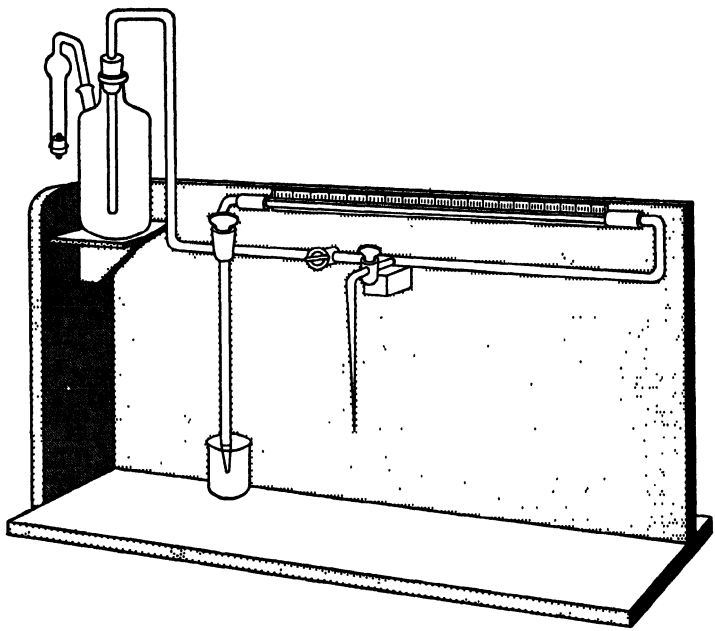


FIG. 17. Conway-type buret. (*Courtesy Crosby Lockwood Co.*)

Conway buret consists of a horizontal calibrated tube with accessories for filling it from a reservoir and for controlling a small hydrostatic pressure, as shown in Fig. 17. The flow rate is adjusted entirely by regulation of the hydrostatic pressure, and the flow is started and stopped by opening or closing a stopcock. This type of buret is relatively simple to construct and allows interchangeability of calibrated tubes, thus altering its range of utility (though not its limiting accuracy since the calibrated tubes are all of the same length). The presence of rubber connections is not favorable, and the buret does not

have the flexibility of some other designs with respect to manipulation.

Calibration of Capillary Burets. All types of capillary burets can be calibrated in a similar manner. Some authors have used titration of known volumes of accurately standardized solutions, a procedure which is not to be recommended since it incorporates in the buret calibration all the errors of the pipets and of the strength of solution. To be satisfactory, the calibration should be directly related to a weight, which at a known temperature can be converted accurately to a volume. The method that has long served for calibration of the Kirk-type buret will be described, since it is based directly on the weight of water delivered, and in an amount which allows accurate weighing. The procedure is as follows.

1. Draw into the buret tube a short thread of mercury approximately the length of one major scale division, and set the meniscus accurately on the 0 mark.
2. Measure the length of thread on the scale as accurately as it can be read.
3. Move the thread so that the meniscus is set on the 1 mark and repeat.
4. Repeat steps 1 and 2 for each major division mark on the scale.
5. Calculate the average length of mercury thread, and the deviation of each individual length as a percentage of the average.
6. Fill the buret with distilled water, and deliver the total buret capacity at the same slow rate as in titration into a small glass-stoppered vessel (Fig. 24).
7. Weigh the delivered water carefully on a good analytical balance, and calculate the true volume according to the figures of Table 5.
8. Divide the true volume of the buret by the number of major divisions to obtain the average volume per division.
9. Since the average volume per division should correspond to the average length of mercury thread, corrections obtained from the mercury thread measurements are applied to each of the major divisions of the buret. The percentage correction is applied in reverse sign to the average volume per division. Note that the shorter the thread length, the greater the volume.

10. Calculate the summation of the volumes from the zero mark to each major buret division.

11. Divide the summation for each division by its numerical designation to obtain a factor by which the buret reading is multiplied to obtain the volume from zero.

12. For convenience in operation, prepare a graph in which the above factors are plotted against the buret readings, so that intermediate factors may be read directly.

This somewhat involved calibration procedure may be avoided if tubing of completely uniform bore is available. It is then desirable to arrange the scale to read directly in microliters, marking it uniformly as in standard types of buret. Owing to the great difficulty of making sufficiently long capillary tubing of fine bore, such burets are not readily available as yet, though some designs of buret marked directly in microliters can be obtained. The objection to them is that the application of the relatively large corrections which are found with most commercial tubing is as time-consuming as the use of an arbitrary scale for which factors are available. The uniformity of capillary tubing of suitable diameter is very much inferior to that of the larger tubes from which conventional burets are constructed.

Titration Vessels

When a liquid sample is small, e.g., less than 0.5 ml., the choice of a vessel in which to contain it during titration is much less simple than at first appears. Flask or beaker shapes are not favorable since they do not allow shaking as with larger samples, and their shape prevents easy access by buret tips, stirrers, or electrodes. For most titrations, the most convenient shape is that of a dish, and with few exceptions this design has proved practical for nearly all types of titration. Such a vessel, constructed of chemical porcelain, is shown in cross section in Fig. 4. The white color of the porcelain aids in recognition of the end point, and the shape of the dish is such that several accessories may be inserted easily at a time. Most titrations allow the dish to be used in the open, but, if an inert or special atmosphere must be maintained, the dish may be readily enclosed in a vessel of special design which allows titrations to be performed under these conditions. Glass dishes of similar design may be substituted for porcelain. Aside from the difficulty of

constructing them, they do not give as easily visible end points with color indicators as are obtained in the porcelain dish.

A criticism of the porcelain dish as a titration vessel is that the thin layer of liquid in the dish does not allow color end points to be observed unless the indicator is kept at a concentration greater than usual. For example, if a large volume of acid is being titrated with base, the operator observes the color by means of light which has passed through a thick layer of liquid. If now a drop of that solution is removed to the titration dish, the color is no longer visible because of the thinness of the light-absorbing layer. The same limitation is present with test tube and other designs of titration vessels. This difficulty may be avoided readily by employing higher indicator concentrations and applying an indicator correction as described in a subsequent section. It was partly corrected by Llacer and Sozzi (26) who used a boot-shaped glass vessel and observed the color change while looking along the axis of the foot portion. Stirring and insertion of accessories were difficult with this design.

Linderstrøm-Lang and Holter (5) adopted micro test tubes as standard titration vessels. They are satisfactory only if the other equipment such as the stirrer is designed specifically to operate in the small available diameter. They likewise require a special holder designed for limited micromanipulation by means of rack and pinions. Their chief advantage is a low surface-to-volume ratio as compared with a dish. Other types of special vessels useful only for unusual types of analysis will be described in connection with the procedures involved.

Stirrers

Stirring of very small volumes such as a single drop of liquid is one of the more difficult operations for which equipment must be designed. The physical limitation of space in which the liquid may move, combined with the surface tension effect, greatly restricts the possible mechanisms. Rotating stirrers are unsuitable for most stirring on this scale,* and vibrating stirring rods must be very much smaller and under better control than is possible with manual operation. The simplest and most effective

* Borsook and Dubnoff (10) report successful use of small rotating stirrers applied to modified Conway (21) diffusion cells which provide a shape intermediate between the micro test tube and the titration dish.

method of stirring is based on the principle of the vibrating rod (12). The design of an effective vibrating stirrer is shown in Fig. 18. A small magnet such as is used in door bells is mounted in a Bakelite block. The magnet operates on a 6-v. alternating current supplied from the line by a small transformer. The house current commonly imparts a 60-cycle vibration to a steel strip attached to the Bakelite block at the upper end and in front of the

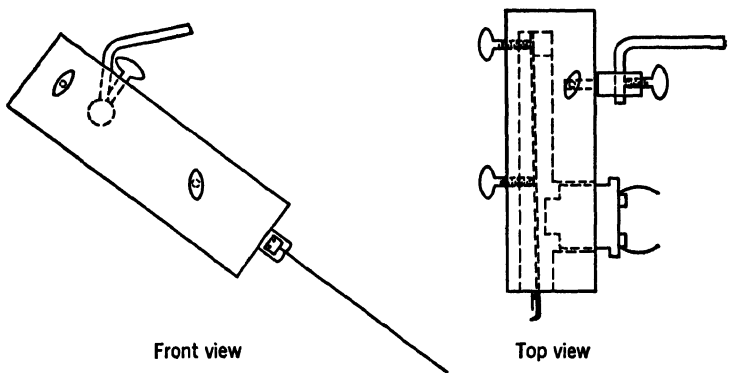


FIG. 18. Magnetic vibrating thread stirrer.

magnet. Mounted on the steel strip vibrator is a thin glass rod held in a small spring clamp. The vibration created by the rapidly alternating current is transmitted to the glass thread which is dipped into the liquid being stirred. Adjustment of the amplitude of vibration is achieved by means of a screw which presses on the metal vibrator. When properly adjusted, no spattering is produced, and the stirring of the smallest volumes is both vigorous and simple. The block carrying the mechanism is mounted on a swivel clamp and is arranged to permit rotation on the clamp, thus giving complete adjustability to the unit.

It is frequently convenient to stir a solution by means of a gas stream, especially when the gas is being used to maintain an inert or special atmosphere over the solution. This may be accomplished in two ways. The gas may be bubbled from a capillary tip directly through the solution (25), or, if a greater volume of gas must be introduced to prevent back leakage of air, the tip may be adjusted just above the edge of the solution so that the gas impinges on the side of the drop (3). This generates

a spinning movement in the liquid drop, the stirring effect of which is enhanced if the buret tip or electrodes are immersed, because these serve as a sort of stationary stirring rod. Bubbling of gas through a small volume of liquid is a questionable procedure because of the relatively large error produced if any spattering or mechanical loss occurs. The method should be adopted only when proper measures to prevent such losses can be taken.

A third general method of stirring small volumes which has proved quite satisfactory with amounts of more than about 0.2 ml. may be termed "pulsing." A small tube carrying a small rubber bulb such as a medicine dropper is dipped into the solution, and the stirring is accomplished by alternately pressing and releasing the bulb which sucks up and expels the solution. This method is particularly useful for stirring liquids kept inside closed containers under inert gas. It may also at times be adapted to titration in a distillation outfit such as is employed for volumetric lactic acid determination (27). The "pulser" tube may usually be incorporated as an integral portion of the apparatus, and in some instances it may utilize tubes already present for other purposes.

The last type of stirrer to be considered is that of Linderstrøm-Lang and Holter (5) which is primarily adapted for small test tubes. A tiny piece of iron enclosed in glass is the actual stirrer. It is actuated from above by a magnet of high capacity which is operated from direct current controlled by an interrupting switch. With each application of current the stirring bead is drawn to the top of the liquid, and it falls back to the bottom when the current is interrupted. Depending on the distance at which the magnet may be placed from the stirring bead, the current may have to be quite heavy, causing considerable heating of the magnet coil. The efficiency of stirring by this type of equipment is certainly less than that of any other methods discussed. The method is considered useful chiefly for special cases in which simpler technique and design are not adequate.

Titration Vessel Supports

It is not usually convenient to place a titration vessel on the laboratory work bench during titration, both because of the difficulty of manipulation and the need to observe the vessel closely to detect end points. When the vessel is a titration dish,

a Conway cell, or in fact any small vessel which may be readily supported, the titration table shown in Fig. 19 is very convenient. It is raised or lowered by rotation of the lower knob, and its general positioning is achieved by its location on a supporting ring stand. It raises the titration dish until the buret tip and stirrer tip are immersed. The table is constructed of chemical porcelain which gives a good titration background and is chemically inert toward spilled reagents, etc. A modified design which is more rapidly operated is shown in Fig. 20. Vertical movement is here obtained by means of a self-locking lever. Otherwise the operation is the same as described for the screw type. A rack-and-pinion gear has also been used for adjustment with considerable satisfaction.

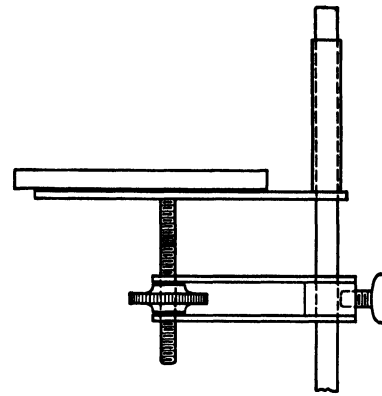


FIG. 19. Titration table, screw adjustment type.

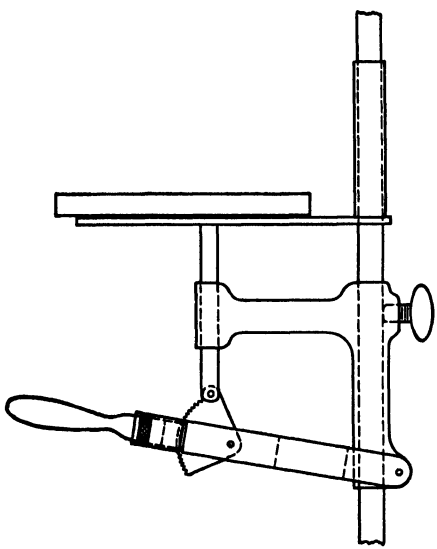


FIG. 20. Titration table, lever adjustment type.

Many variations of titration vessel supports are possible, and no detailed discussion of them is necessary because the analyst with ingenuity may increase the number of designs by an indefinite number. The type of support used by Linderstrøm-Lang and Holter (5) is the only additional one which has in any real sense been standardized and widely adopted. It is shown in Fig. 21. Designed for micro test

tubes, it is not directly applicable to vessels of other types. The holder itself is mounted on racks and pinions to impart vertical movement and, if desired, one or more directions of movement in the horizontal plane. The earliest type utilized a microscope mounting.

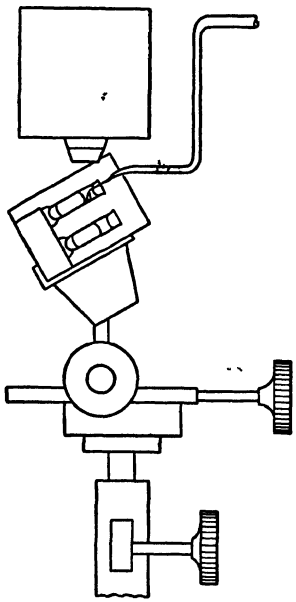


FIG. 21. Support for titration vessels, Linderstrøm-Lang and Holter type.

Illumination

Because of the difficulty of distinguishing end points in small volumes of solution, it is very important that microgram titrations be carried out under optimum lighting. A blue 50- or 60-w. incandescent bulb enclosed in a small shade of the type found on sewing machine lights is very satisfactory when placed not more than 1 ft. above the titration vessel. Properly shaded fluorescent tubes of the daylight variety are quite satisfactory, though their length interferes somewhat with manipulation. For special titrations involving end points which are very difficult to distinguish, colored lights have occasionally been found helpful. The manipulations of microgram analysis should never be performed without careful attention to the illumination.

APPARATUS ASSEMBLIES

Most of the titration equipment described above is suitable for mounting on ring stands, and great flexibility in the arrangement of equipment is therefore possible. It is desirable, however, to avoid the scattering of equipment around the location of the analysis and to improve the appearance of the setup with a carefully designed combination titration outfit such as that shown in Fig. 22, which is commercially available. A cabinet contains a buret so mounted as to retain the flexibility of the usual ring stand mounting, a titration table usually operated by rack and pinion, and a stirrer. An electric micro hot plate may be built into the titration table. Service outlets for gas, water, vacuum,

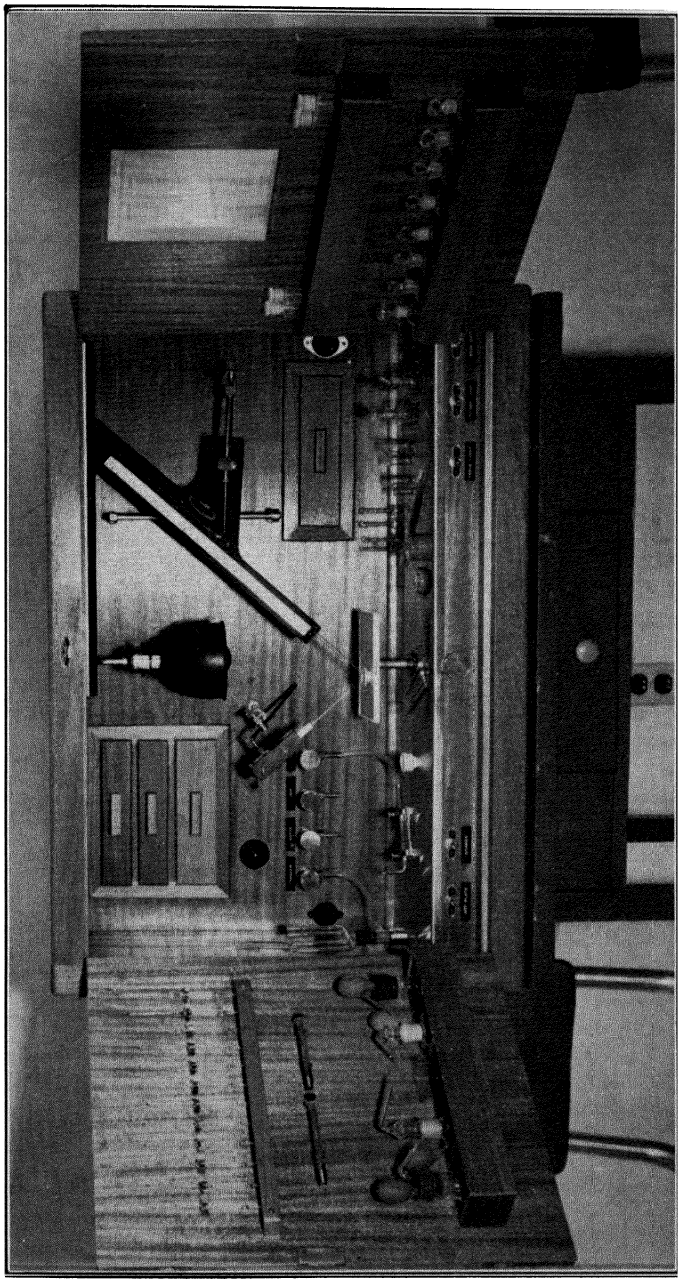


FIG. 22. Titration cabinet assembly. (*Courtesy Microchemical Specialties Co.*)

pressure, and alternating current at 110 and 6 v. are provided. All controls, switches, and rheostats are flush mounted. Racks and drawers are provided for storage of pipets and controls, diffusion and titration vessels, reagent bottles, etc. Both an incandescent titration light and general fluorescent lighting are installed. In general, the efficiency of the analyst reaches the maximum with this type of equipment, for the maximum convenience is provided for all operations.

LIQUID CONTAINERS

The amounts of liquid normally used in volumetric microgram analysis methods are so much less than in conventional analyses that vessels modified to a much smaller size are a virtual necessity. Although stock reagents are often conveniently stored in bottles of about 250 ml., the working reagents are much more conveniently dispensed from smaller containers. Volumetric vessels also require modification to appropriate volumes.

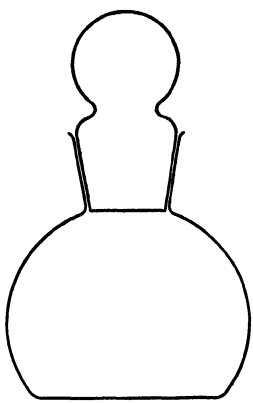


FIG. 23. Reagent flask. (Actual size.)

Solution Bottles

Most standard stock solutions and ordinary reagents are best kept in 250-ml. bottles for the maintenance of stability. Small reagent bottles or flasks such as that shown in Fig. 23 are then used to contain the working solutions. The contents of these bottles range from 10 to 20 ml. Their short necks allow easy insertion of pipet and buret tips. After the bottles have been used for a week or two, evaporation often alters the strength of the contained solution. At frequent intervals, the remaining contents of the vessel is discarded and replaced with fresh material from the larger stock bottle. Even with this degree of waste, the amounts of solution used are practically negligible.

Weighing Bottles

In calibration of burets with water, if the water is delivered into a standard-size weighing bottle, an appreciable percentage of

the total volume is lost by saturation of the contained air with water vapor. To eliminate this error, a small weighing bottle should be used, such as that shown in Fig. 24. The total capacity of this glass-stoppered bottle is about 0.5 to 1 ml. Its short neck allows easy insertion of the buret tip during delivery, and the size of the opening is such that little evaporation occurs with the buret tip inserted, because the latter practically fills the neck.



FIG. 24. Weighing bottle. (Actual size.)

Volumetric Flasks

Most critical of the small solution containers are the volumetric flasks which should have an accuracy more or less comparable with conventional sizes of such flasks if the microgram methods are not to suffer by comparison with larger scale analyses.

The test-tube-type "volumetric flasks" which are widely marketed are utterly worthless as volumetric equipment, since the normal error of setting the meniscus corresponds to several per cent of the total volume. Accuracy can be obtained only by (a) determining the meniscus position with extreme accuracy in a wide tube, which is nearly impossible, or (*b*) providing a narrow stem which allows ordinary errors of meniscus setting without affecting seriously the total volume. The latter procedure is preferred for simplicity. Table 6 shows the necessary internal diameters of stem which must be used with various volumes in order to obtain the accuracies indicated.

TABLE 6. STEM SIZES FOR VOLUMETRIC FLASKS

Volume	*Accuracy for 0.2-Mm. Uncertainty in Meniscus Setting*		
	1%	0.2%	0.1%
1 ml.	8 mm.	4 mm.	2.6 mm.
0.5 ml.	5.6 mm.	2.6 mm.	1.8 mm.
0.2 ml.	3.6 mm.	1.6 mm.	1.2 mm.
0.1 ml.	2.6 mm.	1.2 mm.	0.8 mm.

A small volumetric flask of conventional shape, viz., a bulb with a calibrated stem, is limited in the internal diameter of that stem by the necessity of inserting pipet tips. About the smallest

practical stem size is 2 mm., since a smaller tube not only makes it difficult to insert tips but leads to a serious trapping of air bubbles which are very difficult to displace. If this limitation of 2 mm. is the minimum stem size and an uncertainty of ± 0.2 mm. is assumed in adjusting the meniscus, it is seen that 0.1% accuracy is possible with a 1-ml. flask, almost 0.2% with a 0.2-ml. flask, and about 0.5% with a 0.1-ml. flask. Unless very fine capillaries are employed for filling and adjusting procedures, an 0.2-ml. flask is the smallest that is practical for really accurate



FIG. 25. Volumetric flask, 0.2 ml., glass stoppered.

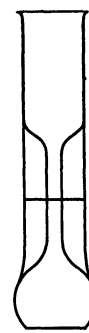


FIG. 26. Volumetric flask, 0.2 ml.

operations, though smaller flasks may serve where the requirements are not critical. When the volume is less than 1 ml., the completely conventional form of flask is not practical because of stem fragility and the difficulty of stoppering. These difficulties are nicely overcome in the design shown in Fig. 25.

The flask is blown from a capillary of 2-mm. internal diameter. The stem is made as short as possible to facilitate entry of pipet tips. The flare at the top also aids in this operation and allows an external ground stopper to be readily fitted. The short stem also aids in displacing entrapped air.

Another design which is not provided with a stopper but is very convenient is shown in Fig. 26. With this flask, nearly the entire volume may be placed in the tube above the capillary, allowing the entrapped air in the bulb to prevent filling. The flask is given a quick flip with the hand which drives the liquid

into the bulb, after which the meniscus is adjusted by careful additions from a pipet.

Mixing in small flasks cannot be accomplished by shaking. After the solution is made to volume, it is possible to mix it by insertion of a vibrating thread stirrer shown in Fig. 18. Another simple method is to insert a small glass bead with a diameter less than the stem, and shake. If the solution is not reactive toward mercury, a drop of this material may be inserted and shaken. The high density of the mercury makes it a particularly effective stirring agent. The solution must be made to volume before any type of stirrer is inserted, because of inevitable loss on its removal, and because of its unknown volume.

POTENTIOMETRIC TITRATION ACCESSORIES

End-point determination by potentiometric methods has been most used in microgram analysis for the determination of chloride ion by titration with silver ion (28–30). Acidimetric titrations employing a potentiometric end-point determinaton have been used in determining amino nitrogen by the formol titration (3, 10) and in studying the use of differential titration equipment (31). The application to oxidimetric titration has been made in the determination of iron by differential titanous titration (16) and in the study of simple differential titrations (31). In every instance, a different form and design of electrode equipment was employed, and it is not possible to describe any single type of equipment which can be considered as standard, or necessarily likely to become standard.

Perhaps the design of differential electrodes (31) shown in Fig. 27, which is a direct adaptation of the apparatus of Hall, Jensen, and Bäckström (32), is more widely adaptable than others; it is certainly one of the very simplest. It consists of a glass tube, 4 mm. in internal diameter and of a length convenient for mounting. To the upper end is attached a short length of rubber tubing, and the lower end is drawn to a tip which should be almost exactly 1.3 mm. in diameter for the best results. On the inside of the tube is mounted a length of clean platinum wire which comes to within 3 mm. of the lower orifice. The wire is sealed through the tube and may be connected with a metal band which becomes a terminal for attachment to the wires leading to the measuring instrument. Another platinum wire electrode is

looped around the outside of the glass tip and also attached to a metal band which serves as terminal. With this arrangement, dipping the glass tip into a solution being titrated causes the immersion of part of each of the two electrodes, and the constricted tip prevents rapid interchange of solution between the inside and outside of the tube. The electrodes are attached to a measuring instrument to determine any potential existing between them. This may be a reasonably sensitive galvanometer, but it is much better to use a potentiometer circuit or a vacuum-tube millivoltmeter such as a *p*H meter. This is desirable because, with the latter instrument, the voltage is measured reversibly without draining appreciable current from the system. If such a current is drained, the small electrodes used may become polarized, and, even more important, the resistance of the cell is usually high enough so that the amount of current which can flow is too little to give satisfactory deflections of the galvanometer.

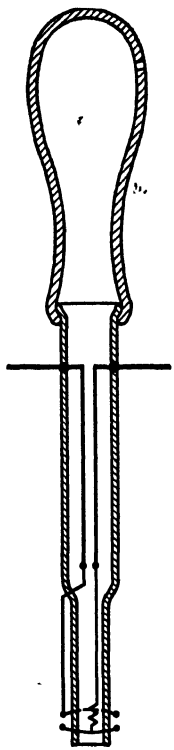


FIG. 27. Electrode vessel for differential potentiometry. (Actual size.)

The titration principle is that of MacInnes (33). If an increment of titrating solution is added to the sample outside the tube, a potential is established between the electrodes, one of which is in the original and the other in the partly titrated solution. If the solution inside the tube is forced out and mixed with the solution outside, that potential becomes zero. If then another increment of titrating solution is added, the potential generated this time is similar to the first, until the end point is approached, at which time the percentage change in composition with a given increment rapidly increases and passes a maximum at the end point. The simple device shown is applicable only to oxidation-reduction systems which operate with bright platinum electrodes. By using quinhydrone, however, it is directly applicable to the

titration of strong acids with bases. Substitution of silver wire for platinum should make it useful for halide titrations with silver salt solutions. The simplicity of design and construction and the wide applicability of the differential principle indicate a general utility for this device, largely unrealized at the time of writing.

Certain special types of electrode find definite application for specific types of titration. The glass electrode, for example, may be designed to operate with very small volumes and is therefore suitable for acidimetric analyses. When the solution volume is sufficiently great, it is possible merely to insert a small glass electrode constructed from Corning 015 glass into the solution to be titrated, along with a calomel or other reference electrode, and conduct an ordinary potentiometric titration (10). Since it is often desirable to titrate in the absence of atmospheric carbon dioxide (end point in the basic region), a device shown in Fig. 28 is at times serviceable (3). A glass electrode of the cup type *B* passes through a block of Lucite *a*, the interior of both being filled with reference liquid, e.g., 0.1 *N* hydrochloric acid solution. Into the solution is inserted an electrode *A*. The sample, which is placed in the cup of the electrode is shielded from the atmosphere by the Lucite hood *c*, which has entrances for the reference calomel electrode *D*, a gas inlet tube *E* which also serves as stirrer, and the tip of the buret *C*. The electrode may be constructed by blowing a bulb on the end of a tube of Corning 015 glass, heating the end of the bulb, and sucking it in to provide a very thin-walled cup. A design similar to that described may be used for other titrations requiring an inert atmosphere. The cup electrode can be substituted by a

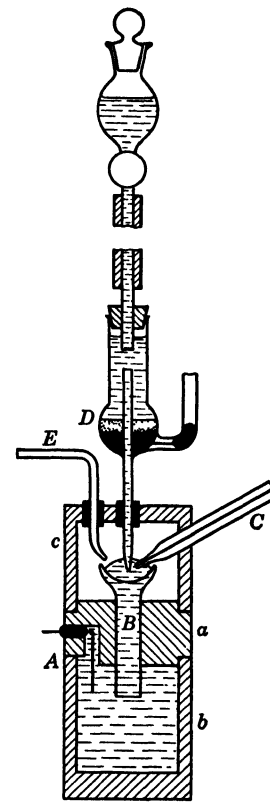


FIG. 28. Glass electrode titration apparatus.

simple cup, and an additional electrode of whatever type desired can be inserted. A titration requiring this kind of precaution is that of ferric ion by titanous ion, in which it is essential that all oxygen be rigidly excluded (16).

Bimetallic electrodes should be peculiarly suitable for microgram potentiometric titrations because of their small size and convenience. Few if any applications of such electrode systems have been made to this scale of titration, though the Clark (34) electrode system has been found suitable for titration of microgram quantities of chloride (30). The electrodes are shown in Fig. 29. They consist of two silver wires, the ends of each having been fused into a small ball. One is amalgamated with pure mercury under dilute nitric acid. Although not an exact bimetallic electrode system, the silver-silver amalgam system behaves similarly in the titration of chloride ion with standard silver nitrate solution.

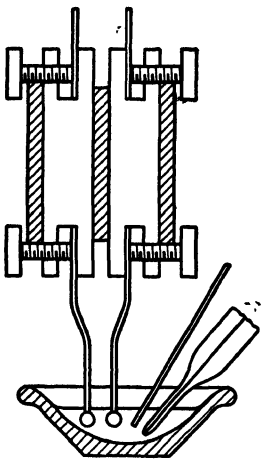


FIG. 29. Micro silver-silver amalgam electrodes. Adapted from Clark type. (Actual size.)

Details of the electrical equipment for measuring potentials in potentiometric titrations are available in several standard texts (35–37).

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CHAPTER 3

Colorimetry Apparatus and Technique

In this chapter is included a general discussion of the application of colorimetric instruments to the microgram and submicrogram scale of analysis. Since the most useful instruments are some of the spectrophotometers, the discussion will center largely around these instruments.

Colorimetric methods are usually somewhat less accurate and less precise than are gravimetric or volumetric procedures. They are, however, often very sensitive and in many instances may be made quite dependable for analysis of a wide variety of constituents in the inorganic, organic, and biological fields. The general applications to the field of inorganic materials (metals) has been well presented by Sandell (1). Broader treatments of the general field of colorimetry have been published by Snell and Snell (2), Yoe (3), and others. A more careful background of this broad field may be obtained from the volume by Gibb (4), and from Kolthoff and Sandell (5). Detailed treatment of biological and clinical colorimetric methods is available in such works as Hawk, Oser, and Summerson (6), Peters and Van Slyke (7), and many others. A recent useful pamphlet on colorimetry has been published by Mellon (8). It is the limited purpose of this volume to include the specialized adaptations of colorimetry to the smallest scale and the necessary theoretical considerations that must be understood in order to obtain dependable ultramicroanalyses by this means.

The volumetric methods described in this volume usually operate in a range somewhat below the usual lower limits of dependable colorimetry, and at the approximate lower limit of the range of the unmodified spectrophotometer. In this range, the latter instrument is much less reliable than when the amount of colored component is greater in amount. It is possible, however, so to modify the spectrophotometric technique as to achieve approxi-

mately the same dependability with submicrogram quantities as the instrument usually delivers with microquantities. Other colorimetric instruments and techniques have also been modified for analysis of microgram and submicrogram amounts, but with considerably less dependability than may be achieved with the spectrophotometer.

The most obvious and one of the widely adopted methods of colorimetry depends on comparing directly a colored solution with a set of solutions containing known amounts of the material in question. This is performed by direct visual observation as in the Nessler determination of ammonia. Alternatively, the standard may be adjusted by dilution or other means to match the unknown. In modern times, colorimetric comparisons and measurements have usually been made with one of various types of instruments which may be classified as follows:

1. *Direct comparison instruments,* e.g., Duboscq colorimeter or wedge-type colorimeter in which the thickness of one or both the solutions being compared may be adjusted until a match is obtained; or simple devices in which dilution or other adjustment may be made to aid the eye in determining a color match.

2. *Photometers,* which measure absolute or relative light intensity, instead of comparing solutions with each other. These may be classified as

A. *Visual,* including both filter photometers and spectrophotometers, and

B. *Photoelectric,* including both filter photometers and spectrophotometers.

Most of the above types of instrument have at some time or other been modified for the analysis of small volumes of solution. This is particularly true of direct comparison instruments, photoelectric filter photometers, and spectrophotometers. Such modifications are not uniformly successful with different types of instrument for reasons which will be developed in the discussion following. Moreover, only a few of them are sufficiently delicate to be classed as strict ultramicromethods, though most are borderline cases. It is not to be expected that the simple color comparison instruments will achieve a high degree of dependability and accuracy when they are used at the highest attainable sensitivity. This fact becomes clear in practice and indicates the extreme desirability of using only those few instruments which

are adaptable to very delicate measurements, if the analytical results are to have a real significance.

LIGHT ABSORPTION OF SOLUTIONS

Colored solutions show a visible color because some constituent of the solution absorbs certain wavelengths of white light more than it does other wavelengths. The visible color is given by those wavelengths that are least absorbed. Most colored materials absorb broadly throughout the visible spectrum; however, some absorb in narrow bands which gives rise to irregular, jagged absorption curves. The latter is termed selective absorption and may be illustrated by Fig. 30, showing the absorption of didymium glass, which, though not a solution, illustrates the type of curve given by some solutions. Selective absorption such as this is encountered most commonly with rather unusual materials such as rare-earth compounds and compounds of the transuranic elements.

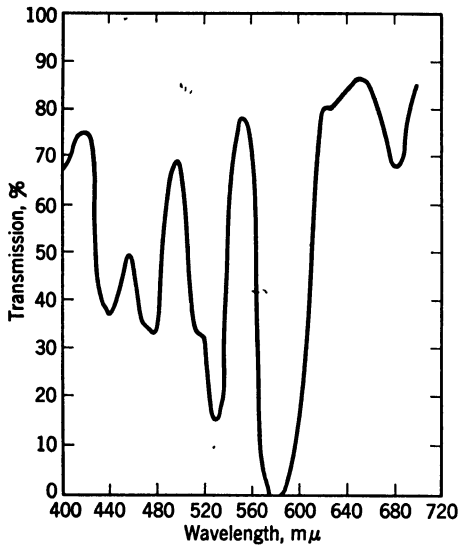


FIG. 30. Absorption curve, didymium glass. Selective absorption.

The more common type of absorption of colored materials in solution is shown in Fig. 31, which illustrates the absorption of dithizone and of copper dithizonate in carbon tetrachloride. It is observed that the absorption is in rather broad bands.

An important fact is that the visible color of the solution is given by that part of the light which is not absorbed, whereas it is the absorbed portion which is significant in colorimetry and spectrophotometry. Some interesting consequences of this fact are evident. Many if not most materials show some absorption

of wavelengths in the violet region. If this is the major absorption, the resulting visible color is yellow, meaning that, if a little violet is removed from white light, the resulting color is yellow. Nearly every common object is more or less yellow, owing to this common violet absorption. Moreover, yellow solutions are difficult to compare, partly because the exact range and amount of absorption necessary to cause yellow coloration is not definite but extremely variable. Another consequence of complementary absorption is that, in order to be truly dependable, colorimetric measurements must rest on color absorbed rather than on color

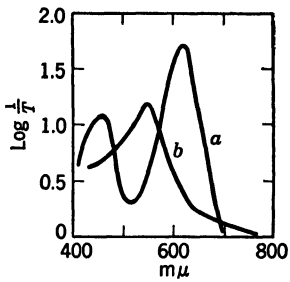


FIG. 31. Characteristic absorption curves. (*a*) Dithizone absorption. (*b*) Copper dithizonate absorption.

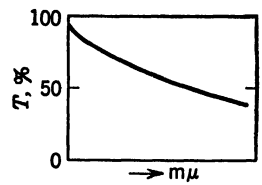


FIG. 32. General absorption. Blue solution.

transmitted. For this reason, visual comparison of colors can never be theoretically dependable unless the incident light is of a single, known wavelength, as with the visual spectrophotometer. Table 7 shows the relation of wavelength to the visible and absorbed colors.

TABLE 7. RELATION OF COLORS IN SOLUTION

Wavelength, mμ	*Hue Transmitted*	*Complementary Hue Absorbed*
400–435	Violet	Yellowish green
435–480	Blue	Yellow
480–490	Greenish blue	Orange
490–500	Bluish green	Red
500–560	Green	Purple
560–580	Yellowish green	Violet
580–595	Yellow	Blue
595–610	Orange	Greenish blue
610–750	Red	Bluish green

Blue solutions tend to show general absorption, i.e., absorption at all wavelengths but greater toward the red end of the spectrum and least in the short wavelengths of the violet end. A typical curve for general absorption is shown in Fig. 32. It is much more common in analytical spectrophotometry to encounter absorption spectra with definite maxima and minima rather than the type of general absorption illustrated.

FUNDAMENTAL CONCEPTS OF COLORIMETRY

Lambert's Law. When light is transmitted through a body, some portion of it is always absorbed. If *any single wavelength* of light is considered, the amount of the monochromatic light transmitted is proportional to the intensity of the incident light. Thus, doubling the light applied should double the amount transmitted.

Then $\frac{I_t}{I_0} = a$ = the fraction of incident light which is transmitted.

I_0 = intensity of incident light.

I_t = intensity of transmitted light.

The value a is a constant for any given wavelength, but it differs with different wavelengths. If the thickness of the colored object is doubled, the incident light of the second half, having been reduced by absorption in the first half, is still further reduced in the same proportion. Thus the absorption is an exponential function. If the thickness l of the object is altered

$$\frac{I_t}{I_0} = a^l$$

Beer's Law. When the colored object is a solution, a similar law may be derived for the effect of concentration of dissolved colored substance. This is termed Beer's law (or the Bouguer-Beer law) and is expressed as

$$\frac{I_t}{I_0} = a^c$$

where c is the concentration of colored substance.

If the equation for Lambert's and Beer's laws are combined and the differential equation for light transmission is set up and integrated, the fundamental equation of colorimetry and spectro-

photometry results:

$$I_t = I_0 10^{-klc} \quad \text{or} \quad \log \frac{I_t}{I_0} = -klc$$

where $I_t/I_0 = T$ (transmission) and k is a constant under stated conditions which is termed the *specific extinction.* If the concentration is expressed in terms of moles, $k = \epsilon$, the *molecular extinction.* The value of the constant is determined by the nature of the colored material, the solvent, the temperature, and the wavelength of the light. For a simple absorber, k or ϵ will not depend on the concentration or the length of solution through which the light passes. If k is not found to be constant with respect to these two factors in a given case, Beer's law does not apply to the system, and something more than a simple light absorber is involved.

APPLICATIONS OF THE ABSORPTION LAW

The most common type of color-measuring instrument for chemical analysis is based on color matching of two halves of a visual field, as in the Duboscq-type colorimeter and less commonly in the wedge type. These instruments operate on the principle that, if the transmission through the two halves of the instrument is equal, i.e., $(I_t/I_0)_1 = (I_t/I_0)_2$ and k is the same for the two solutions being compared, then $c_1 l_1 = c_2 l_2$ or $c_1/c_2 = l_2/l_1$. The assumptions that are not strictly justified in many instances are (*a*) that the transmissions are the same because a visual color match is obtained and (*b*) that k is the same for the two solutions. It need only be pointed out that a combination of yellow and blue wavelengths may be readily adjusted to give the identical visual appearance as a green wavelength. Perhaps even more often, visual comparison of color is in error because the hue is not identical even when the intensity appears to be the same. In this case, it is quite certain that the absorption of the critical wavelength must be different in the two solutions, and the analysis is not correct.

It must also be realized that the substitution of a photoelectric cell for the eye cannot remedy the above difficulties, since that device is totally color-blind except in the sense that it gives a different response to the same intensity of two different wavelengths. The only actual remedy is in the use of light which is more or less nearly monochromatic, whether it be obtained from

a spectrum or from filters. To obtain bands of light which approximate monochromaticity with filters is difficult and requires a large number of different filters, if any significant amount of the spectral range is to be covered. Few, if any, commercial instruments are used with a sufficient number of filters to approach, even remotely, the monochromatic quality of the spectrophotometer.

A corollary of the absorption law, which is developed for single wavelengths only, is that, since k or ϵ is different for every wavelength of light, it is theoretically impossible to obtain accurate analyses from polychromatic light, unless the distribution of wavelengths in the two solutions is the same. This can happen only when the compositions of the solutions with respect to all light-absorbing constituents are identical. Only then can direct polychromatic color comparison instruments give absolutely dependable results, whether the observation is visual or photoelectric. The spectrophotometer (particularly the photoelectric variety) is the only instrument that eliminates with reasonable completeness the uncertainty and error so often inherent in all other types of instrument.

As applied to the spectrophotometer, the absorption law leads to the following conclusions and indications as to its application:

1. Since the spectrophotometer measures directly the ratio I_t/I_0, it is necessary that two other terms of the equation be known in order to obtain the third. The length l of the absorption cell is readily determinable. The concentration may be adjusted to a known value in order to determine the value of k or ϵ. This is ordinarily accomplished by varying the concentration by known amounts and plotting $log\ T/l$ (or log T with constant l) against concentration. If Beer's law applies, this yields a straight line, the slope of which is equal to k or ϵ. This line passes through the origin. It serves as a calibration for later measurements in which c is being determined.

2. The greater the sensitivity of the color-forming reaction, the greater the value of k or ϵ, i.e., the steeper the slope of the straight line plotted as above. For the same reason, the wavelength chosen for the measurement should be one showing maximum absorption with the particular solution, because the greater the absorption, the greater the value of k or ϵ, and the greater the sensitivity of the determination.

3. Presence of light which is not absorbed (e.g., stray light, or wavelengths which are transmitted) decreases the sensitivity of the measurement because it is recorded by the instrument but has no relationship to the concentration.

Consider a beam of light containing two wavelengths, one of which is absorbed (intensity = I_0), the other completely transmitted (intensity = A).

The quantity of light measured will be $(I_t + A)/(I_0 + A)$ instead of I_t/I_0, which alone bears direct logarithmic relationship to klc when Beer's law is obeyed. If all wavelengths of light were equally absorbed (i.e., the absorber truly gray), all the k's for the various wavelengths would be equal and Beer's law would be applicable. In practice this never occurs. Consequently, it is never possible to determine whether Beer's law is actually obeyed by any wavelength if polychromatic light is used for the measurement.

4. The wavelength chosen for measurement must not only be the one which has the maximum absorption, but it should also lie in a region of the absorption spectrum which shows a broad absorption range. This follows from the fact that all practical instruments give a narrow range of wavelengths, 1 to 35 mμ, rather than a single wavelength. Unless this range is appreciably narrower than the absorption maximum, slight variations in setting the monochromator may produce appreciable inaccuracy in reading.

5. The absorption law can be applied to mixtures of absorbing materials, provided their absorption at two or more different wavelengths is sufficiently great to minimize the cumulative errors of multiple readings. It is convenient to define the *optical density* D, as

$$D = \log \frac{1}{T} = kcl$$

With two absorbers, measurements are made at each of two different wavelengths, λ_1 and λ_2. The concentrations of the colored components are then determined by solution of the simultaneous equations

$$D_{\lambda_1} = (k_1)_{\lambda_1} c_1 l + (k_2)_{\lambda_1} c_2 l$$
$$D_{\lambda_2} = (k_1)_{\lambda_2} c_1 l + (k_2)_{\lambda_2} c_2 l$$

DIRECT COMPARISON INSTRUMENTS

The so-called microcolorimeter which is commercially available is a modified Duboscq type of instrument in which small plungers and small cups having a working volume of 1 to 2 ml. of solution are employed. These instruments achieve a reduction in scale over the larger standard instruments inasmuch as the colored component can be concentrated in a smaller volume and therefore increase the total light absorption. Even with this improvement the lower limits of detection are usually of the order of several micrograms, and the accuracy is limited by the inherent difficulties of visual comparison of colors and by the low color intensity of dilute solutions.

Direct comparison of colored solutions in capillary tubes has been employed by numerous investigators as a truly ultramicro-procedure. Thus, Went and Drinker (9) determined the blood volume of small animals by injecting dye and measuring the intensity of the dye color in 0.01 ml. of blood. Probably the most notable series of quantitative analyses depending on direct comparison with standards in capillary tubes of minute solution volumes was that of Richards, Walker, et al. (10), who made extensive studies of kidney excretion in amphibia. As ingenious and useful as these procedures are, they are likely to involve rather large errors except under the most favorable circumstances. Moreover, no special discussion of the techniques aside from the complete method is necessary since only careful handling of capillary tubing is involved.

Of wider utility are modifications of the Duboscq colorimeter for small solution volumes. A simple device of this type was described by Chapman (11) who constructed an entire colorimeter operating with volumes of 0.25 to 0.5 ml. This device, shown in Fig. 33, consists of two capillary cups, 10 cm. long and 2.5 mm. in internal diameter, enclosed in a light-tight box having a lighted compartment in the bottom. The only light emerging at the top is that which passes through cylindrical glass prisms dipping in the cups. Rubber collars on the prisms above the top of the cups eliminate light passing through the cups around the prisms. Vertical adjustment of the prisms is obtained somewhat crudely by counterweighing them and employing friction to maintain the position set. The observer sees two luminous fields

side by side, rather than as halves of a single field. The most interesting feature of this instrument is the manner of making the prisms. They are cut squarely at the bottom, apparently without effort to polish them and with no grinding. The tops of the prisms are ground so as to diffuse the emergent light and equalize irregularities in illumination created by the imperfection of the lower dipping end. The cups are constructed by fusion of a cover slip to the lower end of capillary tubing. The cover slip is heated on a nickel crucible lid coated with lampblack and protected by a Rose crucible lid. A brass wire inside the tubing keeps the end true at the point of sealing.

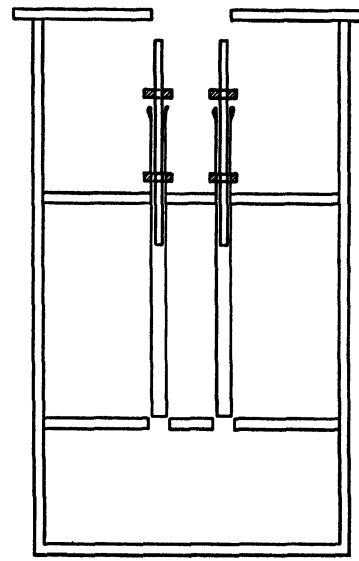


FIG. 33. Chapman microcolorimeter.

In the author's laboratory (12), a conventional Duboscq colorimeter has been modified for use with somewhat smaller volumes, viz., 0.05 to 0.15 ml. The prisms of the colorimeter are removed, and glass rods, about 1 mm. in diameter, are substituted and fastened with a metal bushing. The ends of these rods require grinding and polishing, if results are to equal those obtained with the usual prism. This operation is difficult with the facilities available, and perfect prisms do not result. It was found that a uniform field results when the prism is sufficiently flat, and that the visual field of the instrument is filled even by the small prism. The cups are made 10 cm. in length and with an internal diameter of 2 mm. The bottom end of the capillary tube is ground square and a cover slip cemented in place with balsam. Considerable care is necessary to prevent the balsam from flowing over the bottom window and preventing the uniform passage of light. The instrument is approximately as successful in colorimetric measurement as was the original Duboscq, though the field is not so uniform as could be desired, owing to some irregularity in the

prisms. Considerable care is necessary in operating this instrument, owing to the small size and fragility of the prisms. Analyses of quantities in the submicrogram range can be performed with this type modification of the ordinary colorimeter, and the accuracy is approximately the same as with the standard larger instruments.

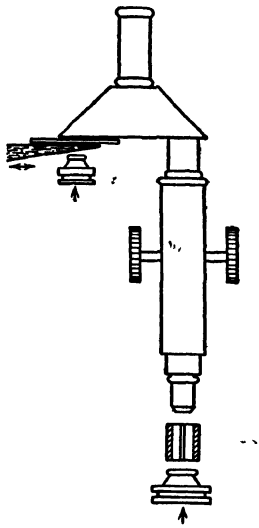


FIG. 34. Somogyi-type microcolorimeter. (Schematic.)

Somogyi (13) described a considerably more elaborate type of colorimeter which he employed for measuring blood volume in small animals by means of dyes. This instrument, the principle of which is illustrated in Fig. 34, combines a microscope with the converging prism system of a colorimeter. The cup containing the unknown is made from a capillary tube 20 mm. in length and holding 0.01 ml. Light passing through the solution is focused by the objective of a microscope from which the ocular is removed and converged by the standard prism system of the Duboscq colorimeter. The standard solution for comparison is carried in a hollow wedge on the other side. Light from a microscope condenser fitted with an iris diaphragm passes through the wedge into the converging prisms. Color matching is achieved by varying the position of the wedge.

FILTER PHOTOMETERS

The possibilities of adapting filter photometers or "photoelectric colorimeters" to very small solution volumes are definitely limited. In nearly all commercial instruments, the light source is rather broad, and the light absorption is measured by the photronic or barrier layer cell without amplification. Insertion of a small cell diminishes the lighted area to a degree inconsistent with the sensitivity of the photometer, unless the cell is rather broad and flat, which shortens the light path to a point at which adequate sensitivity cannot be obtained except with very strong colored solutions. Evelyn and Gibson (14) have described a plunger-type cell shown in Fig. 35, capable of application with as

little as 0.05 ml. of solution. The light path was reduced to as little as 1 mm. The exponential role of cell thickness in determining light absorption leads to the fact that thin cells of this type are useful only for strongly colored solutions in instruments of ordinary sensitivity. Although it is perfectly possible to design a filter photometer of considerably higher sensitivity than those now marketed and to adapt it to measurements with small solution volumes, the developments in this direction have not been extensive.

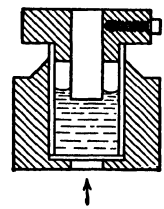


FIG. 35. Micro-absorption cell for the photoelectric photometer, Evelyn Gibson type.

PHOTOELECTRIC SPECTROPHOTOMETERS

Of all the above-mentioned types of instrument, the photoelectric spectrophotometer is by far the most generally useful colorimetric analytical device. It is the only one that can be readily modified to the submicrogram scale of analysis without significant loss of sensitivity, accuracy, or reliability. It consists of a combination of two instruments:

1. A *monochromator,* which is a device for obtaining essentially monochromatic light, and

2. A *photoelectric photometer,* which measures the intensity of light.

The combined instrument must be capable of dispersing white light into a continuous spectrum, isolating from that spectrum a small region which is essentially monochromatic, passing this isolated light through the solution being analyzed, and measuring its intensity with the required accuracy. By comparison with an uncolored blank the absorption of light by the colored absorber in solution is measured.

Monochromators

The monochromator consists of three essential parts: a light source, a dispersion device, and a means of controlling the range (slit width) and wavelength of the radiation emitted.

1. As a general source of light, the tungsten filament lamp provides a range from 350 to 2000 mμ, which includes the entire visible range and the near ultraviolet and infrared. This source is sufficient for most but not all analytical purposes. The hydro-

gen discharge tube covers the range 210 to 400 mμ in the ultraviolet. Infrared light is obtained from special heated filaments which cover a wavelength range from about 2000 to 24,000 mμ. It is essential that the light source give a continuous spectrum throughout the range desired so that any desired region can be isolated from it by the dispersion device.

2. For dispersing the light, glass or quartz prisms and diffraction gratings are employed. Prisms give a high degree of dispersion and do not yield second-order spectra. The degree of dispersion of the prism is continuously variable, being greater with the shorter wavelengths. Quartz prisms make the instrument suitable for the ultraviolet range. The prism is more commonly employed with high grade instruments than are other dispersion devices because of their high resolving power. The diffraction grating is less expensive and gives uniform dispersion at different wavelengths, allowing the use of fixed slits which are not suitable with prism instruments. For this reason, the grating instrument is more simply operated.

3. The slit, which isolates a region of the spectrum, is very important in the over-all design. The narrower the slit, the less light is available, and the narrower the range of wavelengths. Since both a narrow range and high light intensity are desirable, a certain degree of compromise is necessary. The range of wavelengths is a function of the degree of dispersion as well as of the slit width. Since a prism does not yield a constant degree of dispersion throughout the spectrum, the slit width needs to be adjustable. This is not necessary when a grating is employed, but a given slit width produces with a grating a comparatively broader wavelength band because of the smaller degree of dispersion.

The slit width is customarily defined in terms of the range of wavelengths emitted rather than the distance between its sides. Thus, the standard Coleman Model 11 instrument, with a grating and fixed slit, has an effective slit width of 35 mμ. When the wavelength dial is set for 500 mμ, the emitted light varies from 483 to 517 mμ. The Beckman Model DU instrument, having a prism monochromator, has a variable effective slit width, depending on the wavelength setting. The slit is also mechanically adjustable in this instrument. In most regions of the spectrum it is possible to narrow the range to as little as 1 mμ. Thus a

reading of 500 mμ on the dial corresponds to light emission in the range 499.5 to 500.5 mμ. This refinement of the Beckman instrument is one of its chief advantages as compared with other commercially available instruments. Another notable instrument is the Cenco Spectrophotelometer which has a concave grating monochromator and interchangeable fixed slit widths of 20, 10, 5, and 2.5 mμ.

Photoelectric Photometers (15)

Photoelectric photometers are based on either of two types of photoelectric elements: the barrier layer photocell or photronic cell, and the photoemission cell or phototube.

1. The barrier layer photocell usually consists of a copper-cuprous oxide, or selenium cell which generates considerable current when strong light impinges on it. This current is measured directly with a galvanometer and is proportional to the light intensity. It is not simple to amplify this current without a complete change and complication in the instrument. Thus, when the light intensity is low, the response of the photocell is unsatisfactory. This limits its use with micro absorption cells because the amount of light which can be transmitted may be insufficient to operate the cell effectively.

2. The phototube is designed so that light falls on a layer of metal or metal oxide (usually one of the alkali metals), causing electrons to be emitted into the evacuated interior of the tube. A collector electrode (anode) is also present in the tube. If a potential is applied to the metal layer and the collector electrode, the electrons emitted by the action of the light are collected, and a current proportional to the light intensity flows. Though the sensitivity of the tube varies with the wavelength, the cell cannot otherwise distinguish wavelengths and responds linearly with the light intensity.

The current from the phototube is readily amplified, making possible its application to very low light intensities. By variation of the sensitive metal layer, the wavelength range of sensitivity of the tube is altered. Thus, with two or three interchangeable tubes it is possible to measure the radiation intensity over the entire spectral range. The phototube is itself less responsive to light than the photronic cell, but, when amplified, it can be

made to yield a much greater over-all response. It is considerably less simple to operate than the photronic cell.

A comparison of the characteristics of two commonly used instruments is given in Table 8.

TABLE 8. CHARACTERISTICS OF BECKMAN AND COLEMAN MODEL 11 SPECTROPHOTOMETERS

Characteristic	*Beckman*	*Coleman* 11
Light source	Hydrogen discharge tube (210–400 mμ), tungsten bulb (330–1000 mμ)	Tungsten bulb
Dispersion	Quartz prism	Diffraction grating
Effective slit width	~1 mμ	35 mμ
Photoelement	Phototubes with amplifier	Barrier layer photocell
Range of use	210–2000 mμ	350–800 mμ
Radius of smallest absorption cell (calculated)	0.05 mm.	~ 2 mm.

ERRORS IN COLORIMETRY

The errors in colorimetric measurements arise from a number of sources, some of which are traceable to the instrument, and some to the characteristics of the system measured. So many factors of theory, construction, and design enter into instrumental errors that no detailed discussion of them is possible here. Certain well-established facts and considerations of types of instrument will be included.

Instrumental Errors

1. Any instrument that does not utilize monochromatic light fails to measure color intensity accurately in many instances, for the reasons discussed under "Application of the Absorption Law." The spectrophotometer is the only common instrument that utilizes approximately monochromatic light and is therefore the instrument of choice for all colorimetric analysis.

2. Any instrument in which color matches are made by eye is subject to the limitations of the eye, notably its inability to distinguish monochromatic light from similarly colored polychromatic light, its sensitivity, and its individual variation and abberations. The character of the illumination has a large effect on these phenomena and is a critical factor with all visual instruments.

3. Photoelectric instruments are limited by the design of the instrument and by the characteristics and sensitivity of photoelectric cells, vacuum tubes, filters, absorption cells, and other accessories. Since none of these devices can be designed or made perfectly, every instrument is expected to be susceptible to certain variations and inaccuracies. The extent of such variations is relative; instruments that are superior in design and construction will show less variation. As an example of the effect of design on the instrumental characteristics, consider the use of yellow solutions. The light absorbed is in the violet and ultraviolet spectral region when the eye observes yellow. The photronic cell is relatively insensitive in this spectral range, whereas a proper phototube is capable of measuring the absorption in this range approximately as well as in any other. Since so many solutions of interest to the analyst are yellow, this difference is highly significant.

As another illustration, the measurement of color with very small volumes requires small horizontal absorption cells, so that the light beam actually traverses nearly all the solution, thus magnifying the absorption. Any instrument that employs a large light beam, e.g., the General Electric recording spectrophotometer, is not appropriate for this purpose because the necessary cross section of the beam is greater than the possible cross section of the absorption cell. It is evident that the instrument must be carefully chosen with respect to specific requirements.

Deviations from Beer's Law

Most of the non-instrumental errors that arise in colorimetry may be classified as deviations from Beer's law. Fully half the colorimetric methods in common use show deviations of this type, and for some methods the deviations are large. It is therefore essential that the operator have information as to the common causes of such deviations and that every method be checked to determine the presence and magnitude of errors caused by these effects.

Such deviations are caused by the existence of conditions under which the concentration of specific solute and colored compound are not identical.

1. **Dissociation.** If the state of dissociation of a colored compound changes with the concentration, Beer's law will usually not

be obeyed. Thus, if the colored compound is in reversible equilibrium with two or more other components, the color will be altered by a change in the concentration of any of the components involved in the equilibrium.

An example of this effect is the color of picric acid. In its undissociated state, picric acid is colorless, as is also hydrogen ion which is involved in the equilibrium. The picrate ion is colored a deep yellow. Increasing the concentration of hydrogen ion diminishes the color by repressing dissociation, whereas addition of alkaline materials increases it by increasing the dissociation to form the colored anion in accordance with the equation for dissociation.

$$\text{H picrate} \rightleftharpoons \text{Picrate}^- + \text{H}^+$$

It is obvious that the total picric acid concentration will not be determined by a measurement of color unless the hydrogen ion concentration is accurately controlled. Another common example of the influence of dissociation on the color is the case of chromate and dichromate ions. Below approximately *p*H 8, the equilibria involved are

$$\text{CrO}_4^- + \text{H}^+ \rightleftharpoons \text{HCrO}_4^-$$

$$2\text{HCrO}_4^- \rightleftharpoons \text{Cr}_2\text{O}_7^- + \text{H}_2\text{O}$$

Since the color of chromate ion is yellow and that of dichromate orange, an increase in the acidity of the solution shifts the color toward orange. Many other illustrations of this type of effect might be quoted.

2. **Complex Formation.** In many systems complexes are formed. These may be colored or colorless, and equilibria between them may result in different colors as in cobalt chloride (pink), which forms a blue complex in concentrated solution. Ferric iron forms many complexes which are colorless (phosphate, fluoride, etc.). The presence of these complex formers will interfere with complex color development by the ferric ion. On the other hand, the thiocyanate complex of ferric ion is colored. Formation of unsuspected complexes often causes serious deviations from Beer's law.

3. **Suspensions.** When a suspension (colloidal dimensions or larger) exists in the solution being measured, scattering of light

occurs and obedience to Beer's law is not observed, or it is observed only over a limited range. The finer the particle size, the smaller the deviations. Thus, any factor affecting the particle size must be carefully controlled. Factors that may alter the particle size are (*a*) concentration of the reactants, (*b*) temperature, (*c*) time of standing, and (*d*) presence of electrolytes.

Suspensions are found under two different sets of conditions.

(*a*) *Foreign material usually not colored, is present.* This should always be carefully avoided, since its effect is always detrimental.

(*b*) *The colored material measured is a suspension.* A considerable number of the colored materials used for analytical purposes are actually suspensions. This is true of molybdenum blue (for phosphate, silicate, etc., determination), the Nessler compound (for ammonia), Prussian blue (for iron), lead sulfide (for lead), and numerous others.

It is evident that extreme precautions must be taken in these cases to reproduce accurately the conditions of formation of such colored suspensions, and to obtain as fine dispersion as is possible. Protective colloids are often employed for this purpose, since they favor the formation of fine dispersions. Extraction of the colored material is sometimes effective. In any case, the system must be carefully studied, calibrated, and standardized if measurements of this type are to yield significant results.

4. **Chemical Interference.** If there is present more than one material capable of forming color under the analytical conditions, serious deviations from Beer's law may result. This is particularly true if the two color-forming materials absorb in the same region of the spectrum. Thus, arsenate and silicate interfere in colorimetric phosphate determination because both of them form easily reduced compounds with molybdic acid.

Other Errors

In addition to errors of the instrument and errors directly associated with producing deviations from Beer's law, there are certain other sources that the analyst must consider and avoid if possible. Many of these errors are essentially the same as those inherent in volumetric analysis. They require little discussion here.

1. **Errors of Dilution.** In most instances, a sample is considerably diluted by the time the color is formed. Sometimes the color intensity may be too great, and still further dilution is then required. Dilutions always involve some error, and the greater the amount or number of dilutions the greater the possible significance of these errors. Actually, if the apparatus is properly calibrated and operated, the percentage error will not be increased by the dilution, though the absolute error will. Care must always be taken that dilutions are performed accurately.

2. **Temperature Errors.** In addition to the effect exerted by temperature alteration on volume measurements during dilution, temperature also has an influence on the light absorption. Within the range of usual working conditions, this effect is not large enough to be critical in ordinary analyses. If measurements are attempted outside the ordinary room-temperature range, corrections must be applied. This is likely to happen if a solution has been heated to develop a color. The solution must then be completely cooled before measurement.

3. **Range Error.** One of the important errors in spectrophotometry arises from reading transmission in the extreme ranges of the instrument scale. Owing to the logarithmic law of absorption, it is inherent in the system that the highest accuracy is attainable only in the medium range of transmission measurements. Actually the limiting factor is the uncertainty dG, in making the galvanometer reading G. Assuming a steady light source and a linear relation between the intensity and the galvanometer deflection,

$$G = k \cdot I_t \quad \text{and} \quad dG = k \cdot dI_t$$

If l, the length, is constant and Beer's law applies,

$$I_t = I_0 e^{-klc}$$

If the extinction E is defined as $E = \ln (I_0/I_t)$, then

$$E = \ln I_0 - \ln I_t = \ln I_0 - \ln I_0 e^{-E}$$

The accuracy of the determination is dc/c, which may be expressed

$$\frac{dc}{c} = \frac{dE}{E} = \frac{dI}{E \cdot e^{-E}}$$

The error is minimum when $E \cdot e^{-E}$ is maximum. This occurs

when $E = 1$ or $\log (I_t/I_0) = 0.4343$. This corresponds to 36.8% transmission, at which point the range error is at the minimum. If the absolute error in determining the transmittancy is 1%, the error in the concentration c caused by this transmittancy error is about 2.7% at 36.8% transmission. The error increases slowly to 20% transmission and up to 80%, rising much more rapidly outside these limits as shown in Fig. 36.

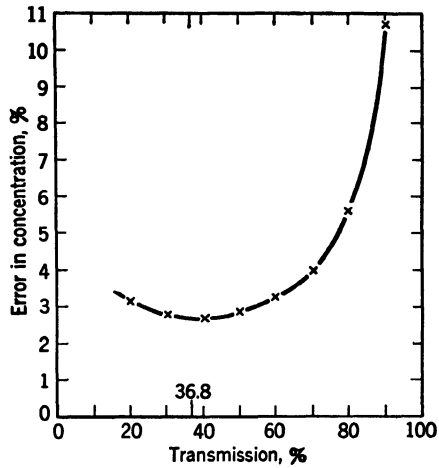


FIG. 36. Range error corresponding to 1% error in transmittancy. From data of Sandell, *Colorimetric Determination of Traces of Metals.*

SUBMICROGRAM ANALYSIS EQUIPMENT

It is evident from the foregoing discussion that the Beckman spectrophotometer may be readily modified for small-scale analysis, and this has been accomplished practically (16, 17). Unmodified, its range of utility extends downward to the order of 1 or a few micrograms of most constituents. Because of its narrow light beam and its very sensitive photometer, it can be operated with attachments that allow a very great reduction in scale of analysis.

As the spectrophotometer is usually operated, color is first developed in a volume considerably in excess of that of the absorption cells. This excess is discarded. Of the remaining colored component which is placed in the absorption cells, light actually traverses only a fraction of the volume, thereby giving only a fraction of the possible absorption. By means of horizontal cells, this inefficiency is partially corrected. However, the smallest horizontal commercial cells commonly used are 5 cm. in length and hold about 7 ml., which is considerably more than the content of the 1-cm.-square cell furnished with the Beckman instrument.

The solution to the problem of obtaining the highest possible

sensitivity and therefore the lowest possible range limit with a given instrument lies in developing the color in a small volume just sufficient to fill a small horizontal cell with an internal diameter not larger than the light beam of the instrument. Thus, all the colored component is utilized in absorbing light, which allows the total amount of colored material to be reduced proportionately.

Absorption Cells

A design for an absorption cell utilizing this principle was described by Kirk, Rosenfels, and Hanahan (16) and is shown in Fig. 37. The cell is made from the plastic, polytetrafluoroethylene (18, 19), known commercially as Teflon, which is characterized by being almost totally insoluble in all known solvents, both inorganic and organic, and by being almost completely unreactive toward all common chemicals. It can be machined and is not subject to distortion at any but high temperatures. Other plastics such as polyethylene and polystyrene are suitable for certain limited applications. Glass cells of similar design have also been manufactured and employed, but they have not yet been developed to a completely satisfactory state.

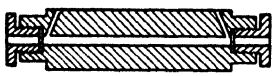


FIG. 37. Capillary absorption cell constructed of Teflon. (½ actual size)

The cell shown in Fig. 37 is made from plastic rod ⅝ in. in diameter, which is about the same as the diameter of certain standard horizontal cells which may then be used with the same cell holder. The internal diameters of 2 and 4 mm. have been used successfully (16), and other sizes, both larger and smaller, are unquestionably practical. The optical path through the cells is 5 cm., though both longer and shorter cells are not excluded. A cell with an internal diameter of 4 mm. and a length of 5 cm. contains less than 1 ml. With such a cell, the colored solution is developed in a 1-ml. volumetric flask.* If the cell is made with a 2-mm. internal diameter, the content will be about 160 λ, and the color should be developed in a 200-λ volumetric flask.* The ends of the cell are constructed with clear windows which may be of glass, quartz, or clear plastic. Glass is best for most purposes because of its non-reactivity with most solutions other than those

* Cf. Chapter 2.

containing fluorides. Plastic windows are more readily made and sealed, but the common clear plastics are not suitable with organic solvents or certain highly reactive chemicals such as strong acids or bases and strong oxidizing agents. In the design shown, the window is held in place by a hollow plug which screws into the body of the tube. Teflon is sufficiently soft so that, if the shoulder is smooth, it operates as a gasket to prevent leakage. Ports for filling and emptying are drilled immediately behind the windows. Filling is performed by pressing of the pipet tip to one port while the cell is held in a slightly tilted position so that air may escape from the upper port. Care must be taken that air bubbles are not entrapped in the cell, a matter which is aggravated by the difficulty of wetting the plastic Teflon with aqueous solution. Visual examination of the interior of the cell should always be made after filling to detect any entrapped air bubbles.

By proper use of these absorption cells, most materials for which good spectrophotometric methods exist may be determined in amounts ranging from about 1 to 100 mγ, i.e., 0.001 to 0.1 γ, which represents a very considerable lowering of the range of colorimetric, or for that matter of all, quantitative methods.

Lowry and Bessey (17) have described another form of absorption cell for analysis of quantities comparable to those indicated for capillary cells. The design of these cells is shown in Fig. 38. The cells are so constructed that the parallel windows through which the light passes are mounted about 1 cm. apart, as in the standard Beckman absorption cell. The sidewalls, however, are thickened so that the space occupied by liquid is 2 mm. wide. Thus, the cross section viewed from the top of the cell is 2 by 10 mm. The cell is 25 mm. high, so that it has a total capacity of nearly 500 λ. By proper masking, it can be used with volumes of about 50 λ. A smaller adaptation only 1 mm. in width which can be used with as little as 30 λ was also described. It is not greatly recommended by its designers because of the increased difficulty of filling and emptying.

This type of absorption cell has essentially the same accuracy as the standard Beckman cell, the light absorption being determined primarily by the length. Because of its 1-cm. length, it has about one-fifth the sensitivity of the corresponding capillary cells described earlier. To obtain the same light absorption as with the latter cells would then require five times the concentra-

tion of colored material. The differences between the two types of cell are in sensitivity which favors the capillary cell, and in ease of filling and adjustment which favors the Lowry and Bessey cell. An additional advantage of the latter cell is that it can be used with the standard Beckman cell holder with only minor modifications. It is particularly advantageous when the color intensity is considerable. It differs from the elongated capillary cells also in that it must be masked to give a light beam width slightly less than the distance between the glass walls.

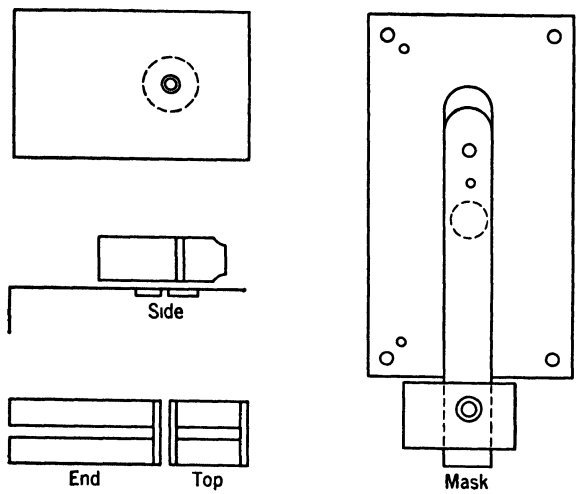


FIG. 38. Microabsorption cell and mask, according to Lowry and Bessey.

Otherwise, extraneous light could travel through the glass and would register on the photoelectric cell. The masking problem with capillary cells has been met by opaque plastic which serves as its own mask. Such cells are more difficult to fill than any type of glass cell because of the poor wetting characteristics of the walls. The analyst will find one or the other type of cell better adapted to his needs, depending on circumstances. Further improvements in capillary cells to avoid some of the present limitations are definitely in order and will probably be developed in the near future.

Absorption Cell Holders

Spectrophotometers are furnished commercially with arrangements for holding one or more forms of absorption cells, which

may be in the form of round test tubes, square vertical tubes, or one of a variety of horizontal tubes with flat end windows. Most instruments have to be modified to accommodate horizontal

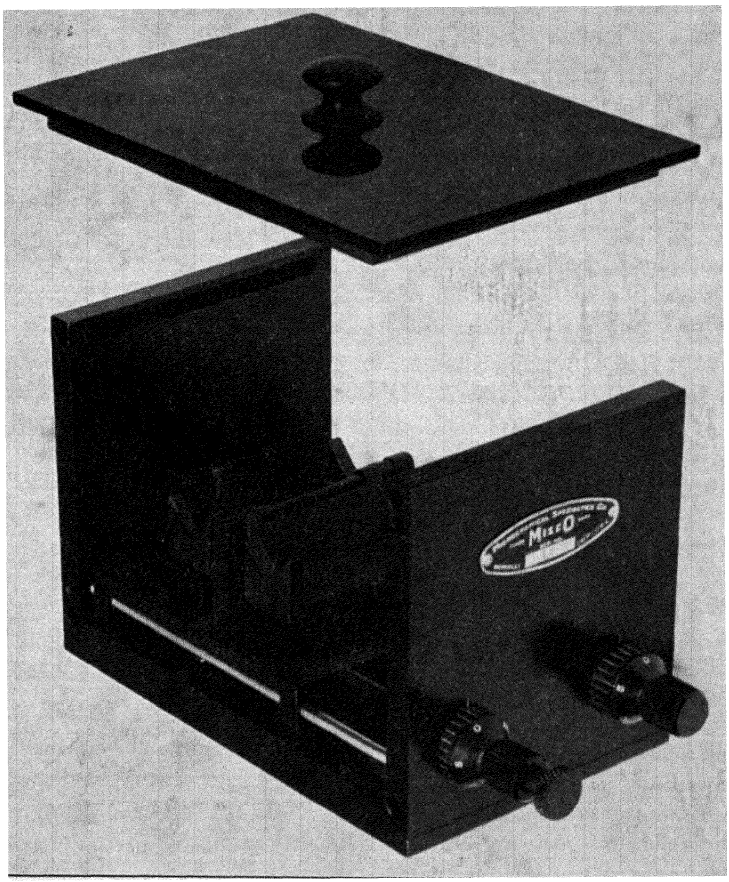


FIG. 39. Capillary absorption cell holder for the Beckman spectrophotometer. (*Courtesy Microchemical Specialties Co.*)

cells with light paths greater than 1 cm. The Beckman instrument is no exception. An attachment can be purchased from the manufacturers for holding absorption cells of 5- and 10-cm. length. This holder is not suitable for capillary cells, however, because it lacks the necessary adjustments for aligning the capillary bore

in the light path. A suitable holder is shown in Fig. 39. It may be used for 5- or 10-cm. cells of the conventional type or for capillary cells. Both vertical and horizontal adjustments are incorporated in order to center and locate the cell in the light path. The plastic capillary cells described above mask out extraneous light because of their opacity, although some light may pass above or below the cell and impinge on the photosensitive element. This light is removed by a simple mask having a hole considerably larger than the cell bore. Because the slit contains an approximate focus of the lamp filament, the light will not show uniform intensity. This makes it necessary to adjust carefully the vertical as well as the horizontal position of capillary cells.

THE SPECTROPHOTOMETER

Since the Beckman spectrophotometer (20) is the instrument which is most readily adapted to precise measurement of submicrogram quantities, its description in some detail is included here. It is designed to operate in the visible, near infrared, and

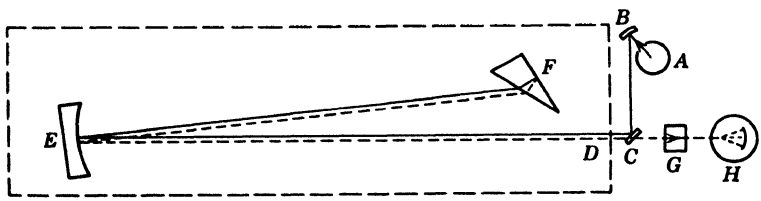


FIG. 40. Optics of the Beckman spectrophotometer.

ultraviolet portions of the spectrum. The monochromator is capable of isolating a band of essentially monochromatic radiation of any wavelength from 220 to 1000 mμ. The amount of radiation passed is such that a relatively simple photometer can be used, and the entire apparatus is compact and rugged. Interchangeability of radiation sources, phototubes, and absorption cell holders gives the instrument a versatility not found in many other commercial instruments.

The Monochromator. The optics of the Beckman spectrophotometer are shown in Fig. 40. One of two interchangeable radiation sources is used at *A*, depending on the spectral range desired. A standard 32-cp. prefocused tungsten lamp (Mazda

2331) is used for the spectral region from 320 to 2000 mμ. A special hydrogen discharge tube is used for the spectral region from 220 to 320 mμ. A 6-v. heavy-duty storage battery serves as the power source for the former, and a special power unit operates the hydrogen discharge tube. The two sources are mounted in interchangeable housings. A mercury discharge tube is also available to check the wavelength setting of the instrument, since it emits a line spectrum of a limited number of known wavelengths.

Radiation from the source *A* is magnified by the collimating mirror *B* and the diagonal mirror *C* and is focused on the plane of the slit at *D*. After passing through the slit opening, the radiation is reflected by the collimating mirror *E* onto the prism *F*. The back surface of the prism is an aluminized mirror, and the radiation is reflected by this mirror, back through the prism, and off the mirror *E* to the slit *D*.

The quartz prism was selected as the dispersion device because it gives a spectrum relatively free of scattered light. The half-prism and reflecting mirror not only require less quartz than a full prism but also obviate several complications which would arise if the full prism were employed. Likewise the use of collimating mirrors for focusing the radiation avoids the necessity of quartz lenses.

Variation in wavelength is secured by rotating the prism with a simple mechanical device. The wavelength setting can be varied slightly by moving the position of the collimating mirror *E*, but in actual practice this fact is not used.

The internal optics are so arranged that the emergent beam is approximately half an inch above the entering beam at the slit *D*. The emergent beam then passes through the absorption cell in the cell holder *G* and into the photocell *H*.

The Photometer. The output of the photocell is amplified by an electronic amplifier whose circuit is shown in Fig. 41. The essential feature of the circuit is that the phototube current is measured by balancing the voltage drop in the 2000-megohm resistor (R10) with the slide wire potentiometer (R1). The indicating device is a sensitive milliammeter. The remaining controls are included to correct for stray currents in the amplifying circuit and to adjust the circuit so that the percentage transmission is read.

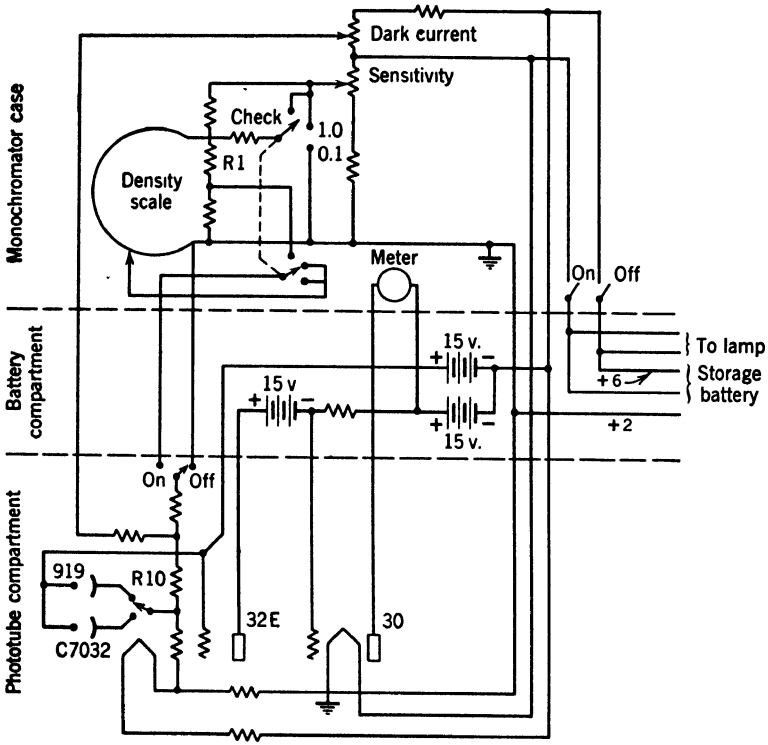


FIG. 41. Wiring diagram of the Beckman spectrophotometer.

Control Panel. The various knobs and dials on the instrument are shown diagrammatically in Fig. 42. The wavelength knob controls the wavelength of the radiation passing through the cell holder to the photocell. The wavelength scale indicates the radiation wavelength being isolated. The percentage transmission knob and scale are used respectively to balance the meter when a measurement is being made and to indicate the percentage transmission reading. Two scales are available, the upper being percentage transmission; the lower, optical density. For analytical work the former scale is usually employed. The optical density values are most often used in certain theoretical phases of spectrophotometry. The third of the large control knobs adjusts the width of the slits.

The amplifier system of the spectrophotometer and the meas-

urement of the absorption or transmission of an object require that the output of the photocell, when amplified, give a constant current. Since the outputs of both the source and the phototube vary with the wavelength of radiant energy, some device must be inserted into the circuit to control either the output of the source, or the photocell, or both. In the Beckman instrument, both outputs are varied. The slits at *D* (Fig. 40), operated by the third large knob on the panel, control mechanically the output of the source *A*. A rheostat marked "Sensitivity" varies the output of the phototube electrically. In practice, the two adjust-

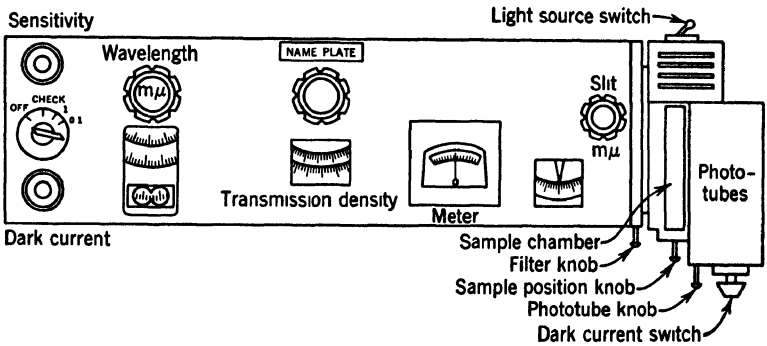


FIG. 42. Control panel of the Beckman spectrophotometer.

ments are used together, the slit width being a rough adjustment, the rheostat a fine adjustment.

In addition to increasing the amount of radiation reaching the phototube, increasing the slit width widens the dispersion range of the radiation. Since this dispersion should be kept as small as possible for accurate work, the slit width should be kept as low as possible. The dial setting is usually between 0.01 and 0.06 mm.

The ammeter between the transmission and slit knobs serves only to indicate a zero or null point, and the several divisions on either side are for convenience only. Throughout the various adjustments of the instrument, the object is to keep the needle at the zero point (i.e., constant current).

On the extreme left end of the instrument are two resistances and the main switch. The dark current resistance balances out any stray current (dark current) in the amplifier. The sensitivity control is essentially a fine adjustment whose purpose is the same as

the slit adjustment, i.e., to control the output of the photocell by reducing the current electrically. The instrument should be operated with this control approximately one turn from its most counterclockwise position, which is its most sensitive position. Operation at the other end of the sensitivity scale, i.e., the clockwise end, requires a wider slit opening and makes the response of the ammeter extremely sensitive to changes in the position of the transmission knob.

The main switch has three "on" positions marked "Check," "1," and "0.1." The "1" position is the true "on" position. The "Check" position is used when it is desired to adjust the meter against the blank. It avoids the necessity for returning the transmission scale to 100 before each measurement. For readings below 10% transmission, the switch is turned to the "0.1" position, and the meter is balanced in the usual manner. The observed scale reading is then divided by ten to give the true transmission. this permits more accurate measurements in the region below 10%.

On the right-hand end of the instrument are three push-pull knobs and a switch. The first of the three knobs controls the filter which is necessary when measurements are made below 400 mμ with the tungsten light source. When the knob is in as far as possible, there is no filter in the light path. With the knob pulled out to its next position, a purple filter is introduced into the light path. This cuts off any stray radiation which may enter the monochromators when the above-mentioned combination of source and wavelength is used. The third and last position is blank.

The second knob controls the position of the absorption cells in the cell holder. This obviates the necessity of opening the cell container while a series of measurements is being made.

The third knob selects the phototube. No one phototube can cover the entire spectral range of the Beckman instrument. Two tubes are employed which are interchanged merely by pushing the phototube knob. When the knob is farthest out, the ultraviolet sensitive tube is in position, and all measurements below 620 mμ should be made with this phototube. For measurements between 620 and 2000 mμ, the red sensitive tube is put in position by pushing the phototube knob in as far as possible.

The off position of the off-on switch shuts off the photocell

TABLE 9. COMBINATIONS OF FILTER, PHOTOTUBE, AND SOURCE IN OPERATING THE BECKMAN SPECTROPHOTOMETER

Spectral Regions, mμ	*Filter Control*	*Phototube Control*	*Source*
220–320	In	Out	Hydrogen
320–400	Out	Out	Tungsten
400–620	In	Out	Tungsten
620–2000	In	In	Tungsten

both optically and electrically from the remainder of the instrument and circuit. It is put in the off position only when the dark current is being checked.

In order to utilize the spectrophotometer for dependable analysis, it is necessary not only that the sources of error and the principles of colorimetry be understood but also that certain generalities and rules of good procedure be followed. Some of the more important considerations are summarized as follows:

1. *Choice of method.* If several procedures or reagents are available for a particular analysis, careful choice between them should be made on the basis of (*a*) specificity of reaction, (*b*) sensitivity, (*c*) character of colored compound (dissociation, suspension, etc.), and (*d*) interferences present.

2. *Accuracy of standard.* No method can be more accurate than the standard used for calibration or standardization of the procedure. It is of great importance that the standard be made as accurately and reliably as is possible.

3. *Matching of cells.* Absorption cells for the standard and unknown, respectively, must be accurately matched for optical path and characteristics. If this is not done, large systematic errors will be observed. It is desirable that every pair of cells be thoroughly checked for matching before use in analysis.

4. *Region of absorption.* Measurements must always be made in a spectral region in which absorption is at the maximum and if possible where the absorption band is broad, as discussed previously. If the best absorption region for the particular colored material is not known, absorption must be measured throughout the spectrum in order to determine the proper wavelength. Ordinarily transmission rather than absorption will be measured. In this case the transmission should be at the minimum. Since the wavelength calibrations of different spectrophotometers do not always agree, instrumental variations make it desirable to

recheck wavelength for the minimum transmission, even when the method specifies the proper wavelength.

5. *Conformity to Beer's law.* In working with a method for the first time or after changes have been made in procedure, reagents, or instruments, it is necessary to establish whether the system conforms to Beer's law. In order to do this simply, a set of standards is prepared, covering the concentration range involved. The transmission of each of these is measured against a blank, and the values of log T are plotted against c. This may be simply done with semilogarithm graph paper. If the system conforms to Beer's law within the range studied, a straight line is obtained. If the line is curved through any portion of its length, Beer's law does not apply through that part. If the curve is highly reproducible, it may still be employed as a calibration. The latter procedure is not desirable and should be avoided if possible.

6. *Range of measurement.* As discussed under range error, the range of transmission in which measurements are made has a very large influence on the accuracy. With any given volume of solution, the color intensity should yield transmissions between 20 and 70%. If, after the color is developed, it is found that the intensity is so low as to yield, for example, 90 to 95% transmission, the error of such a measurement will be expected to be large. Without repetition of the analysis, only one remedy may be applied in this case, viz., the solution may be transferred to an absorption cell having a longer light path. For example, if the solution is changed from a cell with a light path of 1 cm. to one of 5 cm., log I_t/I_0 is reduced fivefold, or the absorption is increased by a power of 5.

Conversely, if the transmission falls below 10%, it would be desirable to transfer to another cell having a shorter light path. If the volumes available do not permit such transfers to be made, it is desirable to make another analysis in which proper cells are chosen for the final measurements.

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CHAPTER 4

General Apparatus and Technique

In addition to volumetric and colorimetric operations, there are many other general types of operation which must be performed by the analyst working with minute quantities. Weighing and gravimetric procedures have been avoided by microgram analysts because of the unavailability of suitable equipment, combined with serious manipulative difficulties. It is inevitable that weighing will assume a greater importance in small-scale analyses with the more general availability of microgram balances. The problems associated with heating, centrifuging, filtering, extracting, and distilling minute quantities are ever present in the laboratory. To some of these a variety of solutions have been described in such works as those of Emich and Schneider (1), Chamot and Mason (2), Benedetti-Pichler (3), and Schneider (4). Their applicability to quantitative operations is often doubtful because they were developed for qualitative, not quantitative, uses. It is certainly necessary that any such technique be capable of quantitative application and that it be so adapted as to integrate with the remaining operations of the analysis to form a smooth procedure.

This chapter describes various general operations and the equipment required. All techniques discussed have been used in connection with quantitative analyses or have been specifically designed with this in mind.

WEIGHING

For the performance of microgram analyses, there are at present few if any published procedures in which weighing of microgram quantities is involved. This means that no organic combustion methods for this range have been developed, and the common gravimetric methods, such as sulfate determination, are still lacking. It may well be questioned whether this situa-

tion will long remain true, for it is evident that microgram combustion methods, for example, might have a very important place in the analytical scheme, particularly for rare and expensive biological compounds.

The load capacity and sensitivity of the microchemical balance, which commonly carries up to 20 g. and has a limit of detection of $\pm 1\,\gamma$, are obviously not suitable for gravimetric microgram analysis or for sample weighings in the microgram range. The operation of the microchemical balance is adequately discussed elsewhere (1, 5, 6) and will not be repeated here.

Microgram Balances

The weighing of microgram quantities has been studied extensively since about 1882, and many forms of balance have been described. The quartz helix balance (7–10) has been widely employed in gas sorption work and for other specialized applications because of its simplicity and the ease with which it may be enclosed inside glass apparatus subjected to vacuum, unusual temperatures, or special gases. Although it is almost ideal for this type of investigation, it is by its nature restricted with respect to its sensitivity as related to its load capacity. A quartz spring which is light enough to be sensitive to small weight differences will not support a large load, and vice versa. By lengthening the spring and increasing the diameter of the fiber, the theoretical limit to the load capacity-sensitivity ratio may be increased almost indefinitely. However, physical space requirements place a practical limit on this type of extension of the use of the quartz helix. The same objection applies to a much greater extent to the Salvioni (11) balance which operates on the same basic principle, namely, the bending of a glass or quartz fiber under load. Here, again, a large fiber which is made long can carry a greater load with the same sensitivity, but the extension in this direction is limited by spatial considerations. The quartz helix is very readily constructed if certain specialized facilities are available, and wide variations in size and capacity are easily arranged by choosing proper diameters of quartz fiber and constructing springs of proper lengths (12).

Weighing on the quartz helix balance is exceptionally rapid, and, because of this and the ease of construction, such balances are extremely useful for rapid weighings of, for example, biological ma-

terials which may lose moisture during weighing and must be handled rapidly (13). For quantitative microgram analysis, the helix balance has only a limited utility because it is usually necessary to weigh a small analytical sample with high sensitivity while the sample is contained in a vessel whose total weight is far from insignificant. Thus a high load capacity and a high sensitivity are required simultaneously, a condition that is met only with difficulty by the quartz helix. Because of this fact, some form of beam balance is usually to be preferred for general application. The analyst should exercise judgment on this point, however, because for many routine purposes a set of helix balances may be mounted at convenient points and used advantageously for specific weighings of a routine nature.

A beam balance allows the large part of the weight to be balanced by counterpoise, and, since the effect of gravity at a particular point is quite constant for a reasonable time, such balancing may be done with very great precision. However, it is not easy to determine large masses with high precision by this means. In general, only the weight of the sample contained in the relatively heavy vessel is of importance in the analysis, so the vessel may be counterpoised and disregarded. It is then possible to employ a delicate method of balancing a small sample or of detecting changes in the weight of the sample. This may be accomplished by application of any one of several general principles based on (*a*) magnetic or electrical forces (14), (*b*) fluid displacement in which the mass is balanced by displacement of a liquid or gas (15), and (*c*) mechanical distortions of construction members, which may include bending (11), stretching, compression, or torsion (16–18). The history of the development of balances weighing in the microgram range is well given by Gorbach (19) and by Emich (20), and the theory of such weighings, particularly as it is affected by the use of fused quartz construction, appears in these sources as well as others (21, 22). In general, balances described in these references suffered from a low ratio of total load to sensitivity, or from operational difficulties caused by such disturbing factors as vibration (19).

Neher (23) described a simple torsion balance made from fused quartz, for which he claimed a sensitivity of from 0.1 to 0.001 γ per division without mirrors or microscopes. It is based on a simple single-member beam fused to a torsion fiber. The

mass applied is brought into exact balance by applying a torque to restore the beam to zero. The position of the dial attached to the rotating end of the torsion fiber is then read. This sensitive but somewhat crude device was modified by Kirk, Craig, Gullberg, and Boyer (24) into a balance which carries loads of several tenths of a gram and has a sensitivity of at least 0.005 γ per

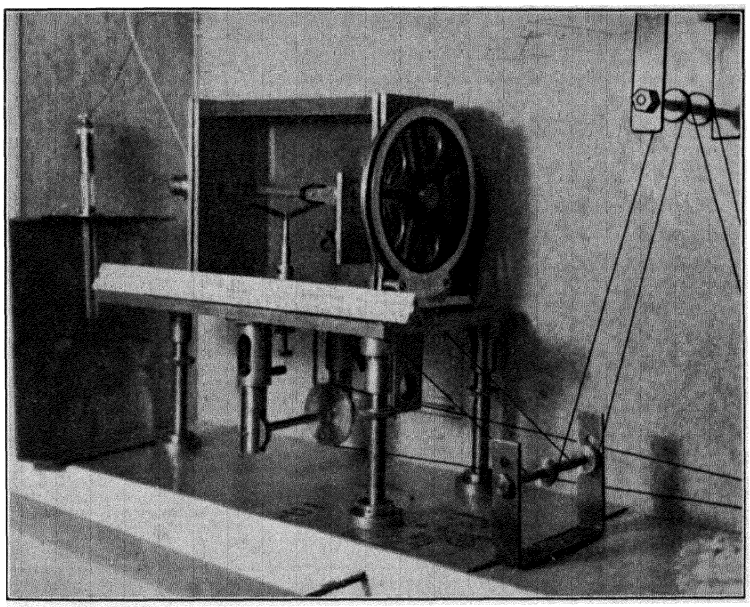


FIG. 43. Quartz microgram balance.

vernier scale division. To the basic design of Neher is added a light but comparatively rigid simple cantilever-type beam, a precise method of determining torque, a comparison microscope to determine the horizontal beam position, and improvements in pan suspensions first described by Steele and Grant (15) and Pettersson (21). The over-all design of the balance is shown in Fig. 43, in which may be seen the internal construction including the quartz parts and most of the microscope. On the front is shown an accurate wheel similar to the vertical wheel of a surveyor's transit, on which the torque necessary to restore the beam to a horizontal position is determined. Below the base on each

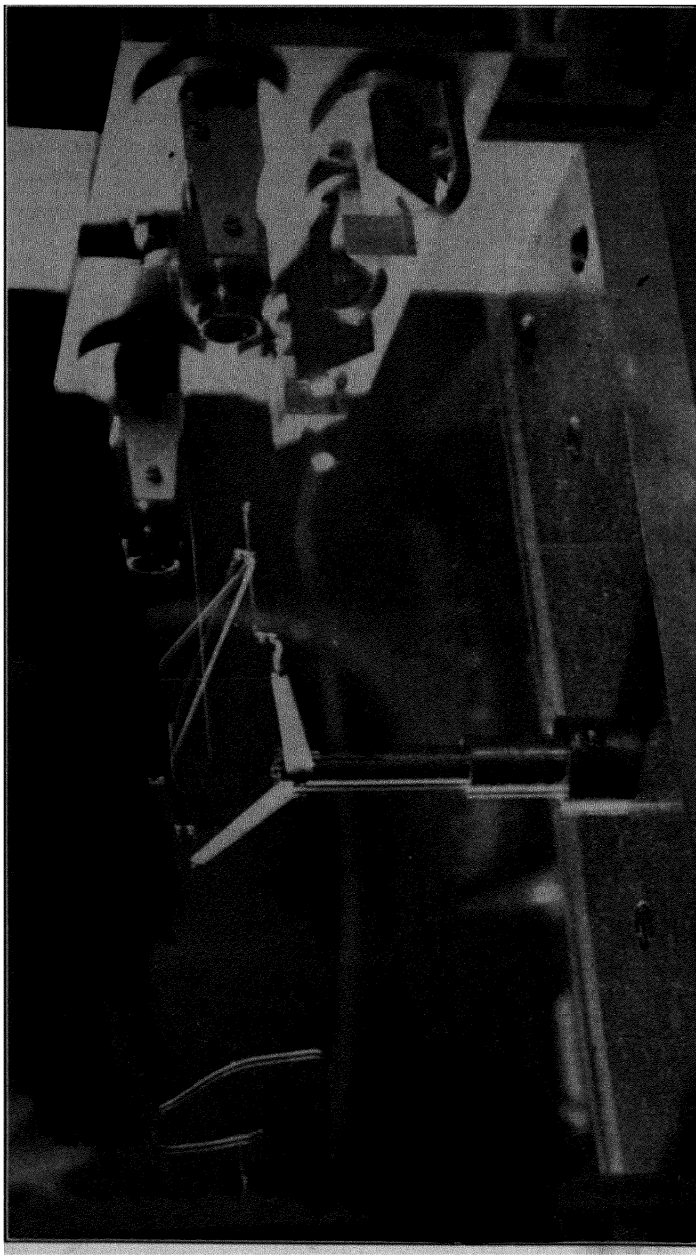


FIG. 44. Quartz microgram balance, interior view.

side is shown a hollow cylinder or pan well in which the pan suspensions terminate and the pans hang. They are reached through a semicircular sliding door. It is thus not necessary to open the balance case to load or unload the pans. Immediately below the front of the base is visible a lens which serves to project the images from the comparison microscope. Omitted from the picture is an insulating cover which carries the necessary small bulbs for illumination and has a window through which the position of the torsion wheel is read. Also omitted is a small ground-glass screen for viewing the projected image of the beam indicator, and the pulleys which operate the wheel and pan release remotely by means of endless cords.

Figure 44 shows the appearance of the inside of the balance case. The fused-quartz cantilever beam, supported on a torsion fiber attached to a bow to maintain constant tension on the torsion fiber, and the elongated pan suspension fibers are visible. Also to be seen are the lenses and prisms of the comparison microscope with which the beam position is determined. The central Y-pillar is the beam arrest, which in later models was designed only for transportation of the balance and not for operation.

Quartz Assembly. The design of the quartz mechanism is shown in Fig. 45. The main horizontal beam member is 10 cm. in length and is constructed from quartz rod about 200 μ in diameter, as is also the vertical member and a short horizontal cross member to which the torsion fibers are attached. The beam is braced by lighter fibers of about 50 to 75 μ. It carries also another horizontal member made from about 15-μ fiber which serves as an index of position in that the comparison microscope is focused on its right and left ends. The beam height is 1 cm., the downward extension of the vertical member being variable since it is used for vertical and horizontal adjustment of center of gravity. To the ends of the beam are attached the pan suspension fibers which are about 5 μ in diameter. As has been shown by Steele and Grant and by Pettersson, a fiber of 6 μ or less acts as a perfect knife edge within limits of about 1 degree of rotation, because of the very low degree of stiffness of such fibers which bend sharply at their attachment without causing lateral displacement of load. Because of the high tensile strength of quartz fibers, one of 5-μ diameter will support several grams of weight. Such loads are not practical in application because of the

danger that the fiber will snap under acceleration when the pans are released. With loads of a few tenths of a gram, no such difficulty has been encountered.

The beam is mounted midway between the ends of the torsion fiber, the over-all length of which is about 10 cm. The sensitivity of the balance is determined largely by the length and especially the diameter of the torsion fiber. The diameter is particularly important because the sensitivity varies inversely with its approximate fourth power. The load-carrying capacity, which is a function of the tensile strength of the torsion fiber, varies approximately as a square power of the diameter. Thus, a small reduction in size increases the sensitivity much more rapidly than it diminishes the total load. Conversely, when the diameter is increased, the sensitivity falls off much more rapidly than the load capacity increases. One end of the torsion fiber is attached to the wheel by which torque is applied; the other end is sealed to a quartz bow of considerable stiffness which serves to maintain a constant tension on the fiber. The bow in turn is attached to a mechanical adjustment which allows alteration of the tension, as well as rotation.

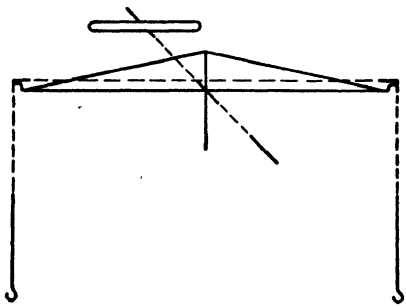


FIG. 45. Quartz assembly, microgram balance. (½ actual size.)



FIG. 46. Pan stirrup, microgram balance. (Actual size.)

The pan suspensions are fibers of about 50 μ, sealed to the 5-μ attachments and terminating at the lower end in a hook on which are carried the pan cradles shown in Fig. 46. These may take a variety of forms, the choice between them being one of convenience of construction only.

All quartz parts are best constructed on a set of jigs or holders which position each member accurately before it is sealed. The jigs may be conveniently constructed of quartz also, as described by Kirk and Craig (22). By this means reproducible beams may be fabricated, and in case of breakage an entire quartz

assembly may be readily replaced. Hand construction as described by Neher (23) is not suitable for this purpose because it is virtually impossible to construct two beams that may be substituted for each other. However, Neher's general techniques are followed in preparing fibers, in making quartz jigs, and for limited operations in beam construction.

Mechanical and Optical Assembly. The quartz mechanism is supported on a metal bow attached to a base and enclosed by a metal case. The dimensions and details should be obtained from the original description (24). Most critical in the mechanical construction is the mounting of the wheel and its accuracy. A vertical wheel from a surveyor's transit is suitable, though the calibration in degrees and the division into quadrants are not desirable. An identical wheel graduated in 2000 equal divisions is preferable. The wheel must be mounted so exactly that rotation does not displace the vernier reading by more than 1 minute from the true scale value. For this reason, a hand-lapped double-cone taper bearing may be used with very satisfactory results. Accurate machine work is essential throughout in order to assure a dust-tight case and proper adjustment and operation of the cam-type pan arrests and of the torsion adjustment and rotation. The details will not be given since the balance is not suitable for construction in the laboratory, in common with the usual analytical and microchemical balances.

The optical system also is not suitable for laboratory construction. Essentially it consists of two 3X objectives, focused respectively on the two ends of the 15-μ indicator fiber, and prism systems which bring the paths of the images through the bottom of the case, converge them into the two halves of a single field, and project them through a 7X Hastings triplet lens to a ground-glass screen about 4 in. square. The circular field on the screen is divided down the center, and the index fiber appears as two horizontal lines, one in each half, which oscillate vertically with respect to each other. When they form a continuous line across the screen, the index fiber is in the exact plane of the objectives, which is taken as the true zero of the balance. The necessary light is brought into the balance case from two small flashlight bulbs by glass or Lucite rods which terminate just behind the index fiber.

Operation of the Quartz Balance. Operation of the balance is considered before the calibration procedure because it is necessary

to operate the balance while calibrating it. The quartz torsion balance described is relatively free of interference from vibration, heating effects, and other environmental conditions that have a large influence on the microchemical balance. It is extremely susceptible to the presence of dust, but the case is designed to eliminate this difficulty almost completely. The most important limitation in its use is the problem of maintaining the weight of the object with the constancy necessary to utilize fully the sensitivity of the balance. Vibration fails to affect it seriously because the torsion-bow suspension damps vibration very quickly. Temperature is of little concern because of the extremely low coefficient of expansion of quartz. Unequal heating of the instrument will cause serious difficulty, however, because of air currents that are established by a local temperature variation. Because of the magnitude of this effect, the balance is usually equipped with two or more coverings with air spaces between.

The only serious hazards in operating the balance occur in loading and unloading, at which times breakage can occur if the pans are handled carelessly. The weighing is both rapid and simple.

The empty pans, which should weigh practically the same, are placed on the pan cradles. All lights are turned on, and the balance is allowed to equilibrate for a short time to dissipate air currents due to unequal heating. The pan release lever is turned to weighing position, and the wheel is adjusted until the projections of the indicator fiber are continuous on the ground-glass screen. If the balance has not reached equilibrium, this position will not be maintained after setting. The pan which is to receive the sample is then removed from the stirrup with a platinum wire fork and is loaded with the material to be weighed. It is replaced on the cradle, the pan-well door is closed, and the wheel is again rotated until the indicator fiber forms a continuous line on the screen. The difference between the two readings of the wheel, before and after the balance is loaded, is referred to the calibration factor to obtain the weight of sample. The entire operation should require no longer than 30 seconds if the balance is first at equilibrium.

The sample weight may not exceed the amount which can be balanced by torsion alone, or, if the pans are not closely the same in weight, the sum of the sample and the difference between the

pans will require balancing by torsion. In balances having a sensitivity of 0.005 γ per scale division, the allowable sample weight has been found to be about 0.3 mg. It is evident that a weight in excess of this limiting value could never be restored to balance by any number of turns of the torsion fiber.

Calibration of the Quartz Balance. A quartz balance of this type shows an arbitrary relationship between mass and wheel readings because of the impossibility of determining in advance the exact properties of the torsion fiber. Theoretically, the response should be linear, however, and in practice this has proved to be true. It is therefore necessary only to determine the factor relating scale units on the wheel to weight units. A single accurate point would locate the position of a straight-line calibration curve passing through zero with the unloaded balance. The determination of such a single point with the necessary accuracy is at present impractical because of the impossibility of making any single weight in the microgram range with an accuracy corresponding to the sensitivity of the balance. Both the unavailability of microgram weights and the difficulty of maintaining them if they were available make the attainment of high accuracy very difficult.

Two methods have been applied successfully to this calibration, and others may be developed in the future. Both are indirect and involve certain errors, the magnitude of which may be approximately calculated.

Method 1. A standard salt solution is prepared to contain an accurately known concentration. Various sizes of sample are evaporated to dryness on the balance pans and used as standard weights. A salt such as potassium chloride, which is of very definite composition and without hygroscopic properties, meets these requirements. The errors inherent in this method are those of microgram volumetric measurement (pipet) and those of making the solution to the original concentration. With care and with multiple samples to reduce random error, this method may be made accurate to 0.1 to 0.2%, but probably no better.

Method 2. This method depends on weighing a rather long piece of fine platinum wire or a quartz thread which is quite uniform and measuring its length as accurately as possible. The wire or fiber is divided into short pieces, preferably of even length, which, because they contain a proportional quantity of the total

weight, may then be used as calibration weights. The errors involved are those of microchemical weighings and the inherent error of cutting the wire or fiber to definite and determinable lengths. The cutting error may be minimized by grouping a sufficiently large number of pieces in different ways so that a mean weight for each group is obtained. Any error in the original weighing will appear in the calibration. Experimental checks have shown that the two procedures agree with each other ordinarily to $\pm 0.5\%$ or better, when carefully performed.

The accuracy of the calibration may be improved with larger numbers of data to minimize chance variations, but, if systematic errors are present, e.g., in pipet calibrations, length measurements, or calibration of weights, they will be retained regardless of the numbers of observations taken. For most purposes an accuracy of 0.5% is adequate for analytical results, and somewhat less than this should be present in the balance calibration. For this reason, the greatest care should be taken in this operation to avoid all errors to the maximum extent possible.

Flotation Weighing

Another principle for certain weighing of microgram quantities is described by Zeuthen (25) and Levi and Zeuthen (26). It is based on the fact that density may be determined with extreme sensitivity and high precision. If the volume of a material can be accurately determined and its density measured, the weight can be calculated from these values. This is true for mixtures of materials of different density as well as for single substances. Two techniques have been described, the first of which depends on the use of the gradient tube of Linderstrøm-Lang (27, 28) and applies only if the object weighed is of known density. It was employed by Levi and Zeuthen to prepare minute gold weights which could then be used for calibration of other weighing instruments. The procedure of utilizing a gradient tube for precise density measurements is discussed in Chapter 10.

The second procedure of Zeuthen involves the Cartesian diver, also originally described by Linderstrøm-Lang (29), which, because of its extreme sensitivity, can be employed for weighings of the smallest quantities with relatively high accuracy, e.g., the sensitivity limit is $\pm 0.01\ \gamma$. All weights are obtained with the

objects immersed, which makes the procedure suitable for weighing single living cells and inert objects that are not damaged or altered by immersion. The Cartesian diver technique is also described in Chapter 8. The application of these procedures for analytical purposes is strictly limited and will not be described in complete detail.

Gradient Tube Weighing. This technique was used by Levi and Zeuthen for the preparation of small gold weights cut with a microtome from embedded gold foil. The gradient tube made from bromobenzene ($d = 1.48$) and kerosene ($d = 0.79$) was established, as will be described later.

An aqueous drop whose volume is accurately known, either by microscopic measurement while suspended or from measurement with a calibrated micropipet, and which contains the piece of gold being weighed is dropped into the gradient tube. Its specific gravity is observed. The tube is calibrated with droplets of mixtures of water and deuterium oxide of accurately known composition. Since the density of the object weighed and of the water in which it is immersed are known, it is possible to calculate the absolute weight by means of a term called the reduced weight, which is defined as the weight of the body minus the weight of the same volume of water. Thus, with a material having the same density as water, the reduced weight would be zero. With gold the reduced weight is relatively high because of the high density, 19.2. The reduced weight is obtained in terms of the volume multiplied by units calculated from the density of the water-deuterium oxide calibration mixtures through the formula

$$\text{Unit} = (d_4^{20}\ H_2O\text{-}D_2O - d_4^{20}\ H_2O)10^6$$

When the reduced weight is established, the true weight of gold is obtained from the formula

$$W = \frac{19.2 \times \text{R.W.}}{19.2 - 0.99}$$

In weighing objects with various specific gravities, the corresponding values of the specific gravity would be substituted for the 19.2 which represents the specific gravity of gold. Measurement of the volume of the drop is troublesome because of distortion of the spherical shape by the heavy objects contained within it and

because the volume varies proportionately with r^3, where r is the radius of the drop. If the volume is measured by the pipet, the sensitivity of the measurement is not greatly different from that obtained by microscopic observation of the diameter. A number of technical difficulties were encountered, chiefly in connection with the loss or gain of deuterium oxide from the gradient tube mixture. It was shown that weighings could be made with an error approximating 1%. Aside from its applicability to the preparation of minute weight standards, no uses of the gradient tube method in analytical work have been described.

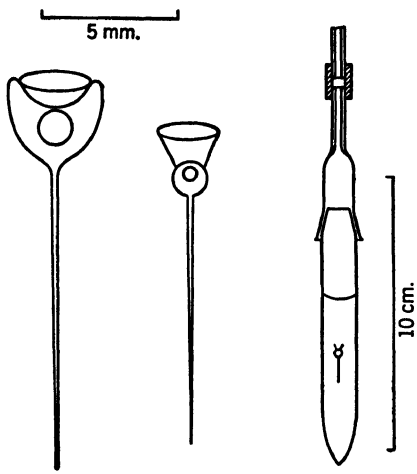


FIG. 47. Weighing diver according to Zeuthen.

Cartesian Diver Weighing. Zeuthen (25) first described the application of the sensitive Cartesian diver technique, discussed in Chapter 8, to the weighing of microgram quantities. The "diver," immersed in a bath and containing a small air bubble entrapped over water or an aqueous solution, is extremely sensitive to pressure changes in the external gas over the immersion bath. At the point at which it is in unstable equilibrium, i.e., suspended between the top and bottom of the bath, any effect within the diver which changes the gas volume of the bubble or the total weight of the diver will be sensitively detected by the vertical movement produced, which is balanced by changing the external pressure.

The application of this principle to weighing is made by comparing the behavior of the diver when carrying the unknown material and when carrying instead a small known weight. Gold weights calibrated by the density gradient tube can be used, but ordinarily polystyrene weights are preferred because they can be manipulated more easily.

The construction of the diver adapted to this purpose is shown

in Fig. 47. The inverted bell was blown from polystyrene dissolved in benzene, the diver proper being made of glass.

The reduced weight of the object, as defined in the preceding section, was obtained from the pressure changes necessary to suspend the diver, with the formula

$$\text{R.W.}_x = \text{R.W.}_{st} \frac{1 - \dfrac{B}{B - P_x}}{1 - \dfrac{B}{B - P_{st}}}$$

where R.W._x is the reduced weight of the object weighed.

R.W._{st} is the reduced weight of the standard.

B is the external barometric pressure expressed in centimeters of water.

P_x is the change in pressure to balance the unknown, expressed in centimeters of water.

P_{st} is the change of pressure necessary to balance the standard weight, expressed in centimeters of water.

The object weighed is immersed in the water of the container during weighing and must be transferred before chemical operations can be performed. For this reason, the diver technique is not practical for many of the purposes of quantitative microgram analysis. It has been successfully employed for weighing cells and tissues, and for this purpose it is more suitable than most open-air weighing methods because drying and subsequent damage to the material is avoided by its immersion.

FILTRATION

When volumes are as small as single drops, quantitative filtration becomes a problem inherently different from that of quantities which may be transferred by pouring. The filter stick of Emich (1) is a near solution to the problem, as perhaps is that of Behrens-Hemmes described by Chamot and Mason (2). Since the precipitate is more commonly determined (by titration) than the filtrate, neither filter is particularly convenient because the precipitate is not readily separated from the filter. A somewhat more useful form of the filter stick is shown in Fig. 48, which is similar to the design described by Kirk (30). It consists of a heavy-walled capillary tube, somewhat expanded at the filter

end and closed with a sintered-glass disk. The diameter of the disk may range from less than 1 mm. up to about 5 mm. With most volumes encountered in volumetric microgram analyses, a diameter of about 2 mm. is suitable. As marketed, these filters contain a rather coarse sintered-glass disk because of the difficulty of sealing or sintering very small filters of fine porosity. Because of the coarse porosity, the filter is habitually used with a thin layer of fine asbestos applied by suction to the outside of the tip. It will then separate both coarse and fine precipitates,

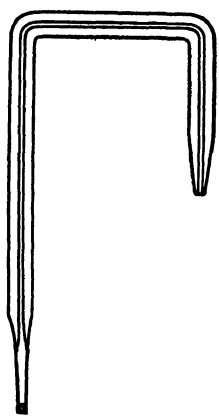


FIG. 48. Sintered-glass filter stick.

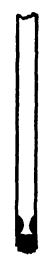


FIG. 49. Filter stick for paper. (Actual size.)

because a suitable porosity can be obtained by adjusting the fineness and amount of asbestos.

Asbestos is prepared as follows: A good grade of asbestos fiber (medium Italian, acid-washed, and ignited grade is preferred) is ground in a mortar until the long fibers are well disintegrated. The asbestos is suspended in water, rather strongly acidified with sulfuric acid, and heated with excess oxidizing agent, usually potassium permanganate, until a permanent pink color is obtained, or with excess ceric sulfate. If a brown precipitate of manganese dioxide is produced, it is discharged with a little hydrogen peroxide. The asbestos is washed thoroughly by decantation with acid and finally with water. It is stored in suspension in distilled water.

When the filter is to be prepared, the asbestos suspension is shaken, and the filter stick with vacuum applied is momentarily inserted into the asbestos suspension to draw a small volume of liquid through and deposit a thin layer of fine asbestos on the surface of the filter disk. This layer adheres throughout subsequent use of the filter, even if the vacuum is discontinued.

Figure 49 shows a simple form of filter stick which is occasionally useful, particularly where the liquid rather than the solid is desired. It consists of a small tube of about 3-mm. inside diameter, with a slight constriction about 2 mm. from the end. Into the end below the constriction is placed a small roll of filter paper. This filter is a convenient substitute for the sintered-glass type when the requirements are simple or the sintered-glass form is not available. It is actually only a slight modification of the Emich filter stick which employs asbestos but is more easily prepared.

CENTRIFUGATION

The only centrifuge that has been specifically designed for microgram analysis is shown in Fig. 50, which is a modification of an earlier type described by Kirk (30). It consists essentially of a streamlined head, an air turbine, and a housing. It operates on 20 to 40 lb. of air pressure and is driven at speeds up to about 25,000 r.p.m., at which speed it generates from five to ten times as much centrifugal force as a standard electric centrifuge running at 3000 to 3500 r.p.m. The high speed of the machine more than compensates for the smaller diameter of the head.

The head of this machine is 3¾ in. in diameter and holds eight cones in a horizontal position. These may be made to hold as much as 0.2 ml. down to as little as desired by using capillary tubing. The fact that the cone is continuously in a horizontal position presents no difficulties because the surface tension in tubes of this size prevents the solution from running out, even when the tube is inverted. The head is constructed from Duralumin surrounded by a steel band against which the pressure of the tubes is exerted. Three designs of suitable centrifuge cones are shown in Fig. 51.

The centrifuge is particularly well adapted to the sedimentation of blood corpuscles, yielding perfectly clear plasma or serum in 3 to 5 minutes. The capacity of each tube is suitable for samples

of finger blood or animal tail blood. The separation of minute precipitates by this machine is also very simple and often quite useful. Usually, a minute or less is all that is required for complete clarification of a solution containing an analytical precipitate.

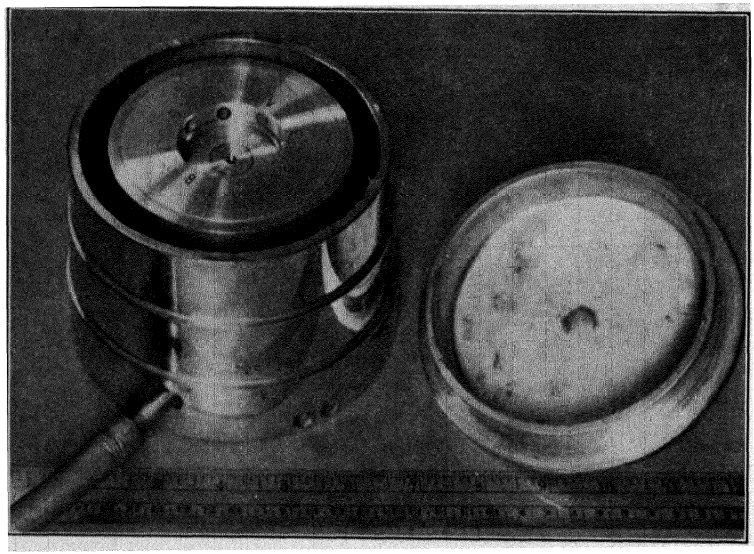


FIG. 50. High-speed air-turbine microcentrifuge.

Ordinary electric microcentrifuges may be adapted for the microgram scale. The cups of such machines are usually too large for cones of a proper volume for microgram quantities. Adaptors may be made to hold smaller cones, or the cone may be constructed from capillary tubing. Although such modifications are valuable for laboratories that do not contain compressed air, they are much less satisfactory than the air-driven machine because of their larger size and slower speed, as well as the difficulties of adapting them to small samples.

DEPROTEINIZATION

Deproteinization of small volumes of blood or other physiological materials may be carried out on the microgram scale in exactly the same manner as is customary on a larger scale, except

that all volumes are reduced proportionately and it is more convenient to centrifuge than to filter the precipitated protein. The addition of reagents is made in the appropriate quantities, and the solution is made to a final volume which is usually either five or ten times that of the sample.

A typical deproteinization is carried out in a tube like that shown in Fig. 52, which is made to fit a standard microcentrifuge, or, for the smallest volumes, a tube such as the third one of Fig. 51, designed for use in the air-driven centrifuge described

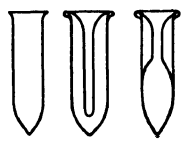


FIG. 51. Cones for air-turbine centrifuge.



FIG. 52. Deproteinization tube.

above. The largest final volume that is conveniently separated with the air centrifuge is 0.1 ml., which would normally require a sample of 10 to 20 λ of original blood. These tubes are used as follows: If two solutions, e.g., sodium tungstate and ⅔ *N* sulfuric acid are used, it is best to pipet one reagent into the tube in appropriate volume. The sample of blood or other fluid is chosen so as to be one-tenth or one-fifth of the deproteinization tube volume. It is added to the tube with rinsing and mixed with the first reagent. The second solution is added slowly with a pipet and with stirring. The stirring may be accomplished by insertion of a vibrating thread stirrer described previously, or it may be obtained even more simply by holding the tube against a vibrating body such as an electric massage vibrator or even the vibrator of a doorbell. This stirs the contents of the tube quite well. After the reagent is completely added and the material thoroughly mixed, the volume is made up to the mark and remixed, and the precipitated protein is centrifuged. Samples of the protein-free solution are removed with capillary pipets from the

tube. Details of solutions are included in connection with the analytical methods given in later sections.

HEATING EQUIPMENT

The most important applications of heat in microgram analysis are for the digestion, evaporation, or distillation of volumes of one or a few drops of liquid. With liquid volumes of this magnitude, very small heat sources are required in order to keep the input of heat under very close control and small in amount.

Gas Burners

The principle of the common Bunsen burner can be applied to obtain flames as small as the head of a common pin. Slightly larger flames than this are more likely to be useful, e.g., those about 3 to 5 mm. in height. The burner shown in Fig. 53 furnishes a very satisfactory Bunsen flame, whose size is regulated by the glass tip which is inserted in the burner and which is readily interchangeable. A glass gas orifice is employed because glass capillaries can be constructed to any required size, whereas metal tips would require drilling of extremely fine orifices.

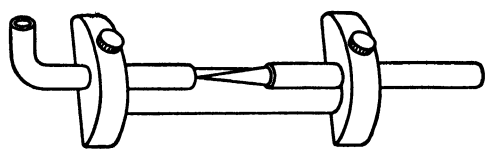


FIG. 53. Sub-micro gas burner. (½ actual size.)

Modifications of burners of this type may be built completely from glass tubing (31). Their light weight gives them an instability which may lead to their tipping over under the torque resulting from a twist in the rubber connection, thus giving preference to the more heavily built metal burner. The ready interchangeability of tips in the metal burner is also an important advantage because the burner can thus produce flames the size of a pinhead and up to an inch or more in height. None of the standard microburners available commercially gives sufficiently small flames for this type of application, with the possible exception of the Pregl burner which does not operate with natural gas.

Glass- and Quartz-Working Torches

Most glass-blowing operations of the microgram analyst are readily performed with standard equipment and commonly known methods (32). At times it is necessary or desirable to construct glass apparatus in very small sizes or with thin-walled capillary tubing. This may involve a type of glass blowing with which the average professional glass blower is relatively unfamiliar, and, particularly, it may require torches providing smaller flames than are obtainable from ordinary burners. This is particularly true with thin-walled capillary tubing, and it is absolutely true in quartz fiber construction, though special torches for this purpose have been described (23). Accessories for micrurgy may require the most delicate glass working in which it may be necessary even to work under the microscope and to employ a flame invisible to the naked eye. These various needs may be met by three types of flame requiring different forms of burner:

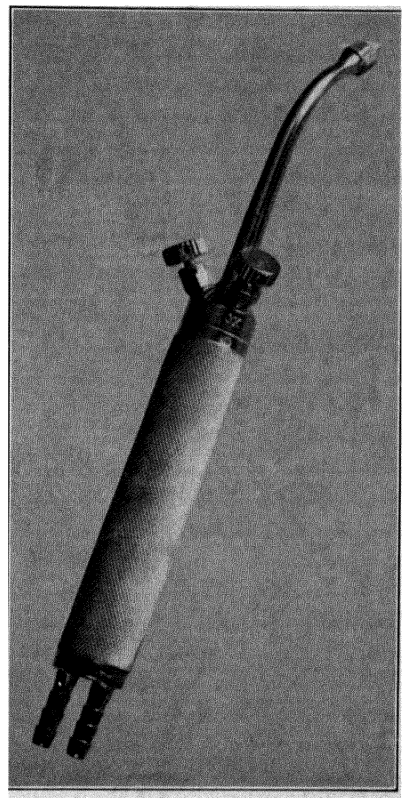


FIG. 54. Microtorch (oxygen-gas). (*Courtesy Microchemical Specialties Co.*)

1. A small, very hot, diffuse flame such as is yielded by a small hand torch operating with gas and oxygen is most valuable for quartz fiber construction and similar purposes. A very advantageous form of such a torch is shown in Fig. 54. It is essentially a standard hand torch, reduced in size so as to be readily manipulated in small work. It is supplied with tips having diameters down to practically microscopic di-

mensions, e.g., 0.02 to 0.002 in. With the smaller tips, flames below the range of visibility may be obtained for work under magnification. The larger flames serve for fusion of quartz rods up to several millimeters in diameter as well as for welding, brazing, and other requirements for very high local temperatures. The torch shown is used exactly as the one described by Neher (23), but it is much more readily operated than the Neher torch because the needle valves are close to the tip, preventing disturbing backfires, and greatly speeding the adjustment of the flame. These torches are not suitable for thin-walled capillary construction because the flame is relatively broad in comparison with its length, causing collapse of thin walls adjacent to the point being worked.

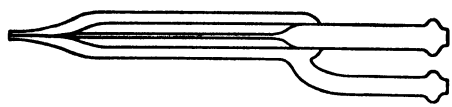


FIG. 55. Glass microblast lamp. (½ actual size.)

2. A microburner of the air or oxygen injection type constructed from glass is shown in Fig. 55. When the burner is used with air, the auxiliary air inlet on the gas line is desirable for good operation with natural gas. With oxygen, this inlet is unnecessary and the gas may be led directly into the outer chamber. The inner tip is made extremely fine, furnishing a very limited amount of high-velocity air or oxygen which produces a very narrow, needle-like flame which is well adapted to working thin-walled tubes, blowing very small holes in glass walls, and similar uses. An air-gas flame is preferred for thin-walled capillary work, including Pyrex glass. Seals of various types may be made in this material, only with a very tiny flame of this type. The amateur glass blower should have little difficulty in constructing this type of torch.

3. The third type of special flame particularly for very small-scale work is a small luminous flame which may be obtained merely by placing a glass tube on the outlet of the gas line and burning a very small amount of gas without introduction of air. The specialized use of such a flame is in the drawing of very fine capillary tubes and tips. It is also valuable for bending small capillary tubes without collapsing or sealing them. If a hotter

flame of the same general type is needed, the microburner of Fig. 53 is satisfactory.

Electric Heating

Electric heating of small volumes has the advantage of allowing smooth and positive control of the heat input. It cannot be localized to the extent that gas heating can, but for many purposes that is unnecessary. Although it is possible to heat small volumes with standard electric hot plates, it is more convenient to use a small, specially designed hot plate adapted to the size of the equipment heated, as shown in Fig. 56. The heating coil is about the size of an ordinary cigar lighter but works at a lower and controlled temperature, achieved with a variable-voltage transformer of 75 to 100 w. capacity. Transformers that drive toy electric trains are inexpensive and entirely satisfactory.

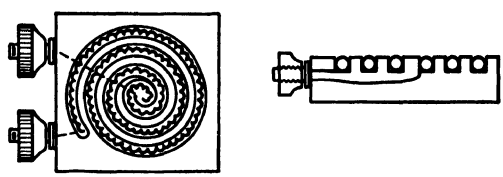


FIG. 56. Micro hot plate. (Actual size.)

The hot plate may be constructed from a Transite block equipped with a Nichrome or Chromel heating unit. The entire hot plate may be encased in a stainless steel or other metal shield for protective or decorative purposes.

One further type of heating is performed most advantageously by electrical means. This is the application of localized and carefully controlled heat to small areas, as is necessary in the construction of certain specialized glass equipment. Illustrations are drawing of capillaries or needles of microscopic size for microinjection and microdissection of cells, and forming indentations in very thin glass membranes as in the construction of certain modifications of the Cartesian diver, small-cup-type glass electrodes, and similar devices. This type of heating is simply accomplished by means of hot platinum wires of various shapes adapted to the specific need. For drawing capillaries and needles, a coil surrounds the glass being drawn. For applying heat at an approximate point, a pointed loop or wire is employed. A convenient

type of holder that can be readily constructed is shown in Fig. 57. Any desired configuration of platinum wire may be substituted for the pointed form shown. The current is supplied from a Variac or similar transformer which gives smooth regulation of the

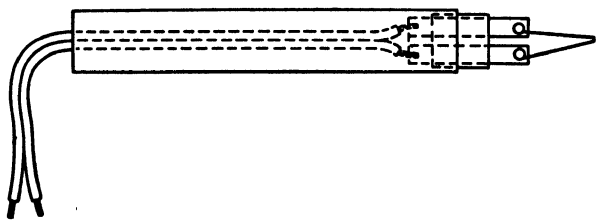


FIG. 57. Hot wire holder. (½ actual size.)

voltage. This type of hot wire holder is very useful in applying and removing diphenylcarbazide which is employed as a cement in quartz fiber construction. Many other applications will suggest themselves in a laboratory where much specialized construction or many operations are carried out.

Ashing Vessels

When non-volatile materials are to be determined, the most convenient method for destruction of the organic matter in small samples of biological materials is unquestionably dry-ashing, either direct or modified by the addition of certain reagents. As compared with dry-ashing of large samples, small samples decompose very rapidly and rarely give rise to difficulties from spattering or frothing. This is true with liquids such as blood as well as with bits of tissue or solid materials. With amounts of liquid not in excess of 0.1 ml., complete ashing is obtained in a small muffle furnace in from 10 minutes to ½ hour, if relatively high temperatures can be used. In order to avoid loss of slightly volatile compounds, it is sometimes advisable to operate at temperatures just under redness, in which case ashing may require somewhat more time. Either operation is likely to be less time-consuming than acid digestion and is to be strongly recommended as compared with dry-ashing of macrosamples.

Animal materials ordinarily leave an alkaline ash that may attack glass or porcelain vessels. Plant materials give less difficulty from this source and usually may be ashed in small

porcelain crucibles or dishes. It is recommended that blood and other animal materials always be ashed in platinum crucibles to avoid formation of insoluble silicates which cannot be readily dissolved in acid. The platinum crucible shown in Fig. 58 is very satisfactory. Its shape is intermediate between that of a dish and a crucible. Suitable dimensions are 12 to 15 mm. in diameter and 6 to 9 mm. in height. A small tab should be left on the side of the rim to serve as a handle in order that the crucible may be held with forceps without contamination of the contents or the forceps.



FIG. 58. Micro platinum crucible. (Actual size.)

Heating of the crucible is best performed with a micro muffle furnace, but it is possible to employ a hot plate to dry the sample, followed by a gas flame for ashing, or to use the ultramicroburner with a very low flame applied cautiously by hand to carry out the entire operation.

EXTRACTION

Two general types of extraction may be utilized in microgram analysis: (1) extraction of solid materials as in the analysis of tissues for their lipid content, and (2) extraction of a liquid sample for some constituent dissolved or suspended in it, e.g., the extraction of phosphomolybdic acid with butyl alcohol or the extraction of ferric thiocyanate with organic solvent mixtures.

Extractors for solid samples have been described by many authors and reviewed extensively by Schneider (4). Few if any of them have been employed in the performance of quantitative microgram analysis, and little information on their performance in this regard is available. The extractor of Hetterich (33), shown in Fig. 59, or some modification of it should be satisfactory for continuous extraction of very small samples. Schmidt-Nielsen (34) extracted and fractionated the lipids in 1 mg. of tissue but used toluene in a sealed tube as a single-batch procedure rather than making a continuous or multiple-batch extraction. Stern and Kirk (35) have employed the extractor shown in Fig. 60 with excellent success in the extraction of sucrose from plant material with alcohol. It is similar to other extractors described in the literature but is probably the only one that has been tested adequately for continuous quantitative extraction of

any constituent in microgram quantities. The method is apparent from the figure except for the heating procedure. To prevent bumping and rapid condensation on the walls, heat is applied by means of a Nichrome wire passed in two or more loops above the solvent level and heated electrically. This wire warms the entire

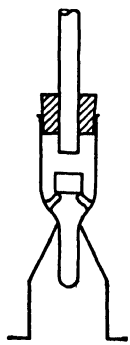


FIG. 59. Liquid-solid extractor. Hetterick type. (Reproduced from Schneider, *Qualitative Organic Microanalysis.*)

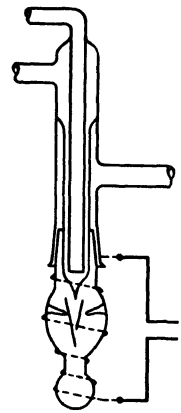


FIG. 60. Liquid-solid extractor Stern-Kirk type. (½ actual size.)

chamber and heats the solvent from above. Boiling is very smooth, and no measurable solvent loss occurs, even over a period of many hours.

Liquid-liquid extraction which appears simple to perform may nevertheless be quite a difficult operation when the volumes are excessively small. With volumes of the order of 1 or a few drops, no large apparatus is suitable because of the loss incurred in wetting the walls with the liquid. Special apparatus has been described which is distinctly superior for these extractions as compared with the use of batch extractions in small tubes.

Heavier-than-Water Solvents

Extraction with heavier-than-water solvents, e.g., dithizone salt extractions with chloroform, is readily accomplished with a modified micro separatory funnel as shown in Fig. 61. The chamber of this funnel may be designed to contain any desired

quantity but should rarely exceed a capacity of about 5 ml. It is formed in a long gradual taper, terminating in a capillary sealed to a capillary stopcock. The top of the funnel is fitted with a three-way stopcock, one arm of which is attached to a controlled low vacuum. The funnel is mounted rigidly on a ring stand where it remains throughout all operations. If desired, the stopcock can be substituted by a gooseneck stem, as shown in Fig. 62.

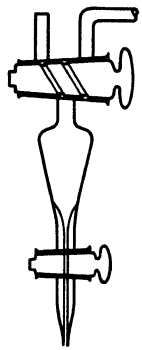


FIG. 61. Capillary separatory funnel. (½ actual size.)

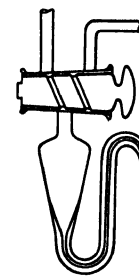


FIG. 62. Gooseneck capillary separatory funnel. (½ actual size.)

The material to be extracted and the solvent are both sucked in through the tip by means of the vacuum line. The mixing is carried out by allowing a controlled stream of air to flow through the solution while vacuum is applied at the top. Some care is necessary to prevent loss by spattering. After the material is extracted, the solvent phase is withdrawn by opening the upper stopcock to the atmosphere and separating the phases at the tip of the funnel rather than at the lower stopcock. The next batch of solvent is drawn in directly with vacuum as before.

Lighter-than-Water Solvents

With solvents that are lighter than water, a separatory funnel is distinctly unsatisfactory, owing to the necessity of removing the lower aqueous phase before the upper solvent phase can be removed. The aqueous phase must then be replaced for a second extraction, resulting in marked loss of material by wetting several

vessels. When the volumes are very small, a separatory funnel is completely impractical for this purpose.

Depending on the amount of liquid to be extracted, two different types of extractors may be employed advantageously. The first of these may be used with volumes down to about 0.5 ml. It was described by Kirk and Danielson (36) and is shown in Fig. 63. It consists of an extraction chamber which is drawn to a long gentle taper and attached through a capillary tube and stopcock to a side chamber. Inserted at the top of the extraction chamber is a small separatory funnel with a long capillary tip which extends into the capillary opening at the bottom of the chamber. The chamber is also attached to a three-way stopcock, one arm of which is attached to a controlled low vacuum, the second to a closed rubber bulb. The small separatory funnel is fitted with a ground joint at the top so that it may be readily removed from the extraction chamber.

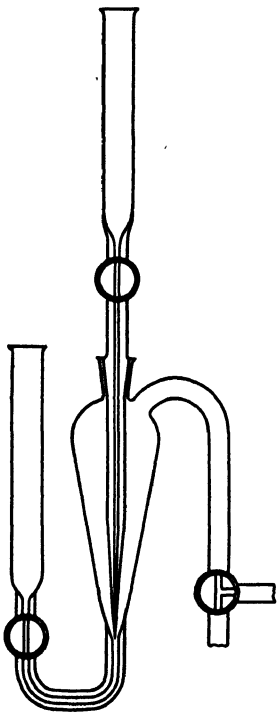


FIG. 63. Liquid-liquid extractor. (½ actual size.)

The sample to be extracted is drawn in from the side chamber by vacuum followed by the solvent. The two phases are mixed by drawing a slow, controlled stream of air through the liquid. Separation of the phases is performed by discontinuing the air flow, which allows them to separate. The rubber bulb is then used to force the lower aqueous phase into the side chamber to a point at which the interface is directly below the capillary tip of the upper separatory funnel. The stopcock to the side chamber is then closed, and that to the small separatory funnel opened, after which the solvent phase is forced upward into the separatory funnel where it is stored. The aqueous phase is then drawn back into the extractor and the second batch of solvent drawn in after it. Extraction is performed as before, the second

batch of solvent being added to the first in the upper separatory funnel. When extraction is complete, this funnel is lifted out of the apparatus and the contents are transferred to whatever receiving vessel is employed for the next step of the analysis. The extraction apparatus is cleaned by inserting into the side chamber a capillary attached to an evacuated bottle which serves as a liquid-collecting trap. The apparatus is washed out and the washings removed to the trap bottle by suction as before, after which it is ready for the next extraction.

When the amounts of liquid to be extracted are small, i.e., of the order of 0.1 ml. or less, a batch extraction method is likely to be highly inefficient because of the difficulty of completely separating and recovering the solvent. At the same time, the surface of such a small amount of liquid may be made very large as compared with the volume, thus allowing rapid diffusion from one phase to another. In order to utilize this principle effectively, the extractor shown in Fig. 64 was designed (37). It consists of a small hemispherical bulb blown in the side of a capillary tube whose internal diameter may be 0.5 mm. or smaller. The tube is drawn to a filling tip, and, if desired, also carries one or more washing reservoirs as shown. The tube terminates in a delivery tip enclosed in a ring-sealed tube which is attached to a syringe controlled by means of a screw. Below the delivery tip a receiving vessel is attached by means of a rubber connection. The entire apparatus except the syringe is mounted by suspension so that it may be shaken with the finger during extraction.

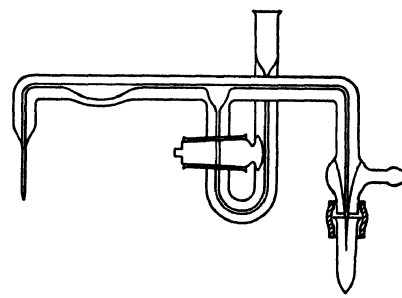


FIG. 64. Capillary liquid-liquid extractor for lighter-than-water solvents.

The sample to be extracted is drawn into the hemispherical bulb by rotating the wheel which controls the syringe plunger. The solvent is placed in the same vessel to rinse it and is then drawn into the extractor. The heavier water phase settles in the bulb, and the lighter solvent is drawn slowly over it while the apparatus is shaken to hasten the attainment of equilibrium.

Each small volume of solvent passing over the aqueous phase extracts the material in question and passes into the capillary from which little or no back-diffusion can occur. Thus the effect of a continuous extraction is achieved in that a large number of very small-volume batches are passed over in succession. When the solvent must be washed, the washing well is filled with wash liquid which is effective for a similar reason. More than one such well may be used, or the well may be omitted if it is unnecessary.

Another form of capillary extractor which is useful particularly when the solvent volume must be kept small is shown in Fig. 65 (37). It may be used only as a batch extractor and can be applied to solvents both heavier and lighter than water. If the mixing chamber is sufficiently large, mixing may be obtained by air bubbles. If it is smaller, rotation of the tube in the ground joint causes mixing. Either phase may be withdrawn by manipulating the pressure control and rotating the tube properly. It should find extensive application in such procedures as those involving extraction of phosphomolybdic acid with butyl alcohol, where the amount of solvent must be kept small as compared with the aqueous phase.

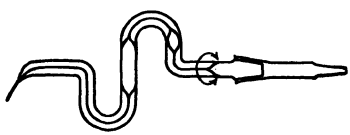


FIG. 65. Capillary liquid-liquid extractor.

The application of the two types of capillary extractors have been little explored as yet, but it is evident that many possibilities exist, both in ordinary removal of materials from mixtures, and as specialized analytical tools in combination with chemical analysis methods.

DISTILLATION

As a quantitative microgram procedure, ordinary distillation has been employed to a limited extent only. The well-known microdistillation methods which have proved so valuable in qualitative and preparative operations are quite completely described elsewhere (1, 4). Most of them are not well suited for quantitative operations, in which a volatile constituent is usually distilled and collected in an absorbent. A common illustration of this type of technique is the determination of nitrogen in the form of ammonia after the Kjeldahl digestion. Experience shows that

ordinary distillation is less suitable for this determination than is diffusion by some modification of the method of Conway (38). That it can be used successfully has been shown by Kirk (31) who used the distillation apparatus shown in Fig. 66. The distillation was accompanied by aeration through the boiling solution to prevent the transfer of large quantities of water. The vapors were condensed in a simple tube wrapped with wet filter paper

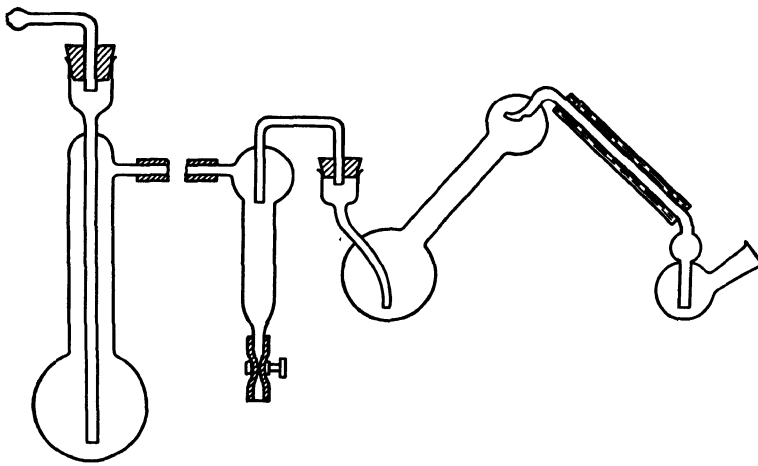


FIG. 66. Distillation apparatus. Steam and/or aeration attachments.

which furnished enough cooling for the small amounts of material distilled. The ammonia was collected in standard acid in the lower reservoir, in which it could be titrated without transfer. This type of apparatus can be used for other distillations, with or without aeration. A modification was designed for the determination of lactic acid in which acetaldehyde formed by oxidation of the lactic acid was distilled and collected in bisulfite solution (39). Other adaptations of the technique will occur to the ingenious analyst.

Isothermal Distillation

By far the most widely useful distillation technique for microgram analyis is that commonly known as the diffusion method. It was originally developed in detail by Conway (40) for determination of urea and ammonia and has been completely described

by the same author (38). This type of operation is properly considered as an isothermal distillation, since the temperature of the entire system is uniform and the gradient in concentration is created by chemical means. A typical Conway diffusion cell modified for microgram quantities is shown in Fig. 67. The cell,

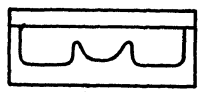


FIG. 67. Plastic diffusion unit, Conway type. (⅔ actual size.)

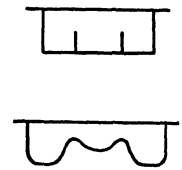


FIG. 68. Glass diffusion units, Conway type. (⅔ actual size.)

which may be constructed from glass or a plastic such as Lucite, contains two chambers. The volatile constituent is released in the outer chamber and collected in an absorbent in the inner chamber. Thus ammonia may be distilled into standard acid, chlorine into potassium iodide solution, etc. The cell may be constructed in a variety of sizes ranging from a diameter of 1½ in. down to perhaps half this much. The larger cells are more convenient to manipulate and may be readily constructed on a lathe from Lucite rod. Smaller cells are readily constructed from glass. Two designs are shown in Fig. 68. They are subject to breakage and are not preferred to Lucite cells except where strong reagents are needed, as in the determination of chloride by oxidation to chlorine which is then subjected to diffusion. A plastic such as polytetrafluoroethylene, which is completely inert toward virtually all reagents, would be markedly superior to either glass or Lucite. This material can be machined readily, is not subject to easy damage, and is almost completely inert chemically as well as

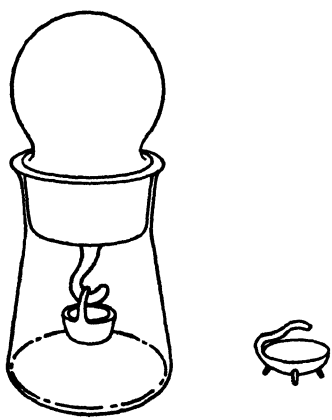


FIG. 69. Kjeldahl diffusion unit, Kirk-Bentley type. (⅔ actual size.)

being insoluble in aqueous or organic solvents. Because it is opaque, clear covers of glass or plastic would be required. Such cells have not been described but are known to be entirely satisfactory.

Special designs of equipment for isothermal distillation have been described chiefly in connection with the determination of nitrogen following the Kjeldahl digestion. Bentley and Kirk (41) used a vessel shown in Fig. 69, which, because of the necessity of transfer of the digest, has been superseded for total

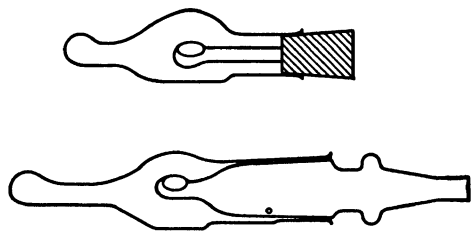


FIG. 70. Kjeldahl digestion-diffusion unit, Tompkins-Kirk type. *Top,* atmospheric pressure type. *Bottom,* vacuum type. (½ actual size.)

nitrogen analysis by vessels in which both digestion and diffusion may be carried out. This design is suitable for general application, however, and offers a possible substitute to the Conway-type cell when somewhat larger volumes must be used than are convenient with the latter vessel. Needham and Boell (42) described an ingenious combination digestion-diffusion vessel which was, however, slow in operation. This difficulty was overcome by the somewhat similar design of Tompkins and Kirk (43), which is shown in Fig. 70. Digestion could be performed in the small end of this vessel and diffusion carried out after the vessel was laid on its side to utilize the larger surface of the flattened bulb. Most important in all diffusion operations is maintenance of a maximum surface combined with a small depth of liquid. This is achieved admirably in the Conway cell and to a very satisfactory extent in some other designs. The theory of diffusion analysis is covered in detail by Conway (38).

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CHAPTER 5

Microgram Titrimetry

With few exceptions, titrimetric analytical procedures are divisible into (1) acidimetric, (2) oxidimetric, and (3) argentimetric methods. All three of these general classes have been utilized for microgram analysis. The first two listed are employed much more widely than the third and will receive greater attention.

ACID-BASE TITRATIONS

Acid and base may be used to titrate each other with amounts as small as the practical limitations of end-point detection allow. If the sample is large enough in volume so that the color change can be perceived with the eye, it can usually be titrated. The limitation of concentration of reactants must also be considered inasmuch as the titration of the necessary amount of indicator alone might conceivably require more reagent than that needed for titration of the sample, and in very dilute solutions end points are rarely sharp. It has been found that the lowest practical concentration of reactants with which one can readily use color indicators in very small total volumes is about 0.01 N and that indicators are more favorably employed with somewhat stronger solutions, e.g., 0.02 to 0.03 N. The smallest total volumes that are conveniently titrated by the ordinary capillary buret methods are of the order of 0.05 ml., only a portion of which need be original sample. Thus, a volume of 10 λ may be diluted to 50 λ and titrated without unusual difficulty. Ten microliters of 0.01 N acid would correspond to 0.1 ϵ. This may be considered as the practical lower limit of acid-base titration with color indicators, though somewhat less has been used under special conditions.

When potentiometric end points are utilized, the lower limit of application of acidimetry may be reduced somewhat, but there is little evidence on this point. It is possible to titrate solutions as dilute as 0.001 N, and possibly the total sample may be below

0.1 ϵ. In general it is not simple to use electrodes of adequate size with volumes less than about 50 λ, which corresponds to 0.05 ϵ, when the solution is 0.001 *N*. Here also, considerable diminution in electrode size and total volume may be achieved by special means.

Titration of weak acids in microgram quantities is less satisfactory than titration of strong acids. When an alkaline solution must be kept in contact with air, as will occur close to the end point in titrating a weak acid, absorption of carbon dioxide occurs and obscures the end point. This effect of carbon dioxide on small volumes is enhanced where the ratio of surface to volume is large and is greater in titration dishes than in test tubes or flasks. Special precautions to avoid this error are necessary when the end point is on the alkaline side of neutrality.

Many applications of acidimetry and alkalimetry to actual analyses have been made. The best-known methods are the determination of total nitrogen or ammonia by diffusion or distillation, and the amino nitrogen determination.

Indicators

Color indicators for microgram titrations in acidimetry are the same as those for larger-scale analysis except that certain ones are less satisfactory on the small scale. Phenolphthalein titrations are difficult because of the carbon dioxide effect mentioned above. Another factor of importance is the indistinct end point of some indicators, e.g., methyl orange, which in small volumes does not allow easy recognition of the color change. Titration and drop errors, discussed in Chapter 1, must always be considered because of their very considerable significance in microgram titrations. In all titrations of strong acids and bases, methyl red or a combination of this indicator with methylene blue (1) or another indicator to modify the type of color change observed (2) have been most advantageous. The transition point is slightly on the acid side of neutrality (*p*H about 6), which prevents any notable effect of carbon dioxide absorption, and the color change is very sharp. The titration error with methyl red is small because its color transition takes place very close to neutrality. In most titrations of strong acids and bases which are used in actual analyses, methyl red is the preferred indicator.

Several procedures, e.g., the determination of ammonia, require

back-titration of excess acid. In all these methods the methyl red is mixed with the acid before use in a suitable amount to give a clearly observable color change in the small volumes. By this procedure the amount of indicator is standardized for any given volume of acid, and the correction for drop error remains constant and is completely corrected by a control titration.

Preparation of Solutions

Acids. The most generally useful standard acid for microgram titrations is 0.02 *N* sulfuric acid. Particularly in diffusion analysis, the evaporation of the acid during the diffusion process may cause loss of acid if hydrochloric acid is used. Both hydrochloric and sulfuric acids are made in the usual way from suitably pure reagents. Before standardization, enough methyl red is added to the acid to give a clearly perceptible color when a drop is placed in a titration dish. The acid may then be standardized against a primary standard* or against carefully standardized sodium hydroxide solution, with a 10-ml. buret graduated in 0.02-ml. divisions. For most purposes, the acid need not be accurately standardized, since it is usually added in excess and titrated back with standard alkali solution. In these cases, which include analysis for total nitrogen, urea, ammonia, and amide nitrogen, only the alkali is carefully standardized, and the acid is merely measured carefully in both control and analysis. The indicator-containing acid is kept in a tightly stoppered bottle, usually of 250-ml. capacity, from which it is transferred to a 10-ml. reagent flask to be used as required and discarded when significant evaporation has occurred.

Alkali. Standard sodium hydroxide solution, approximately 0.02 *N*, is the most useful of the various alkalies which may be employed in microgram titrations. For all ordinary analytical purposes, the solution may be prepared by the Sørensen technique (3), i.e., by appropriate dilution of saturated sodium hydroxide solution from which the sodium carbonate has been allowed to precipitate. Freshly distilled water from which carbon dioxide is essentially absent should be used for the dilution. Since saturated solution contains about 72 g. of sodium hydroxide per 100 ml., 11 ml. of this solution diluted to 1 l. will make an approximately 0.02 *N* sodium hydroxide solution.

* Cf. Chapter 1.

This solution is carefully standardized against primary standard,* with a 10-ml. (or larger) buret. In most of the methods in which this alkali solution is used, the accuracy of the analysis is determined largely by the accuracy of this standardization. As with the acid, the stock solution should be stored in a tightly stoppered bottle and used to replenish a small reagent flask which is emptied and refilled from stock at rather frequent intervals. When the solution is stored for considerable periods of time, the inside of the stock bottle should be coated with paraffin.

If the nature of the analysis requires that the alkali be absolutely free from carbonate, a small amount of barium chloride is added to the sodium hydroxide solution before standardization. Any carbon dioxide absorbed from the atmosphere will be precipitated immediately, and, if only the clear solution is used for titration, the absence of carbonate is assured. It must be remembered that the presence of barium carbonate precipitate is a direct indication that some change in normality has occurred, and restandardization of the solution will be necessary at reasonably frequent intervals.

Titration Procedure with Color Indicator

The acid sample, which in most methods already contains the proper amount of indicator, is measured into a clean, dry titration dish as follows. The pipet, which is clean and dry, is fitted into the coupling of a pipet control. With the pipet in the right hand and the thumb and forefinger on the plunger of the control, the tip is immersed in the acid and the plunger withdrawn with rotation until the meniscus of the acid sample is barely past the mark on the pipet. The tip is withdrawn and wiped with a small cotton wad or a piece of absorbent paper such as cleansing tissue. Wiping is performed by wrapping the wipe around the pipet above the tip and drawing it over the end with a rapid stroke so as to leave the meniscus undisturbed. The meniscus is then adjusted exactly on the mark by tapping the pipet tip with the finger, or with a clean cover slip if the material contained in the pipet is hazardous. If the outside of the tip has become wet in this process, it is wiped again as described above, and the sample is transferred to the titration dish. A drop of water is suspended on the tip of a micro wash bottle, and the tip of the pipet is

* Cf. Chapter 1.

inserted in the drop which is drawn completely into the pipet and slightly above the mark, thus rinsing the pipet both outside and inside. The wash water is added to the acid, and the washing is repeated a second time. The drop of water must at all times be less in volume than the capacity of the pipet, so that it may be removed completely. Otherwise, acid on the outside of the tip may be left behind.

With small pipets, either syringe-controlled transfer pipets, self-adjusting, or self-filling pipets, it is usually better to place the droplet of wash water in advance on a block of paraffin or a paraffin-coated surface rather than to suspend it from the wash bottle tip. This allows the use of smaller and more easily controlled drop volumes.

If indicator is not already present in the acid, it is added quantitatively from a 5-λ pipet, with 0.1% or more dilute solution of methyl red. The quantity of indicator is maintained constant in a series of titrations in order to obtain an exact correction for the drop error, which varies with the amount of the indicator.

The rinsed pipet may be used without being dried for a succeeding sample if it is rinsed first with the acid to be measured, all of which is drawn out with an absorbent wipe, leaving the walls wet with the acid but no excess. It is often preferable to dry the pipet after rinsing, particularly when a different solution is to be measured. This is accomplished by inserting the pipet tip into a manifold arm with a rubber connection as shown in Fig. 71, and drawing through it, with vacuum, air which has been passed through a cotton filter attached to the upper end of the pipet as shown. Failure to filter the air may often cause the accumulation of dust which in some methods interferes seriously with the accuracy of the results.

After the acid sample is measured, the capillary buret is filled with standard alkali, the meniscus adjusted to zero, and the buret tip wiped with a wad of cotton or absorbent paper, using a quick stroke that does not withdraw liquid from the interior of the tube. The buret tip and stirrer tip (Fig. 18) are adjusted over the titration table (Figs. 19 and 20) in close proximity and at the same level. The dish containing the sample is placed directly below, and the table is raised until the two tips are dipping into the sample. If insufficient volume of liquid is present to cover

the tips, a drop or more of distilled water may be added. The stirrer is started, and caustic solution is added from the buret at a slow and reasonably constant rate which should require 4 to 5 minutes for complete emptying of the buret. As the end point is approached, a slight color change will be observed near the tip of the buret. Addition is then made with caution until the end-point color (canary yellow when methyl red is used) is just reached, throughout the entire solution. At times the circulation of liquid in the dish is not uniform, and a little of the original color will persist around the edge of the liquid. If this happens, the dish is rotated to provide complete mixing, and the titration is completed. The buret is read immediately, and the scale reading is converted to volume by means of the calibration graph.

The stirrer is stopped, the titration table lowered, and both stirrer and buret tips washed into the dish with a few drops of water. The buret tip must be wiped as described above before it is refilled from the stock solution.

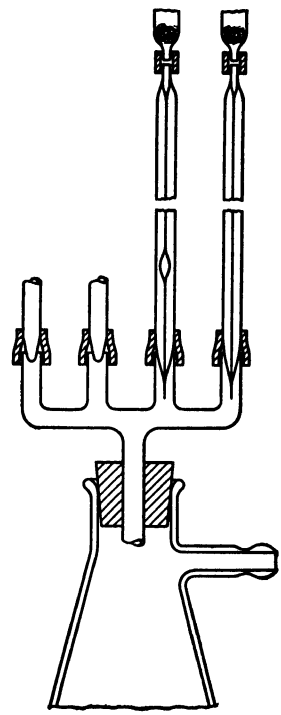


FIG. 71. Drying manifold for pipets.

Titration of Weak Acids

In titration of a weak acid in which the end point is on the alkaline side of neutrality, interference by the carbon dioxide of the air may be significant. Such titrations of microgram quantities are often performed with phenolphthalein as indicator, the procedure essentially as described above. It is necessary, however, to minimize the contact of the alkaline solution with the air, and the latter part of the titration must be carried to completion as rapidly as possible and stopped at the first appearance of pink throughout the entire solution. Fading after that is disregarded, since it is caused by absorption of carbon dioxide. Somewhat greater confidence in the results may be achieved with a device that prevents access of carbon dioxide, such as that shown in Fig. 72. Stirring is

achieved by impinging a stream of carbon dioxide-free gas through the entrance tube on the edge of the liquid in the titration dish. This produces a rotary motion in the liquid which stirs it quite effectively. The gas is most conveniently obtained by passing an air stream through a tower of soda lime or ascarite. Tank nitrogen is quite satisfactory as a gas source, also. Alternative methods require potentiometric equipment, as described below.

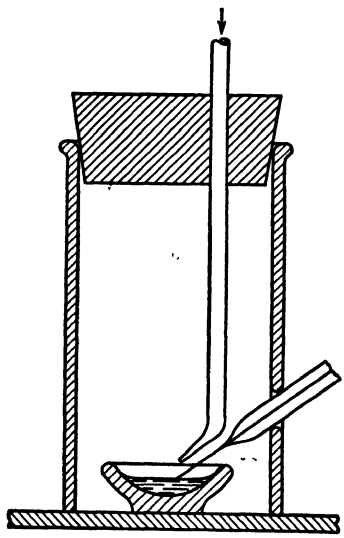


FIG. 72. Hood for titration under inert gas. (Actual size.)

Potentiometric Titration of Acid

Differential Procedure. The sample should contain only strong acid if it is to be determined by potentiometric titration with a quinhydrone electrode in air. If the *p*H at the end point is greater than 8, atmospheric oxidation of quinhydrone takes place to a degree which prevents accurate determination of the end point. Absorption of carbon dioxide in the alkaline region may also interfere seriously.

The sample containing the acid to be titrated is measured into a titration dish as described previously, and the buret and stirrer tips are inserted. The tip of the differential electrode described previously (Fig. 27) is also inserted until the outside electrode is nearly covered. The leads from the differential electrode are attached to a potentiometer of medium accuracy (e.g., Leeds and Northrup student type) attached to a lamp and scale galvanometer, or more conveniently to an electronic instrument such as the Beckman *p*H meter. The Beckman instrument is more satisfactory than a simple potentiometer circuit, because of the relatively high resistance of the differential electrode system described when it is used with dilute electrolyte.

A little quinhydrone is added from the flat end of a toothpick or from a microspatula, and the stirrer is started in order to saturate the solution with the quinhydrone. If the approximate

titration value can be estimated, it is convenient to add about three-quarters of this amount of caustic solution before the potential value is read. If it is not known, the standard alkali is added in increments, usually not more than five small divisions on the buret. After each addition, the potential is observed and recorded. The solution is then expelled from the electrode tube by placing the finger over the end of the rubber tube and compressing the latter several times until the potential is reduced approximately to zero. At the beginning of the titration, the potential observed after each addition will be small and approximately constant. As the end point is approached, the potential rises rather sharply; it diminishes after the end point is passed. With a quinhydrone electrode, few readings may be expected after the end point is passed since the solution is then alkaline and the quinhydrone is subject to serious air oxidation above about *p*H 8.

The potential readings are plotted against the increments of volume added, and it will be observed that there is a sharp peak in the curve in the region of the end point. The apex of this peak is usually taken as the end point, but, as Dubnoff and Kirk (4) have shown, the true end point is one-half increment earlier than the exact maximum in the curve. This difference is not important in the titration of rather large samples but becomes highly significant on the microgram scale, and the end point should always be calculated by subtracting one-half increment from the value of the extrapolated peak of the curve.

The precision and accuracy of the differential potentiometric titration is somewhat better than that obtained with color indicators. With as small amounts of acid as 0.2 ϵ, standard errors could be made as low as 0.3% in a series. With color indicators the standard error with so small a sample is expected to be 0.5 to 1%. Titrations can be carried out with more dilute reagents, which should improve somewhat the above figures, since neither drop nor titration error are involved with potentiometric end points, and the deviation found is due to technical variations only.

Glass Electrode Procedure. For the titration of weak acids particularly, the differential quinhydrone electrode is not suitable, and a glass electrode is definitely to be preferred. This requires a reference cell. If the apparatus shown in Fig. 28 is employed, it is convenient to maintain the solution in a controlled atmosphere

such as carbon dioxide-free air, or nitrogen. Since carbon dioxide from ordinary air interferes to a considerable extent when the solution is basic in reaction, it is desirable to employ a convenient carbon dioxide-free gas. This may be obtained by passing air through soda lime or ascarite, or by using commercial nitrogen from a tank.

The sample containing the acid to be titrated is placed in the cup of the glass electrode shown in Fig. 28, and the cap *c* is placed on the apparatus. The reference electrode, a simple calomel cell of small dimensions (5, 7), is inserted so that the tip is immersed in the sample. The gas stream is turned on through the tube *E* at a rate which is sufficient to spin the solution in the dish. The buret tip is inserted through the port provided, until it is also immersed in the solution. Titration is made by adding increments of standard alkali from the buret and measuring the potential between the glass and calomel electrodes with a standard type of potentiometer circuit or, better, with a Beckman or other *p*H meter. The potentials are plotted against the increments added to give a titration curve of the usual shape. Alternatively, they may be plotted differentially by subtracting successive readings and plotting the potential differences against the increments of solution added. This yields a typical differential curve of the type discussed in the preceding section. The only important application so far published for this type of titration is in the formol titration of amino acids, though other applications to weak acid systems are certainly possible. It should be noted that this is essentially the only potentiometric titration method which is thoroughly suitable for high precision analysis when the *p*H of the end point is in a distinctly alkaline range. Phenolphthalein may be used with considerable success when the titration is performed rapidly to minimize the action of atmospheric carbon dioxide, or when the solution is jacketed and kept in a carbon dioxide-free environment as discussed in a preceding section. Under these conditions, the color indicator method for weak-acid titration is not greatly inferior to the glass electrode and is considerably more convenient.

OXIDIMETRIC TITRATIONS

Oxidimetry is probably more widely applicable in microgram analysis than is acidimetry. The choice of reagents and the

method of use is somewhat different from that of macroanalysis. For example, iodometry, which is particularly valuable for a wide variety of ordinary analyses, has only limited application in microgram analysis. This is because the volatility of iodine in a sample is accentuated by the high surface-to-volume ratio when the sample is small. It is not often possible to release iodine and titrate it with thiosulfate on the microgram scale. On the other hand, it is entirely practical to titrate a reducing agent with a standard iodine solution, an operation which is infrequently employed on the larger scale.

Ceric sulfate is considered the most satisfactory general oxidizing agent for microgram quantities. Not only is the reagent very stable, but the indicators, ferrous *o*-phenanthroline and Setopaline C, are very sensitive and satisfactory. Permanganate and dichromate are useful only occasionally.

Ellis (8), Kirk and Tompkins (9), and, more recently, Solomon, Gabrio, and Smith (10) employed ammonium hexanitratocerate in perchloric acid, which is a very powerful agent capable of oxidizing oxalic acid in the cold. It should be used with nitro-*o*-phenanthroline ferrous sulfate as indicator, as emphasized by the latter authors (10). The relative instability of this reagent as compared with ceric sulfate has been a deterrent to its general application, particularly in microgram analysis.

Of the various reducing agents, ferrous ammonium sulfate (Mohr's salt) probably has the widest utility because of its stability and ease of application in connection with ceric sulfate. Potassium ferrocyanide and some other reducing agents find application in special cases. The oxidation-reduction reagents which are most dependable for a wide range of analysis are ceric sulfate and Mohr's salt. Because of the very sensitive indicators available for oxidation-reduction titrations, the reagents may be diluted more than is possible with acids and bases. In most cases they are used in 0.01 N solution, and under some circumstances it is possible to dilute them even more than this. By comparison, the end points in acidimetry with small volumes are definitely inferior.

Indicators

With ceric sulfate and potassium permanganate titrations, ferrous *o*-phenanthroline (11) is the most common indicator.

Setopaline C (12) finds application for the same titrations but is less well known. It is particularly valuable when the solution at the end point is yellow, as when ferrocyanide is titrated to ferricyanide.

Ferrous *o*-phenanthroline (Ferroin) is made in 0.025 *M* stock solution (11) by dissolving 1.485 g. of *o*-phenanthroline in 100 ml. of 0.025 *M* ferrous sulfate solution made by dissolving 0.695 g. of ferrous sulfate heptahydrate and diluting it to 100 ml. This stock solution is commonly diluted to 0.0025 *M* before use, particularly when it is added from a pipet to an individual sample. If the indicator is added to the reducing agent as described below, it is unnecessary to dilute it. This indicator has a relatively large blank titration, because of the necessity of oxidizing the complex from the ferrous to the ferric state with a color change from brick red to sky blue. Since comparatively large amounts of indicator are used in microgram titrations and an excess of ferrous ion is usually present, this "drop error" will be large and must be corrected. A sample of the indicator may be titrated and a proportional correction made for the amount added in a titration. In many instances it is possible to standardize the conditions of the titration and the volume of indicator in such a way that the correction is applied automatically. For example, if the titration is made by addition of an excess of ceric sulfate and back-titration with Mohr's salt, a separate titration of the same volume of ceric sulfate and the same amount of indicator will involve the same correction. When the two titers are subtracted, the amount of Mohr's salt needed to change the indicator will be the same in both and will cancel out in the remainder.

Ferrous *o*-phenanthroline may not be added to ceric sulfate stock solution as is done in acidimetry with methyl red. The indicator under these conditions is destroyed. It may, however, be added to the Mohr's salt solution in which it is stable. If this procedure is followed, the Mohr's salt will require restandardization after addition of the indicator. The small variations in titration value of a given amount of ceric sulfate will not be sufficient to cause measurable variations in the indicator blank, since the ferrous phenanthroline complex is now essentially a part of the reducing agent and included in the standardization value.

Setopaline C is prepared in 0.1% aqueous solution. The solution has a dark-blue color initially, and during titration it changes

from yellow to bronze when oxidized with ceric sulfate. It is stable only in dilute ceric sulfate for a short time and is not well suited to back-titrations of that material with a reducing agent. It is most applicable when ferrocyanide is titrated directly with ceric sulfate, as in the determination of glucose. The color change is then from lemon yellow to bronze and is very sharp and distinct in a yellow solution such as ferricyanide. This indicator exhibits practically a zero drop error under all circumstances tested, in contrast to ferroin. When used with permanganate instead of ceric sulfate, it must be added just before the end point is reached. The color change is then from blue-green through lemon yellow to bronze. Similarly, it has been employed in titrations of oxalate with hexanitratocerate (9). The end-point change is then from yellow to red, passing through a bronze that fades to yellow as the end point is approached. The final red color has been found most suitable as the end point.

Setopaline C is relatively unstable and may be used only for a few days without renewing or regenerating the solution. Regeneration consists of heating the indicator on the steam bath for about ½ hour, after which it behaves like a fresh preparation. This may be done repeatedly. The indicator is usually added to the solution to be titrated by means of a small (e.g., 5-λ) pipet, in order to keep the amount constant. Since there is a negligible blank, it is actually important to do this only in order to produce a uniform color of end point, rather than to standardize and correct a drop error.

Other color indicators that have been reported for oxidation-reduction titrations with ceric sulfate and other strong oxidants are Alphazurine FG and GG (13), erioglaucine and eriogreen (14), ruthenium dipyridyl (15), methylene blue (16), and diphenylamine or its sulfonate (16). The application of these indicators on the microgram scale is as yet unreported in the literature, and, in fact, some of them are almost certainly not satisfactory, though others may well be.

Preparation of Solutions

Ceric Sulfate Solutions. Ceric sulfate solution in sulfuric acid, also designated as sulfatoceric acid, requires special precautions when being prepared for microgram titrations. It is a curious fact that a solution that gives normal end points and

titration values with rather large amounts (e.g., 10 ml.) at times fails to give a correct end point or titration value with amounts of 10 to 100 λ. It has also been observed that the solution that is 0.1 *N* will be entirely dependable, and, when diluted to 0.01 *N*, it may no longer be satisfactory even with milliliter titration volumes.

Despite these difficulties, it is possible to specify conditions of preparation that yield perfectly dependable solutions which may be employed in 0.01 *N* or more dilute solution, and in volumes as small as can be conveniently dispensed with capillary equipment. In fact, properly prepared ceric sulfate solutions may be considered as perhaps the most valuable and dependable reagent available to the microgram analyst. Before the reagent is made, all water and sulfuric acid to be used are redistilled from an all-glass distilling apparatus. Concentrated sulfuric acid offers little difficulty in redistillation with the proper type of equipment. A standard distilling flask of about 50- to 100-ml. capacity containing a ground-glass stopper has sealed to it a glass air condenser about 2 ft. in length. Sulfuric acid distilled in this vessel will bump and fume before reaching its correct boiling point, owing apparently to its water content. After all the water is removed, the acid distills smoothly and rather rapidly.

Stock Solution, 0.1 N. For the preparation of 1 l., approximately 65 g. of anhydrous ceric sulfate is weighed and placed in a 600-ml. beaker. To this is added 60 ml. of concentrated redistilled sulfuric acid. To this is added *cautiously* 50 ml. of redistilled water, and the solution is digested close to the boiling point over a low flame for about ½ hour. More redistilled water is then added cautiously, a little at a time with stirring, until the solid is all or practically all dissolved. The solution is cooled and decanted carefully into a 1-l flask, with care not to transfer any of the undissolved solid which may remain, and made up with redistilled water to approximately 1 l. All measurements are approximate because of the variation in composition of commercial ceric sulfate preparations.

The solution, prepared as described, will usually be satisfactory as stock solution for the preparation of more dilute titrating reagent. The dilution to 0.01 *N* or other solution of suitable strength is made with approximately 2 *N* sulfuric acid which is

prepared from redistilled water and acid as described. After dilution, the solution must be tested for microgram titrations. The end point with ferrous *o*-phenanthroline should be sharp and definite. If it is not, the diluted solution is placed on a steam bath and left overnight or from 10 to 12 hours, after which it will nearly always yield excellent results. The same treatment applies if the solution through slight contamination, fails to yield sharp and definite end points at a later date. Standard ceric sulfate solutions must always be stored in an all-glass vessel. Any organic matter, as from dust, corks, rubber stoppers, or tubing, must be prevented from contacting the solution. The diluted solution is standardized as described below.

In the preparation of ceric solutions, the preferred starting material is anhydrous ceric sulfate. Ceric ammonium sulfate has not been successful for microgram titrations despite numerous attempts. Ceric oxide may be used, but it must be converted to sulfate before it is dissolved, making the procedure rather difficult.

Standardization of Ceric Sulfate Solutions. Ceric sulfate solution may be standardized by any one of several methods and with a variety of primary standards. Regardless of the primary standard chosen, the titration should be made on a larger scale than that for microgram titrations, chiefly because greater accuracy is attainable and solid samples of primary standards may now be weighed and titrated directly. The standard may be put in solution and the sample measured volumetrically, but, if this is done, most of the solution is wasted and may better be used with larger equipment for the sake of obtaining extra accuracy. The same considerations apply to the standardization of other oxidizing and reducing agents.

Oxalate. Sodium oxalate of primary standard purity or oxalic acid in a comparable state of purity are convenient standards for ceric sulfate standardization. In solution, both reagents show some instability and should be made fresh before use. A sample of about 0.1340 g. of pure, dry sodium oxalate (rational equivalent weight, 66.98) is weighed with the maximum accuracy of the analytical balance and dissolved to make exactly 200 ml. of 0.0100 *N* solution which is used immediately to standardize 0.01 *N* ceric sulfate. If 0.100 *N* ceric sulfate stock solution is to be standardized, 0.6698 g. of sodium oxalate weighed in air without

a buoyancy correction and dissolved to make exactly 100 ml. yields an exactly 0.1 *N* solution.

Oxalate standardizations require also a ferrous ammonium sulfate solution made as described below and of about the same strength as or slightly stronger than the ceric sulfate solution. For the standardization, two identical 10-ml. samples of ceric sulfate are measured carefully into titration flasks of about 50-ml. capacity. The first is titrated immediately with ferrous ammonium sulfate solution added from a calibrated 10-ml. buret graduated in divisions of $\frac{1}{50}$ or $\frac{1}{100}$ ml. A drop of 0.025 *M* ferrous *o*-phenanthroline is added shortly before the end point. This titration, when carried out in duplicate or triplicate, establishes the ratio of normalities of ceric sulfate and Mohr salt solutions. To another identical sample of ceric sulfate in another titration flask is added a carefully measured sample of the sodium oxalate solution, somewhat less in quantity than the ceric sulfate. The solutions are mixed and heated for 5 minutes at 50° C. The excess ceric sulfate is then titrated back with the Mohr salt solution as before, with a drop of ferrous *o*-phenanthroline as indicator. The difference between the titration values when oxalate is present and absent is equivalent to the oxalate oxidized and serves to yield the normality of the ferrous ammonium sulfate in terms of the oxalate standard. From this the normality of the ceric sulfate is calculated on the basis of the first titration.

Ferrocyanide. Another valuable primary standard is pure potassium ferrocyanide trihydrate, $K_4Fe(CN)_6 \cdot 3H_2O$ (rational equivalent weight, 422.15), used in solution as described subsequently. Ceric sulfate solutions are standardized against this reagent by titration of a standard ferrocyanide solution directly, with Setopaline C as indicator. A 10-ml. buret is convenient for this titration, as discussed in the paragraph above.

Ammonium Hexanitratocerate Solution. This solution is a considerably more vigorous oxidizing agent than ceric sulfate when made from ammonium hexanitratocerate dissolved in 2 *N* perchloric acid (17). It is, for example, capable of oxidizing oxalates directly and rapidly without a catalyst, which is not the case with ceric sulfate. The reagent is quite difficult to maintain in stable condition and is not simple to prepare. If the solid ammonium hexanitratocerate is dissolved in 2 *N* nitric acid, its strength is somewhat less than in perchloric acid, but it is still

greater than that of ceric sulfate (sulfatocerate). The stability in nitric acid is probably somewhat greater also than in perchloric acid. A liter of 0.1 *N* solution would require 202.1 g. of salt which is dissolved in 2 *N* perchloric or nitric acid. It has been found difficult to stabilize the solution without considerable loss of strength (9), though Smith, Sullivan, and Frank (17) claim that the solid salt may be used as a primary standard in oxidimetry. Ellis (8), Kochakian and Fox (18), Tompkins and Kirk (9), and particularly Solomon, Gabrio, and Smith (10) used hexanitratocerate in perchloric acid for direct titration of oxalate with satisfactory results. With catalysts, such as manganous ion, the superior stability of ceric sulfate can be realized without serious increase in difficulty of technique or loss of time. Ferrous *o*-phenanthroline may be the indicator for titration with either hexanitratocerate or hexaperchloratocerate. The indicator, ferrous nitrophenanthroline, which changes color at a higher oxidizing potential (1.25 v. versus 1.14 for ferroin) has been particularly recommended for use with these reagents (10, 19) because of the more satisfactory end-point potential.

Other Oxidizing Agent Solutions. Permanganate, dichromate, and other oxidizing agents have been little used in microgram analysis. So far as known, preparation for such specific purposes is not different from preparation for their more common applications. Potassium permanganate (9) may be employed for determination of oxalate; it will also titrate ferrous salts and similar reducing agents on the microgram scale. With potassium permanganate, the usual precautions such as complete preliminary removal of manganese dioxide from the solution by filtration through sintered glass and storage in the complete absence of organic material are essential. It should be noted that permanganate requires an indicator for microgram titrations because the excess required to produce a visible coloration of the small volumes employed gives an excessively high drop error.

Iodine solutions are occasionally employed in microgram analysis. Their preparation involves no unusual precautions, though the stability of such solutions is always poor. A very convenient preparation is carried out by using the very stable potassium iodate solution, which has been carefully standardized, to generate iodine solutions of known strength as needed. Addition of a measured quantity of the iodate solution to an excess of mildly

acidified potassium iodide with subsequent adjustment of the volume allows accurate iodine solutions to be prepared in small amounts from day to day. At times, it is possible to develop the iodine in the solution being titrated by using potassium iodate as titrating reagent rather than an iodine solution. The material being titrated must be slightly acid in reaction and must contain an excess of potassium iodide. This technique is satisfactory if no side reactions of iodate with the reducing agent take place. It can, for example, be performed in the presence of thiosulfate ion.

Reducing Agent Solutions. *Ferrous ammonium sulfate, 0.1* N. This solution is prepared by dissolving 39.2 g. of high-quality ferrous ammonium sulfate hexahydrate in approximately 0.1 *N* sulfuric acid. The latter is prepared by diluting 3 ml. of concentrated sulfuric acid to 1 l. with redistilled water. The ferrous ion is relatively stable at this acidity, undergoing little air oxidation and no appreciable hydrolysis. Oxidation of stock solution may be avoided completely by storing the solution under illuminating gas or nitrogen. The smaller quantities which are removed from time to time for use in titrations may be kept in air for a few days and renewed frequently from the stock solution. Since the common strength for microgram titration is 0.01 *N*, the stock solution is diluted with nine parts of redistilled water. Alternatively, 0.01 *N* stock solution may be used, in which event it definitely should be kept under illuminating gas.

Potassium ferrocyanide solution, 0.1 N. A solution of 42.215 g. of primary-standard-grade salt, weighed in air without corrections, in 1 l. of solution, gives a normality of exactly 0.1 without standardization. Potassium ferrocyanide trihydrate of suitable purity may be prepared by recrystallizing the salt from water two or three times and drying it to constant weight over wet calcium chloride. Alternatively, the salt may be dried over an aqueous solution saturated with sodium chloride and sucrose. The dried material should be kept in a tightly stoppered container to prevent changing the water of hydration resulting from variations in humidity of the air. A solution made from this salt should be alkalinized with about 0.2 g. of sodium carbonate per liter and stored in a dark bottle. A fresh solution is preferable for each standardization, thus avoiding the necessity for storage. If primary-standard-grade material is not available, the weighing

is made only approximately and the solution standardized against standard ceric sulfate solution.

Titration Procedure with Color Indicator

The technical details of oxidimetric titrations are in nearly all respects identical with those of acidimetric titrations. Unlike the latter, either reagent may be added from the buret without difficulty from atmospheric carbon dioxide. The indicator may be added to the reducing agent as described above, but not to the oxidizing agent. Alternatively, it may be measured from a small pipet into each sample being titrated. The reagents may be more dilute than in acidimetry because of the superior end points obtained.

Potentiometric Titration Procedure (Differential Electrode)

The differential potentiometric titration method discussed earlier for acidimetry was actually applied to the quinhydrone oxidation-reduction system. Other oxidation-reduction systems may be titrated in an analogous manner. Instead of the addition of quinhydrone to establish the oxidation-reduction system, the reacting materials may yield their own potentials which are used for measurement of end point. Few applications of small-scale potentiometric oxidimetric titrations have been described. Dubnoff and Kirk (4) employed a differential potentiometric end point in the titration of ferric ion with titanous ion. Owing to the necessity of rigid exclusion of oxygen in any system containing titanous ion, the arrangements were much more complicated than those described in the above section for quinhydrone electrodes. The details are given in connection with reductometric iron determination.

A simple differential electrode is applicable to titrations involving ferrous-ferric, cerous-ceric, and other similar reversible systems. It may not be employed dependably with permanganate, dichromate, hydrazine or hydroxylamine, oxalate, or other oxidizing and reducing agents which give rise to irreversible systems. It is often possible to titrate an irreversible reducing agent with a reversible oxidizing agent, or vice versa, since the potential produced is a direct function of the ratio of reductant to oxidant in the reversible system.

It is probable that the future will see many applications of

potentiometric methods to oxidation-reduction systems containing only microgram quantities. At present, the direct experimental evidence is very limited.

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CHAPTER 6

Metallic Constituents
Titrimetric Methods

Methods have been developed for the titrimetric determination of microgram quantities of several common metallic ions. With a few exceptions, these methods are at least as sensitive as any colorimetric procedure described for the elements in question and considerably more accurate. At times they involve a more complex procedure than corresponding colorimetric methods, but they remain completely practical in operation. Quantities of somewhat less than 1 γ of sodium, potassium, calcium, and iron can be determined with an accuracy which is in general better than 1%. Except for iron, the alternative colorimetric methods are not completely satisfactory, at least for this range of operation. The three elements, sodium, potassium, and calcium, each require separation by means of quantitative precipitation and final determination by titration of the precipitate formed. These methods are applicable to all types of sample when the initial preparative stages of the analysis are adapted properly.

SODIUM DETERMINATION

The titrimetric determination of microgram quantities of sodium is a comparatively elaborate procedure. It is also one of the most delicate methods so far developed for metallic constituents, being capable of determining as little as 0.1 γ of sodium with an accuracy approaching or equaling that of the corresponding milligram methods. Chemical methods for sodium determination nearly always depend on precipitation and estimation of the triple acetate of sodium and uranyl with zinc (1), magnesium (2), or manganese (3). The microgram procedure (4) is no exception, being based on a proper adaptation of the precipitation of sodium zinc uranyl acetate, which is separated by filtration and determined by reduction and oxidimetric retitration of the uranyl contained in the precipitate. With amounts

of sodium in excess of 1 γ, the error may be kept less than 1%. With proper arrangements to control interference, the method is applicable to nearly all types of samples.

Lindner and Kirk (4) developed the microgram method described below and obtained the results summarized in Tables 10 and 11 with known sodium salt solution and with blood respectively.

TABLE 10. SODIUM CHLORIDE SOLUTION

Number of Determinations	*Average Sodium Found*	*Sodium Calculated*	*Standard Error**	
	γ	γ	γ	%
5	4 127	4 13	0 0030	0 07
8	2 509	2 51	0.0033	0.13
8	0 979	0 98	0.0023	0 23
5	0 520	0 52	0 0032	0 62
5	0 128	0.13	0.0041	3.18

* Calculated from the formula $S = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

TABLE 11. BEEF BLOOD PLASMA

Diluted (1:100) Plasma Sample	*Added Sodium*	*Number of Determinations*	*Average Sodium Found*		*Standard Error*	
λ	γ		γ	Mg/l. Original Blood	Mg/l.	%
26.63	..	3	0 877	3292	10 7	0.3
..	0 52	3	1 393	3277	19 6	0.6
....	1.105	3	1 980	3284	18 1	0.6
64.73	..	3	2.134	3297	6.2	0 2
....	0.52	3	2.632	3262	33 6	1.0
....	0.98	3	3.117	3302	4.7	0.1
....	....	..	Mean	3286	7.0	0.2

Principle of Method

Samples containing organic material may be treated by ashing or acid digestion to eliminate organic interference. This is necessary with most biological materials or with samples that contain relatively large amounts of organic acids or proteins. The latter constituents must be completely absent. Phosphate requires complete separation, and, if large amounts of potassium are present, the quantity of this element must be reduced to an amount less than that of the sodium. Sodium is precipitated as

sodium zinc uranyl acetate, with a precipitation method modified from that of Barber and Kolthoff.

The formation of minute amounts of the precipitate obtained from microgram quantities of sodium is very slow, requiring up to 24 hours for its quantitative formation. The precipitate is separated by a filter stick, washed, dissolved, and determined oxidimetrically. The uranyl ion is reduced to uranous ion (IV) by means of metallic cadmium and titrated back to the six-valent state with standard ceric sulfate solution. Ferrous *o*-phenanthroline serves as indicator.

Reagents

Several precautions are necessary in preparing reagents because of the likelihood of contamination with minute amounts of sodium from the finger tips and from impure chemicals, as well as with sodium dissolved from glass apparatus. All water used in making reagents must be redistilled from an all-glass apparatus.

1. *Zinc uranyl acetate solution* (1) is prepared in two parts: (*a*) Uranyl acetate dihydrate, 10 g.; 30% acetic acid, 6 g.; water to make 65 g. (*b*) Zinc acetate trihydrate, 30 g.; 30% acetic acid, 3 g.; water to make 65 g.

Each solution is warmed separately until the salt is completely dissolved and the solutions are combined. After standing 24 hours, the reagent is filtered through a sintered-glass filter covered with a layer of fine asbestos, or through a $\frac{5}{3}$ grade of Jena or equivalent Pyrex sintered-glass bacteria-proof filter without asbestos. If any visible turbidity appears in the solution on standing, as may happen from solution of sodium from the glass, the reagent must be refiltered. The reagent is best stored in a plastic bottle, e.g., Lucite, or in a paraffin-lined glass bottle to prevent solution of sodium from the glass.

2. *Wash solution.* Ninety-five per cent alcohol is saturated with sodium zinc uranyl acetate and filtered before use as described under 1.

3. *Asbestos.* Italian medium-fiber washed and ignited asbestos is ground in a mortar until no coarse strands remain. It is treated in 1 to 2 *N* sulfuric acid suspension with successive small portions of ceric sulfate solution until the suspension assumes a persistent faint yellow color. It is then washed with 1 to 2 *N* sulfuric acid solution and finally with water and stored in an all-glass con-

tainer. The asbestos may be washed by decantation or, better, by passing the liquid through a coarse sintered-glass filter.

4. *Five per cent sulfuric acid solution,* made by diluting 5 g. of redistilled sulfuric acid with redistilled water to make 100 ml.

5. *Standard ceric sulfate solution,* approximately 0.01 *N.** This should be standardized against primary-standard-grade sodium oxalate or potassium ferrocyanide.

6. *Indicator solution.** *o*-Phenanthroline ferrous sulfate, 0.0025 *M*. Aqueous Setopaline C, 0.1%, may be substituted.

7. *Standard sodium chloride solution,* made from carefully purified and dried salt. A convenient strength may be made by dissolving 0.254 g. of the salt in redistilled water to make 500 ml. of solution. This will contain 2 γ of sodium in 10 λ. Reagent-grade salt dried for several hours at 130° to 150° C. is satisfactory.

8. *Cadmium spirals,* made by cutting a thin ribbon from stick cadium on a lathe. The spirals are flattened on the bottom, forming a disk with a total surface of about 2 $\overline{\text{cm.}}^2$ and with one end extended upward and serving as a handle.

Procedure 1. In Absence of Interfering Materials

Solutions with no phosphate or protein and with only traces of organic acid or small amounts of potassium are analyzed as follows.

A porcelain titration dish which has been carefully cleaned with sulfuric-chromic acid mixture and rinsed thoroughly with distilled water without contact with the fingers is employed as precipitation vessel. The dish may be held on a glass fork or with special forceps. Otherwise, sufficient sodium may be introduced from the finger tips to invalidate the analysis. The sample, which may contain 0.5 to 10 γ of sodium, is measured into the clean dish with a capillary pipet which is rinsed as usual. The sample is evaporated carefully over a micro hot plate while being stirred with the magnetic thread stirrer (Fig. 18) until just short of dryness. While the sample is still warm, 1 ml. of zinc uranyl acetate reagent is added, stirred, and warmed gently for about 5 minutes. The dish is then removed from the hot plate and placed under a Petrie or evaporating dish which also covers a dish of water. These precautions prevent evaporation and entrance

* Cf. Chapter 5, Oxidimetric Titrations; also Chapter 1.

of dust. The sample is allowed to stand for 12 to 24 hours to complete the precipitation, which may be very slow with minute amounts. During this time of standing, it is very essential that the volume in the dish should not be reduced by evaporation which will allow reagent to crystallize with the sodium zinc uranyl acetate.

After precipitation is complete, the supernatant solution is removed through a sintered-glass filter stick coated with fine asbestos.* The dish and precipitate are washed thoroughly with the alcohol wash solution, and the asbestos is removed to the dish with a drop of 5% sulfuric acid which also washes the end of the filter. The filter is also washed with a little water to remove any residue of dissolved precipitate.

The uranyl ion is then reduced by placing the dish on a micro hot plate, inserting a cadmium spiral, and warming while stirring with the magnetic thread stirrer for about 5 minutes. The cadmium spiral is removed and washed thoroughly with a little redistilled water, and the dish is allowed to cool about 5 minutes, after which the sample is ready for titration. Standard ceric sulfate is employed in the usual manner with a measured quantity of ferroin indicator. A blank must be run under identical conditions but with no added sodium. The titer of the blank is subtracted as a correction.

The end point of the titration may be reached slowly because of the low rate of reaction of ceric ion with uranous ion. If this difficulty is serious, it may be avoided most easily by adding a measured excess of ceric sulfate solution and titrating it back with standard ferrous ammonium sulfate solution. The content of uranyl ion in the precipitate has been found to parallel the sodium content quite closely, even with the most minute samples. This may not be true of the weight of the precipitate or possibly of its other constituents. It is known that the water of crystallization shows a tendency to be variable and thus to narrow the useful range of application of the gravimetric methods based on this precipitate (1).

Procedure 2. Samples Containing Organic Interference Only

Occasional biological materials may contain organic acids, proteins, or other organic interfering materials which must be

* Cf. Chapter 4, Filtration.

destroyed, but not phosphate or large amounts of potassium (greater than the amount of sodium). Such samples are ashed in a small platinum dish in a micro muffle furnace at 450° C. Most materials ash very rapidly when the sample is very small, though occasional samples may require an hour or more at this temperature to burn off all carbon residues. The clean ash is dissolved in a minimal quantity of dilute (about 0.1 *N*) hydrochloric acid, transferred by means of a pipet, with rinsing, to a porcelain titration dish, and analyzed as in Procedure 1.

Procedure 3. In Presence of Organic Matter and Phosphate

Most samples of biological material contain both organic interference and phosphate but insufficient potassium to cause difficulty. This is true of blood and some urines. Samples of this type are ashed as described in Procedure 2, the ash dissolved in about 50 λ of 0.1 *N* hydrochloric acid, and transferred with rinsing by means of a capillary pipet of about 0.1-ml. volume to a centrifuge cone (0.2 ml.). With a microspatula or toothpick, a little solid calcium hydroxide is added (5) and mixed thoroughly by agitation of the cone. After the sample stands for 2 to 4 hours, a drop of water is added and the tube centrifuged in the air-driven microcentrifuge until the precipitated calcium phosphate is well packed. The supernatant solution is transferred quantitatively with washing of the precipitate to a porcelain titration dish by means of a capillary transfer pipet. It is evaporated on the hot plate and analyzed as described in Procedure 1.

Samples containing large amounts of potassium may be treated with perchloric acid or saturated ammonium perchlorate solution in a similar manner. Since a liquid is added, the volumes must be measured and allowance made for the dilution. The precipitation of potassium under such conditions is not quantitative, but the amount is reduced to a point at which it no longer interferes (5). Evaporation of the sample nearly to dryness before the perchlorate is added improves the yield. Since it is very seldom that a sample contains enough potassium to cause interference, this treatment is usually unnecessary.

Remarks

It was shown by Lindner and Kirk (4) that the reduction of uranyl ion involved a slow rate of reaction under certain conditions and the possibility of hydrogen peroxide formation. Amalgamated metal re-

ductors in general are less rapid than metallic cadmium or zinc and lead to greater formation of hydrogen peroxide. The reduction is appreciably accelerated by the application of raised temperature. The formation of trivalent uranium in the reduction probably takes place to a slight extent, as has been shown to occur with larger samples, but constant stirring of the small volume of solution in the presence of air prevents any measurable amount of uranium (III) from accumulating. The additional difficulty which may be encountered is due to the slowness of the reoxidation to uranyl ion. In determining sodium, the titration is carried out under optimum conditions for obtaining correct results because of the standardized system and the absence of interfering materials. The direct determination of uranyl ion in a complex solution, as in the ordinary determination of uranium, is usually less satisfactory with this procedure because of the presence of interfering materials and the variability of the conditions of the titration.

Another possible source of error lies in the slight variability of composition of sodium zinc uranyl acetate when it is precipitated from media of different concentrations. The original gravimetric method of Barber and Kolthoff (1) was severely restricted in its range of application because of this variability. It has been shown, however, that the ratio of uranyl ion to sodium ion in the precipitate is independent of this effect over a very wide range of concentration. The greatest care is necessary to avoid introduction of sodium from perspiration on the finger tips. At no time prior to the titration must the dish or any other object which comes in contact with the solution be handled directly with the finger tips. For this reason, a fork made from glass rod is recommended for handling the dish. In addition, the analyst must exercise scrupulous care to avoid contamination of stirrers, buret tips, or other equipment which is in contact with the solution.

POTASSIUM DETERMINATION

The determination of potassium on amounts as small as 0.7 γ and in the presence of twenty times as much sodium was accomplished by Cunningham, Kirk, and Brooks (6). The method depended on the precipitation of potassium as the chloroplatinate, the chloride content of which was determined volumetrically. The somewhat more sensitive method which depends on precipitating a double salt of potassium with sodium, silver, or other cobaltinitrite is probably not well suited to the accurate determination of microgram quantities of potassium. Although no complete study has been made of the cobaltinitrite method in the very lowest ranges, it is well known that the precipitated

double salt of potassium cobaltinitrite varies in composition, depending on the content of sodium and other constituents of the liquid phase in the sample (7). The fact that the most critical portion of all microgram analytical methods which utilize precipitation is the reliability of composition of the precipitate makes it unlikely that dependable results would be obtained with the cobaltinitrite method.

Principle of Method

Potassium is determined by a modification of the milligram procedure described by Bullock and Kirk (8), in which potassium is separated in the form of chloroplatinate, the latter being determined by argentimetric titration of the chloride contained in the precipitate, after reduction of the platinum. In order to determine the minute amounts of chloride involved in the microgram scale of analysis, it was necessary to resort to potentiometric titration instead of color indicators, as used by Bullock and Kirk. The precipitation also requires alterations in the standard technique in order to yield quantitative results with the small amounts employed. This is carried out in 80% alcohol with filtration, instead of evaporation to dryness and washing away of the other constituents with a wash solution as in the milligram procedure.

Reagents

1. *Chloroplatinic acid,* 4% in 80% alcohol. This reagent should be made in small lots since it is not indefinitely stable and may deteriorate after a week's use, owing to reduction of the platinum by reducing substances which form in the alcohol on standing.

2. *Ethyl alcohol,* 80% by volume, saturated with potassium chloroplatinate. An excess of the finely divided potassium chloroplatinate is added to 80% alcohol and the mixture shaken occasionally for 12 hours, after which it is centrifuged to separate all suspended material. The clear supernatant solution is removed carefully to avoid disturbing the precipitate and is used for wash solution. The solution deteriorates on standing and should not be kept for more than about a week. The remaining precipitate is saved to saturate later lots of wash alcohol.

3. *Ethyl alcohol,* 95%, saturated with potassium chloro-

platinate. This wash solution is made as described for 80% alcohol wash solution, but with 95% alcohol.

4. *Sodium formate solution,* 0.2 *M,* prepared by dissolving 1.36 g. of pure sodium formate in redistilled water.
5. *Ethyl alcohol solution,* 80% by volume.
6. *Standard silver nitrate solution,* 0.01 *N.*
7. *Sulfuric acid,* 1 *N* in redistilled water.

Special Apparatus

Silver-silver amalgam electrodes (6) of a modified Clark (9) type, as shown in Fig. 29, are used. The potential is measured by means of a standard potentiometer circuit.

A water bath consisting of a 100-ml. beaker fitted with a metal cover drilled to receive small test tubes (8 by 20 mm.) is shown in Fig. 73.

Procedure with Samples Containing Only Inorganic Potassium Salts

The sample, containing from 0.5 to 10 γ of potassium and free of ammonium ions, is accurately measured in the usual manner into a small test tube 8 by 20 mm. and evaporated by being heated in the boiling water bath. After dryness is reached, the tube is removed and 80% alcohol is added in sufficient quantity to dissolve the potassium salts. The tubes should be stoppered to prevent evaporation while the precipitate is being dissolved. The potassium is precipitated from the alcoholic solution by adding a volume of 4% chloroplatinic acid in 80% alcohol equal to the volume used to dissolve the salts. The tubes are stoppered and rotated to mix the solution and are allowed to stand 12 hours at room temperature to complete the precipitation. The excess reagent is then filtered from the potassium chloroplatinate precipitate with a filter stick prepared as shown in Fig. 74. The filter is dipped into an asbestos suspension, and a layer 1 mm. thick is applied by suction. This is pressed into place with the finger nail. A second layer of fine asbestos is deposited by reinserting the filter stick into the washed and purified asbestos suspension, made as described previously,* until the cone is about 2 mm. in thickness. Filtration is carried out by inserting only the tip of the filter pad into the solution

* Cf. Sodium Determination, Reagents.

so that no precipitate ever reaches the glass portion of the filter stick. The precipitate is washed twice with 50 λ of 80% alcohol and once with 50 to 100 λ of 95% alcohol, both of which are saturated with potassium chloroplatinate. The filter pad is then removed to a porcelain titration dish. Precipitate remaining in the test tube is also transferred to the same dish by dissolving it in a small volume of warm water, the transfer being accomplished by means of a capillary transfer pipet. The tube and pipet are rinsed twice with a few drops of warm water.

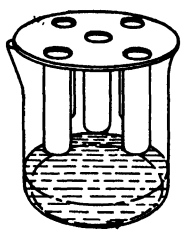


FIG. 73. Water bath arrangement for potassium analysis.

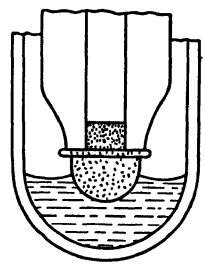


FIG. 74. Filter arrangement for potassium chloroplatinate separation.

The platinum in the precipitate is reduced by adding to the dish a drop of 0.2 *N* sodium formate and evaporating it to dryness on a steam bath. After it cools, a few drops of water are added to dissolve the chlorides, and the solution is acidified with two drops of 1 *N* sulfuric acid. The titration of the chloride is carried out exactly as described in Chapter 7, Chloride Determination.

For each atom of potassium there are three chloride ions in the molecule of potassium chloroplatinate. Therefore, the number of microequivalents of chloride must be divided by 3 to obtain the microequivalents of potassium. The results must be corrected by a blank determination carried out in the same manner as the analysis. The blank value found by Cunningham, Kirk, and Brooks was equivalent to about 0.05 γ of potassium. It originated from a little potassium chloroplatinate in the wash solutions and from the sodium formate which was not completely free of traces of Cl^-. In determining the blank it is necessary to keep the volume of solution as small as practical during the

titration because of the very low concentrations involved. This procedure may be applied in the general analysis of biological materials, at least until the ratio of sodium to potassium is 20. With amounts greater than 2 γ of potassium the error should not exceed 0.5%, and down to 0.7 γ it should not be greater than about 3%.

Procedure with Biological Materials

In order for a method to be of value in analysis of biological materials, it must (1) effectively determine potassium in the presence of a variable amount, and often a large excess, of sodium salts, and (2) include means for elimination of all interfering organic materials without loss of potassium. The second point has led to considerable difficulty on all scales of analysis, and many of the published methods are deficient with respect to the first.

The elimination of organic matter, chiefly protein, from the sample has led to a wide diversity of opinion and technique. The sample is often ashed, usually at temperatures ranging up to 650° to 700° C. Some investigators find that potassium is lost to some extent at this temperature (10). A multiplicity of wet digestion methods have been recommended. These are always time-consuming, even with small samples. Various deproteinization methods have been used (11), and some analysts have adopted the unwise course of precipitating potassium in the presence of protein, as in serum. Although any of the destruction or removal methods may apply under certain specified conditions and with all the necessary precautions, it should be emphasized again that with very small samples no procedure for destruction of organic material is so simple and rapid as ashing. No loss of potassium was detected (6) when the ashing temperature was not allowed to exceed about 400° C., though Linderstrøm-Lang (12) records a slight loss of alkali halides at 330°. At 400° a clean ash may be obtained with small samples in small platinum crucibles heated in a micro muffle furnace. The thinness of the dry layer combined with ready access to air allows this relatively low temperature to burn away organic matter in such samples as blood serum without difficulty. It cannot be stated positively that all types of biological sample yield to this treatment. After ashing, the ash is dissolved in a

little 0.1 *N* hydrochloric acid and subjected to analysis as described for simple solutions above. No comparative study of methods is available for destroying organic material for potassium analyses on the microgram scale.

It has been proved repeatedly that potassium chloroplatinate is the most reliable precipitation form when the amount of other metallic constituents is high or variable. Sodium variation, particularly, causes wide fluctuations in composition of potassium sodium cobaltinitrite. The corresponding silver cobaltinitrite (13–15) is more uniform in composition and less soluble, but it also suffers from variations in composition and from the fact that silver also precipitates halide ions and may coprecipitate a variety of other ions such as phosphate. The potassium salts of perchloric acid, dipicrylamine, tartrate, perrhenate, and picrate are more soluble than is the chloroplatinate. It is probable that microgram analyses can at times be profitably carried out with one or more of the above reagents, usually colorimetrically (16), but their advantages for volumetric analyses are at the very least considered dubious.

Other Methods

Brief mention was made of the use of cobaltinitrite precipitation for the determination of microgram quantities of potassium. Methods based on this principle have not been adapted to accurate volumetric determinations in the range covered by the chloroplatinate method and are unlikely to be so satisfactory, because of the variability of composition of the precipitate (7). Effects of this kind are likely to be greater with microgram samples than with larger amounts. Several colorimetric methods for comparatively small amounts of potassium have been described.

Another principle employed for the final determination of potassium chloroplatinate which has been reasonably successful depends on the conversion of potassium chloroplatinate to potassium iodoplatinate by treatment of the former precipitate with potassium iodide in neutral solution. The resulting iodoplatinate may be titrated with sodium thiosulfate according to the following equation:

$$K_2PtI_6 + 2Na_2S_2O_3 = K_2PtI_4 + 2NaI + Na_2S_4O_6$$

This titration has proved less satisfactory in the author's

laboratory than has the potentiometric chloride titration described above. However, Norberg (17) applied it successfully to the determination of potassium in amounts as low as 4 γ with an accuracy of 1 to 2%. His method consists of adding to the sample an aqueous chloroplatinic acid solution and evaporating it to dryness in a small conical tube. The precipitate is washed free of extraneous materials and excess reagent by means of pure absolute ethyl alcohol which is separated from the precipitate with a special pipet, the tip of which is under microscopic observation to prevent removal of precipitate. The washed precipitate is dissolved in a hot neutral buffer solution in a period of 6 to 12 hours, after which it is treated with potassium iodide solution to convert it to iodoplatinate. The latter is titrated with standard thiosulfate solution to the complete loss of the red color which is characteristic of potassium iodoplatinate. Special illumination with a green light is used to render the end point distinct.

The increasing availability of microgram balances will probably make possible the accurate gravimetric determination of microgram quantities of potassium. The desirability of such a procedure as compared with either volumetric or colorimetric analysis is doubtful.

CALCIUM DETERMINATION

Virtually all procedures that have been successful in the determination of very small quantities of calcium have been based on precipitation of the calcium ion as calcium oxalate, its separation, and titration of the oxalate with a standard oxidizing agent, or by converting the calcium to calcium carbonate and titrating acidimetrically (18) or iodometrically (19). The gasometric determination of calcium oxalate (20) has not been applied to microgram quantities, nor have gravimetric methods up to the present time. Oxidimetric titrations are so favorable for application to the microgram range of analysis that nearly all successful methods in this range depend on them. A variety of oxidizing agents have been used, including potassium permanganate (21, 22), ceric sulfate (23–25), and perchloratocerate (26–28). The last two are to be preferred to permanganate, and between them there is little preference. Perchloratocerate may be applied to the direct titration of oxalate, as contrasted with ceric sulfate, but it is more difficult to prepare, stabilize, and use, which largely

eliminates the advantages of the direct titration. Variations of technique in preparing the calcium oxalate and separating it are also available. Because the method of Lindner and Kirk (29) adheres to the technique described in detail in this volume, it will be given first. It should be noted that the equipment and technique apply equally to numerous other methods for calcium analysis.

Principle of Method

The sample, which has been ashed or deproteinized, is treated with a considerable excess of ammonium oxalate in very slightly acid solution, to precipitate calcium oxalate. When the acidity is adjusted to a *p*H of 5 to 6, phosphate and magnesium in an amount equivalent to the calcium will not interfere. The precipitate is separated by filtration, dissolved in acid, and treated with a measured excess of standard ceric sulfate solution, the unused part of which is determined by back-titration with standard ferrous ammonium sulfate solution. The method is capable of determining as little as 0.5 γ of calcium with an error not greater than 2%. The average error of the method throughout the range studied (0.5 to 12 γ) was less than 0.5%.

Reagents

1. *Sodium acetate solution,* saturated, prepared by dissolving the pure salt in redistilled water to saturation and filtering it through a fine sintered-glass filter.

2. *Ammonium oxalate solution,* 4% by weight, made with redistilled water and filtered through fine asbestos on a sintered-glass filter.

3. *Asbestos suspension.* A sample of Italian medium-fiber washed ignited asbestos is ground moderately fine in a mortar, boiled with acid ceric sulfate solution, and washed thoroughly with redistilled water on a sintered-glass filter as described earlier. The asbestos is finally suspended and stored in water in a glass-stoppered bottle.

4. *Wash solution,* prepared by diluting concentrated ammonium hydroxide solution with ten volumes of redistilled water and saturating it with freshly precipitated and washed calcium oxalate. Before use, this solution is filtered through a sintered-glass filter coated with a fine asbestos pad.

5. *Ceric sulfate solution,* standard 0.01 *N*. This solution is made as previously described* and standardized against pure sodium oxalate. It must be stored in a clean glass-stoppered bottle.

6. *Ferrous ammonium sulfate solution,* standard 0.01 *N*. This solution is made and standardized as previously described.* It is stable for a considerable time if stored in a dark bottle. Under illuminating gas or other inert gas, it remains stable almost indefinitely. Portions withdrawn from it are stable in air for about 2 weeks.

7. *Indicator solution.* 0.0025 *M* *o*-Phenanthroline ferrous sulfate is prepared by diluting a 0.025 *M* stock solution prepared as previously described.*

8. *Calcium salt solution,* standard. If a standard calcium-containing solution is required for checking of the procedure or any other purpose, it is made from reagent-grade calcium carbonate dissolved in the correct amount of dilute redistilled sulfuric or hydrochloric acid, just sufficient to complete solution. Exactly 0.0500 g. calcium carbonate dissolved to make 100 ml. of solution will contain 1 γ Ca^{++} in 5 λ of solution. The calcium carbonate should be thoroughly dried before use by being placed in a desiccator over phosphorus pentoxide or another desiccant of approximately the same vapor pressure, for a few days.

9. *Sulfuric acid solution,* approximately 6 *N*. This solution is prepared by adding 18 ml. of concentrated sulfuric acid slowly to 50 ml. of redistilled water and diluting the resulting solution to 100 ml.

Procedure with Protein-Free Solutions

The sample containing soluble calcium, no protein, and no more magnesium than calcium may be used for direct precipitation of calcium oxalate. It is measured with a capillary pipet in the usual manner, and the pipet is rinsed with redistilled water into a porcelain titration dish. If the sample is not already acid, enough dilute hydrochloric acid is added to acidify the sample definitely. The dish is placed on a micro hot plate, and a magnetic thread stirrer is inserted. While it is being warmed and stirred, a volume of 4% ammonium oxalate solution equal to the sample volume is added. About 0.1 ml. of saturated sodium acetate

* Cf. Oxidimetric Titrations, Chapter 5.

is then added to the solution which is by now moderately warm. Heating is continued just below boiling for about 5 minutes, with continuous stirring. If the volume decreases appreciably, the evaporation loss is replaced with a drop or more of redistilled water. The dish is then set aside, covered with a Petrie or other protective dish to exclude dust and minimize evaporation, and allowed to stand for at least 12 hours.

The calcium oxalate, which will have precipitated quantitatively, is separated by means of a sintered-glass filter stick with a disk of about 2-mm. diameter, coated with a thin layer of fine asbestos removed from suspension as previously described. The dish and precipitate are washed thoroughly with about 30 drops of wash solution. After removal of suction, the asbestos pad is returned to the dish by washing with a drop of 6 *N* sulfuric acid. The filter stick is washed with several drops of the acid, followed by several drops of water. The dissolved calcium oxalate in the dish is heated on the hot plate for 2 minutes with stirring and allowed to cool about 5 minutes. A measured amount of standard ceric sulfate solution is measured into the dissolved oxalate from a capillary pipet, which is rinsed with redistilled water. The amount to be taken depends on the quantity of calcium in the sample and should be as nearly as convenient a twofold excess, though the amount is not critical. With amounts of about 10 γ of calcium, 50 λ of 0.01 *N* ceric sulfate solution is the optimum quantity. If the amount is below about 5 γ of calcium, this may be reduced to 25 λ of standard ceric sulfate solution. The mixture is stirred in the cold for about 5 minutes to complete the oxidation of the oxalate. A 5-λ sample of *o*-phenanthroline ferrous sulfate indicator is added, and the excess ceric ion is determined by titration in the usual manner with standard ferrous ammonium sulfate.

A blank is run with all the reagents, distilled water substituted for the sample. The blank titration value gives a corrected normality ratio of the ceric sulfate in terms of the standard ferrous salt. The difference between the blank determination and the analytical titration is equivalent to the amount of oxalate in the precipitate. The difference in microliters multiplied by the normality of the ferrous ammonium sulfate solution is equal to the number of microequivalents of oxalate in combination with calcium in the precipitate. The ratio of the two standard re-

agents derived from the blank value usually deviates very slightly from the normal titration ratio, owing to slight impurities or systematic technical errors.

Procedure with Biological Materials

The interfering materials often present in biological materials are phosphate, small amounts of magnesium, and protein. Plasma containing high cholesterol and fat concentrations (28, 30) has also been reported to cause interference. Interference by phosphate and magnesium is controlled by adjustment of the *p*H of the solution to avoid precipitation from alkaline solution. The effect of protein, which is commonly disregarded (31, 32) has been shown by Van Slyke and Sendroy (20) and others (33) to be definite though small: (*a*) because the protein tends to protect the colloidal state of some of the calcium oxalate causing it to be lost through the filter, and (*b*) because some of the protein is retained on the precipitate and consumes oxidizing agent. For these reasons, it is definitely preferable to remove protein before determining calcium. With small samples, as noted earlier, ashing is extraordinarily simple and rapid. Alternatively, deproteinization with trichloroacetic acid (20) has been used with success, as has wet digestion (34). Ashing and digestion with oxidizing acids also destroys cholesterol which occasionally prevents accurate analysis (28, 30).

The biological material if liquid, such as blood, urine, cerebrospinal fluid, or similar material, is measured with a capillary pipet into a small platinum dish previously described. Tissue or other solid or semisolid material must be weighed in a tightly closed container if wet weight is desired, or dried and the dry weight obtained. The solid sample also is placed in the platinum ashing vessel. Siliceous vessels such as porcelain or Vitreosil are not suitable because of the formation of insoluble silicates which prevent re-solution of the calcium. The sample is ashed in the micro muffle furnace at a temperature that does not exceed dull redness and may be kept below 450° C. The resulting ash is dissolved with small successive portions of dilute (about 0.1 *N*) hydrochloric acid, each of which is transferred with the capillary pipet into a porcelain titration dish. The dissolved ash is treated exactly as described in the preceding section except that no extra acid need be added.

Liquid protein-containing samples may be analyzed by precipitating calcium oxalate directly from the sample with a sacrifice of from 2 to 5% in accuracy. With blood plasma or serum, the sample is diluted with one volume of water, and no acid is added, nor is sodium acetate required. Samples which are essentially free of protein such as urine, may be analyzed by direct precipitation of calcium oxalate with little or no loss in accuracy. It should be emphasized, however, that the little increase in time and difficulty that is associated with ashing makes it definitely preferable to follow this procedure with all biological materials.

Remarks

The most important possible source of error in the method given lies in the possibility that some extremely fine calcium oxalate crystals in the wash solution pass the filter used to prepare the wash solution. These may then be retained when the analytical precipitate is filtered, because of the greater tightness of that filter. The effect is completely eliminated by using a very fine filter in preparing the wash solution. This may be accomplished conveniently by means of a fine asbestos suspension on a sintered-glass filtering funnel. Similar difficulties may also arise from incompletely dissolved ammonium oxalate crystals in preparing the precipitating solution.

Alternative Procedures

Oxalate Titrations. Ellis (26) used ammonium hexanitratocerate solution in perchloric acid for the direct titration of oxalate ion on the microscale. His results were not completely confirmed by Kirk and Tompkins (21) who experienced difficulty in stabilizing the reagent, in destruction of the indicator by small local concentrations of the oxidant, and in the slowness of the reaction. On the milligram scale the method was successful but less satisfactory than ceric sulfate used in excess. Kochakian and Fox (27) have more recently specified conditions under which the titration is more satisfactory on the milligram scale. They have not tested it with microgram amounts. Similar results are reported by Salomon, Gabrio, and Smith (28), who also worked in the milligram and submilligram ranges. Their lower limit of test was about 10 γ of calcium, with which quantities a precision of about $\pm 10\%$ was obtained. It is probable that the techniques described by the above investigators could be applied

successfully to direct titration of microgram quantities of calcium oxalate, with some possible advantages over the use of excess ceric sulfate. The instability of ammonium hexaperchloratocerate to light, particularly at normal and raised temperatures, and its relatively rapid change in normality even when stored under careful conditions appear to neutralize most of the advantages of substituting a direct for an indirect titration. Kirk and Tompkins (21) demonstrated that the application of permanganate to the titration of microgram quantities of oxalate was practical. It was, in several respects, inferior to ceric sulfate for the purpose and will not be discussed here. Another practical possibility for milligram quantities is manganous sulfate as catalyst for the direct titration with ceric sulfate (35). It has yet to be tested with microgram quantities. A variation in the determination of oxalate by excess ceric sulfate was introduced by Rappaport and Rappaport (23) who determined the calcium in 0.2 ml. of serum by addition of an excess of ceric sulfate to the oxalate and evaluation of the excess iodometrically. Aside from the introduction of extra reagents and steps in the procedure, accurate iodometry on the microgram scale is exceptionally difficult because of the rapid loss of iodine from small volumes of solution having large surfaces.

Acidimetric Procedures. Calcium oxalate may be determined by heating it to a temperature of 475° to 525° C which converts it to calcium carbonate which may then be dissolved in standard acid, the excess of which is determined alkalimetrically or iodometrically. Alternatively, the calcium carbonate may be dissolved in boric acid and titrated with standard acid. The acidimetric procedure was apparently applied first to quantities down to about 10 γ of calcium by Nordbo (18). Siwe (36) and Van Bergen and Hill (18) further tested and applied the method. Finally, Sobel and coworkers (18) have published a series of studies of the method, in which are described most of the possible technical modifications. The method of Sobel and Sobel (37) for analysis of 0.1 ml. of serum follows.

Principle of Method. Calcium oxalate is precipitated directly from blood plasma or serum by saturated ammonium oxalate. The precipitate is separated by centrifugation, washed with ammonium oxalate solution, and converted to calcium carbonate by ignition at 475° to 525° C. The excess ammonium oxalate is

destroyed in this process. The calcium carbonate is dissolved in hot 10% boric acid, cooled, and titrated with standard hydrochloric or sulfuric acid, using a capillary buret and methyl red-methylene blue indicator. In the range of 6 to 12 γ of calcium, the average deviation was about 1.5%.

Solutions. 1. *Ammonium oxalate solution,* saturated. A solution is approximately saturated at 40° to 50° C with pure salt and allowed to cool to room temperature.

2. *Ammonium oxalate solution,* 0.5%, made by dissolving 0.5 g. of ammonium oxalate to make 100 ml. of solution.

3. *Standard acid solution,* 0.01 *N* hydrochloric or sulfuric acid.

4. *Boric acid solution,* 1%, made by dissolving 10 g. of recrystallized boric acid in 500 ml. of 95% alcohol, and diluting 1 l. with distilled water. The final solution is filtered if it is not clear.

5. *Boric acid solution,* 10%, made by dissolving 10 g. of recrystallized boric acid in 100 ml. of distilled water with heat. Since this is a supersaturated solution, it must be reheated and used hot. To each milliliter of the hot solution is added 3 drops of indicator before use.

6. *Indicator solution,* 0.2 g. of methylene blue and 0.5 g. of methyl red dissolved in 50 ml. of 95% alcohol and diluted to 100 ml. with distilled water. The solution is filtered if not clear.

Procedure. The sample, 0.1 ml. of serum or similar material, is measured into a special centrifuge cone 69 to 71 mm. in length with a conical portion about 23 mm. in length ending in a flat bottom about 2 mm. in diameter. The wall thickness is 1.2 to 1.5 mm., and the tube is rimmed to be held in a special rack. It must be carefully cleaned before use with sulfuric dichromate mixture, rinsed thoroughly, and dried in an oven. To the sample is added 0.1 ml. of saturated ammonium oxalate and 0.1 ml. of distilled water, mixing the solutions by means of gentle tapping. The tube is stoppered and allowed to stand for 3 hours. The stopper is then removed and the tube centrifuged at about 2000 r.p.m. for 15 minutes. The supernatant fluid is removed by a pipet with a fine tip and equipped with a rubber bulb. Care must be taken to remove none of the precipitate. The precipitate is washed once with 0.3 ml. of 0.5% ammonium oxalate, being mixed with the wash solution by tapping as before and centrifug-

ing as described. After the wash liquid is removed with the pipet, the tube is placed in an oven at 110° C until dry and in a muffle furnace maintained between 475° and 525° C for 30 minutes. This converts the calcium oxalate to carbonate and decomposes the excess ammonium oxalate. A sand bath may be substituted for the muffle furnace. After it cools, the tube is immersed in a boiling water bath, and 50 λ of hot 10% boric acid containing indicator is added. The boric acid is prepared immediately before use by heating the boric acid suspension to solution and adding 2 or 3 drops of indicator to 1.0 ml. of the acid. Heating for a few minutes dissolves the calcium carbonate. After the solution cools, it is titrated with standard 0.01 *N* hydrochloric or sulfuric acid solution, with a capillary buret. The end point is determined by comparison with a similar tube containing 0.05 ml. of the same boric acid solution containing indicator to which is added 0.25 ml. of water. Titration is continued until the indicator colors in the two tubes match. The calcium present in the sample is equal to the acid added in terms of equivalents.

ULTRAFILTERABLE CALCIUM DETERMINATION

Determination of that portion of blood calcium which is in ultrafilterable form has been extensively investigated and employed to some extent in clinical analysis. It is recognized that a portion of the blood calcium is attached to proteins of the blood and is removed by an ultrafilter which is not permeable to the proteins. Other physiological fluids such as cerebrospinal fluid have also been investigated by this means, but to a much lesser extent. In dealing with blood samples from small children particularly, the quantities of blood necessary for the conventional techniques are quite difficult to obtain. The same is true to an even greater extent if it is desired to perform investigations with small laboratory animals. With microgram techniques, it is possible to determine the ultrafilterable and non-diffusible fractions of calcium with amounts of blood as small as 0.1 ml. without important difficulty. Moreover, filtration on this scale is probably simpler and certainly as rapid as larger-scale filtration. The method for accomplishing the microgram determination of ultrafilterable calcium was developed by Johnson and Kirk (38).

Principle of Method

A flat collodion ultrafiltration membrane of small dimensions is supported by means of a special glass tube clamp. Blood plasma or serum is forced through this filtration membrane by positive air pressure. The filtrate, which accumulates in the glass tube below the filter, is removed for analysis for calcium. The total calcium of the blood minus the ultrafilterable calcium of the filtrate gives the non-filterable fraction.

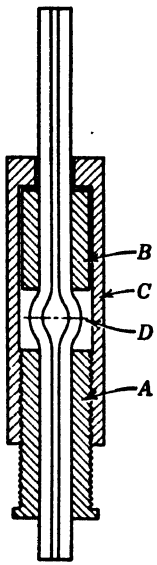


Fig. 75. Capillary ultrafilter. (⅔ actual size.)

Procedure

An ultrafilter is constructed as shown in Fig. 75. Two 2-mm. heavy-walled glass capillary tubes are expanded on the ends and ground perpendicularly so as to meet, as shown at *D*. One is cemented into a threaded brass collar *A* and the other to an unthreaded brass collar *B*. A sleeve *C* is threaded to *A* and slides freely on *B*. Thus, by means of the sleeve, the ground ends of the glass tubes can be compressed on each other. Between them, at *D*, is placed a sheet of collodion suitable for ultrafiltering the solution in question. This collodion filter will be held quite tightly between the two ground-glass surfaces when the sleeve *C* is tightened. The glass tubes are cemented into their collars by means of Krönig cement (1 part white wax and 4 parts rosin) or other suitable laboratory cement.

Many variations in the method of making ultrafiltration membranes have appeared (39). The mixture found suitable (38) was a solution of Parlodion dissolved in a 40–60 mixture of absolute alcohol and dry ether and containing 5% by volume of ethylene glycol. The membrane may be cast in a test tube in the usual way and disks cut from the cylindrical side of the casting, or, preferably, the disks may be cut from flat membranes cast on a piece of flat glass floated in mercury.

The filter (or filters) after assembly are charged with about 0.1 ml. of blood serum or plasma which is inserted with a long-

tipped pipet into the upper tube in contact with the membrane. The tube containing the blood is connected by means of a rubber tube with a large bottle which is tightly stoppered and connected to the water pipe and to a manometer. Water is run into the bottle until the manometer indicates a pressure of 150 to 200 mm. of mercury pressure in the line. If there are no leaks in the system, no further attention is necessary until the requisite amount of filtrate has collected below the membrane. At times, some leakage is encountered around the ground connection. In this case it is desirable to seal it on the outside with some Krönig cement. The reservoir of pressure is sufficient to maintain a considerable number of filters under pressure for many hours without attention.

Some blood serum or plasma from the original sample is analyzed for calcium as described in the section above. The ultrafiltrate which collects directly below the membrane of the ultrafilter is removed by insertion of a pipet with a long narrow tip and is analyzed in exactly the same manner as the original sample. The ratio of the two concentrations of calcium multiplied by 100 gives the percentage of diffusible calcium in the sample. It should be noted that this technique is applicable to the determination of other constituents which may be bound to protein and rendered non-diffusible through collodion, provided an analytical method is available for the determination of the two fractions.

IRON DETERMINATION

Iron is frequently and conveniently determined by several colorimetric procedures, as described later in this volume. In larger amounts, i.e., milligram to decigram amounts, a variety of volumetric procedures are available, two of which have been adapted to the analysis of microgram amounts. One of these, described by Dubnoff and Kirk (40), employs direct titration of ferric ion with titanous chloride, the end point of the titration being indicated by differential potentiometry. The other procedure, described by Kirk and Bentley (41), depends on the reduction of the iron to the ferrous state with liquid cadmium amalgam, after which it is titrated with ceric sulfate. The second procedure described below is much simpler from the technical standpoint though not more accurate than the first.

Oxidimetric Method

Principle of Method. The sample, which is free of other easily reducible substances and of oxidizable organic materials, e.g., the solution of an ash in iron-free 5% sulfuric acid, is measured into a volumetric flask of 0.1- or 0.2-ml. capacity. The small liquid sample must contain at least 1 γ of iron. It is reduced with 3% cadmium amalgam which is used to stir the solution. A large aliquot of the reduced solution is removed with a pipet and inserted into a measured excess of standard ceric sulfate solution. The excess after stirring is titrated back with standard ferrous ammonium sulfate solution.

Reagents. 1. *Sulfuric acid,* free of iron, 5%.

2. *Cadmium amalgam,* 3%, made by dissolving 3 g. of metallic cadmium in 100 g. of mercury and storing the resulting amalgam in a narrow test tube, tightly stoppered.

3. *Ceric sulfate solution,* standard 0.01 *N*, made as described previously.*

4. *Ferrous ammonium sulfate solution,* standard 0.01 *N*, made as described previously.*

5. *Indicator solution,* 0.0025 *M* phenanthroline ferrous sulfate, made as described previously.*

6. *Sodium hypochlorite solution,* free from iron. Commercial hypochlorite was satisfactory. Used only in ashing highly proteinaceous samples.

Procedure for Solutions Free of Interfering Materials. The sample, which is free of organic material and easily reducible substances other than iron, such as the solution of an ash or a simple inorganic solution, and which contains at least 1 γ of iron in a volume less than 0.2 ml., is measured by a capillary pipet into a volumetric flask of 0.1- or 0.2-ml. capacity (Fig. 25). If the sample is not already as acid as 0.1 *N*, a little 5% sulfuric acid is added. The acidity is not critical, but the sample must contain enough acid to persist throughout and after the reduction. When an ash is dissolved, it is placed in solution with 40 to 75 λ of the acid. The sample is made to volume in the flask, and to it is added a drop (about 30 λ) of cadmium amalgam. The flask is stoppered and shaken vigorously for about 10 minutes, making sure that the drop of amalgam penetrates the liquid in the stem to the meniscus repeatedly. Thus, vigorous stirring from the

* Cf. Oxidimetric Titration, Chapter 5.

amalgam is produced, and the iron is reduced quantitatively. The amalgam is left in the stem to prevent oxidation from air while a measured volume, usually 50 λ of standard ceric sulfate, is delivered to a titration dish on the titration table. The stirrer is inserted and placed in operation. A pipet is chosen having a volume somewhat less than the volume of the flask, e.g., 75 λ for an 0.1-ml. flask, 100 to 150 λ for a 0.2-ml. flask. The flask is opened, the amalgam shaken down, and a measured aliquot is withdrawn with the pipet as rapidly as possible. It is discharged into the ceric sulfate solution, and the pipet is rinsed. The excess ceric ion is immediately titrated back with 0.01 *N* ferrous ammonium sulfate solution which may contain ferrous *o*-phenanthroline as indicator. Alternatively, the indicator is added separately.*

A control is run by performing all operations on acidulated water, keeping all reagent volumes the same as in the determination. The control titration minus the titration resulting from the analysis of the sample is equivalent to the iron contained in the aliquot, i.e., the volume difference in microliters multiplied by the normality of the ferrous solution gives the microequivalents of iron. This is multiplied by the ratio of total volume to aliquot volume to give the microequivalents in the sample.

Procedure for Biological Solutions. Biological materials that can be weighed or measured with a pipet are transferred into a micro platinum crucible. Whole blood contains more iron than is conveniently determined and should be diluted with five volumes of water before sample measurement. This also has the effect of hemolyzing the red corpuscles. Serum and plasma commonly have so little iron as to be difficult to determine on this scale, which would require samples of at least 1 ml. When the protein content is high as it is in blood, a little hypochlorite solution added to the water used for dilution dissolves any clot that forms and aids materially in oxidizing the protein rapidly. The wet sample is dried carefully over a micro hot plate, inserted in a micro muffle furnace at a temperature just under redness, and heated until a white ash is obtained. This requires only a few minutes, and heating should be gentle and not continued longer than necessary, since it may then form an insoluble iron oxide

* Cf. Chapter 5.

which is difficult to dissolve, except by fuming with sulfuric acid. The ash is dissolved, and the iron determined as described above.

Remarks

The method has been used satisfactorily between the ranges of 2 and 15 γ of iron with an average error of about 1%, maximum about 3%. Slightly smaller and considerably larger amounts can undoubtedly be determined, the smaller with less accuracy, and the larger by increasing the amount or concentration of the critical reagents as much as necessary.

The most serious source of error in the method, assuming that the reduction and titration are performed carefully, is in the volume relations. The volumetric flask may have an appreciable error, as discussed in Chapter 2, and the total error may be increased if the aliquot measurement error is additive. The reduction itself is somewhat tiresome because of the long manual shaking necessary, and this factor may tempt the analyst to shorten the reduction period, with consequent lowered recovery. The simplicity of the method as compared with alternative methods recommends it for routine use when the available samples are in the proper range, and particularly when the number of determinations is not large enough to justify more elaborate equipment. Few materials can interfere, except organic matter, and in the ordinary biological system there is no other known interference of importance.

Reductometric Method

Principle of Method (40). When the sample is highly dilute, e.g., 1 part of iron per million to ten million, and the volumes are such that as much as 1 γ of iron can be obtained in the sample, it is advantageous to titrate the ferric ion directly with titanous ion according to the equation

$$Fe^{+++} + Ti^{+++} \rightleftharpoons Fe^{++} + Ti^{++++}$$

The entire system in which the titration is performed must be quantitatively free of oxygen, which imposes a difficult technical limitation on the method. The end point is accurately determined by differential potentiometry, made necessary by the fact that no color indicator is sufficiently sensitive for this extreme range of dilution. Special equipment is necessary not only for the end-point detection but for the addition of reagent, since this also must be protected very carefully from oxidation by the oxygen of even traces of air.

The solution to be titrated is placed in a special differential titration vessel, such as that shown in Fig. 76, and swept free of

all traces of air with purified nitrogen. It is titrated with titanous chloride or sulfate solution using the differential electrode system to detect the end point corresponding to the complete reduction of ferric to ferrous ion.

Apparatus. The titration vessel of Fig. 76 consists of two chambers, the larger of which, *A*, contains most of the sample being titrated. In this solution is immersed the tip of the capillary buret and a platinum electrode. The small chamber *D* contains another similar platinum electrode. The solution contained in it is the retarded portion of the sample undergoing titration, since all added reagent flows into the solution in chamber *A*. The two chambers are connected by a capillary tube *a* and the bottom by another capillary *b*. Pure nitrogen flows into and bubbles through the two chambers from tubes *c* and *e*. If the plunger *d* is raised, the gas entering the chamber through *c* serves as a gas lift pump similar to that described by MacInnes and Dole (42). Thus when it is desired to mix the solutions, the plunger is raised; when the mixing is complete, the plunger is lowered, and the next addition of reagent is made. The paired electrodes are connected through a tapping key and variable resistance to a galvanometer having a sensitivity of about 25 μv. per millimeter.

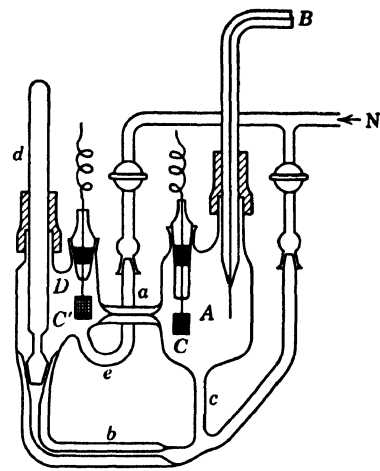


FIG. 76. Differential titration vessel for use with inert gas.

The buret is an upright type similar to that of Linderstrøm-Lang and Holter (43), with a reservoir connection allowing filling without exposure of the buret contents to air. Other designs of buret can undoubtedly be adapted, but it is essential in this case that a reservoir filling principle be employed. The design of the buret is shown in Fig. 77. Both the reservoir and the titration vessel are swept with nitrogen which is passed over hot copper (700° C.) in a micro combustion tube heated with a micro combus-

tion furnace of the type used in microchemical determination of carbon and hydrogen. At intervals the copper oxide formed in purifying the nitrogen stream should be reduced by being heated in a stream of hydrogen. Some commercial nitrogen gas may contain several per cent of oxygen and oxidize the copper rapidly.

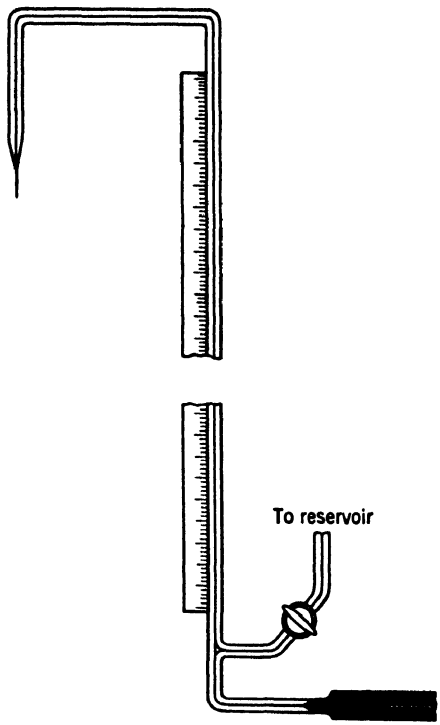


FIG. 77 Reservoir buret for use with inert gas over solution.

Almost completely oxygen-free nitrogen is available commercially and may be substituted.

Solutions. 1. *Titanous chloride*, standard solution, made by dilution of commercial concentrated titanous chloride solution, which is furnished usually in 20% strength. The dilution is made by drawing into a clean evacuated bottle a convenient amount of freshly boiled iron-free 0.5% hydrochloric acid solution, followed by a volume of commercial titanous chloride solution sufficient to produce about 0.002 *N* solution. If this solution

is stored under nitrogen which has been completely freed of oxygen, it will stay comparatively constant for several days. It is standardized against a very dilute standard iron solution.

2. *Ferric iron solution*, standard stock, may be prepared readily from pure iron wire, dissolved in iron-free hydrochloric acid solution and diluted to 0.001 *N*. This solution serves to make a more dilute standard as needed, ordinarily in the strength of 0.00002 *N*, which standardizes the titanous solution. So dilute a standard is not stable and must be made fresh daily. The solution is made with iron-free 0.5% hydrochloric acid.

3. *Trichloroacetic acid solution*, 10%. Trichloroacetic acid is purified from iron by distillation and made in 10% solution with redistilled water. It is used for deproteinizing blood and other proteinaceous solutions.

Procedure. The solution containing ferric ion is measured and transferred to the titration vessel and made distinctly acid but not more than 0.1 *N* in hydrochloric acid. Ordinarily the volume of sample will be relatively large, e.g., 1 to 5 ml., since this method is useful particularly for the most minute traces of iron, rather than for ordinary concentrations. The volume of liquid is increased if necessary to cover the electrodes, and the vessel is connected with the buret freshly filled with standard titanous solution. The purified nitrogen stream is started through both chambers by manipulation of the two stopcocks in the gas line. The flow should be such as to produce mild but definite stirring. The tapping key is closed, and, if the galvanometer does not register zero, the solution should be mixed for about ½ hour by raising the plunger. The plunger is then seated, and titanous solution is added slowly from the buret, the tapping key being closed from time to time until significant deflections are observed. The solution is then remixed, and additions of reagent are made in increments. The galvanometer is read after each reagent addition, and the buret and galvanometer readings are recorded. The solution is remixed after each set of readings. The deflection of the galvanometer will be observed to increase for a time and then diminish with successive increments. This indicates that the end point is passed, and the titration is terminated.

End-Point Calculation. The galvanometer readings are plotted against the buret readings, giving a curve similar to that shown in Fig. 78. Ordinarily the peak of the curve is taken as the end

point, which in most titrations introduces no appreciable error. However, in titrations for trace constituents, as in this instance, a significant error is introduced by this procedure, when the retarded portion of the solution is small with respect to the main body. If the greatest reading observed corresponds exactly with the peak of the curve, it would represent the flat portion of the titration curve, passed symmetrically in one increment. But the

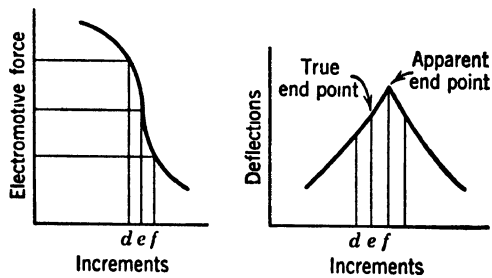


Fig. 78. Differential titration curve.

buret reading at that point is as far past the true end point, i.e., midpoint of the inflection, as the previous reading was short. Therefore, an error of 0.5 increment is involved by reading the peak of the curve. This would not be true if the retarded portion of the solution were of the same volume as the titrated portion, since the one would be as far behind the end point as the other was past. Consideration shows that, if the retarded liquid is small with respect to the main portion of liquid titrated, this correction is always required for accurate determination of end point, regardless of the actual points of the titration curve represented by the individual readings. This was shown experimentally as well as theoretically by Dubnoff and Kirk. The method is accurate to 1 to 2%, even with dilutions as great as 1 part in 1 to 5 million, and with amounts of as little as 1 γ of iron.

Procedure with Proteinaceous Solutions. Solutions such as blood serum or plasma, which contain mere traces of iron and considerable protein, require removal of the protein prior to titration. This is accomplished by deproteinizing as previously described, adding one volume of the serum or plasma to two parts

of iron-free 10% trichloroacetic acid solution. The filtrate or centrifugate is titrated directly as described with titanous salt.

Remarks

The differential electrometric titration described illustrates a possibility for the more difficult analyses carried out with traces, and on the microgram scale. Differential titrations are one of the most sensitive means of detecting end points at extreme dilutions. For research purposes, particularly where accuracy and sensitivity rather than rapidity are essential, methods of this type may provide a successful technique to which there are few, if any, alternatives. This determination, which is too complex for routine application, is considered in some detail because it provides an illustration of the possibilities of this approach to certain unusually difficult analytical problems. For most purposes, colorimetric methods described in a later chapter achieve almost as satisfactory analyses for iron with much less difficulty.

Other Metals. Volumetric procedures for determination of other metals in the microgram range are few in number and for the most part unsystematic. Allcroft and Green (44) have described a titrimetric method for determination of arsenic with a lower limit of almost 5 γ and an error of $\pm 5\%$. Considerably smaller quantities can be determined by various colorimetric methods with little, if any, loss in accuracy. The titration of dithizone in the analysis of various heavy metals has been used, e.g., by Hibbard (45) for determining traces of zinc. The technique has not been widely applied, partly because of its low accuracy. The range of analysis is also higher than the true microgram range. With dithizone also, smaller quantities of metals are more readily determinable by spectrophotometric procedures. Stock et al. (46) have adapted micrometric measurement of minute mercury droplets to the determination of quantities of that metal in the submicrogram range. Their procedures are novel and extremely sensitive but do not appear to yield an accuracy comparable to various other procedures available for determination of mercury, e.g., spectrophotometric determination. Reith and van Dijk (47) used a volumetric dithizone titration for mercury in a slightly higher range, i.e., 5 to 100 γ.

The possibilities for development of true microgram titration methods for other metals are numerous and relatively unexplored. If a satisfactory macrotitration procedure for a metal exists, it is

usually adaptable to the microgram scale if the proper technique is applied.

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CHAPTER 7

Non-Metallic Constituents
Titrimetric Methods

Inorganic non-metallic constituents of the biological system are more commonly and more reliably determined as a rule by volumetric methods than are the metals or the organic constituents. For both metals and organic constituents many of the most sensitive and dependable methods are colorimetric. It is because of this difference that titrimetric microgram analytical methods are more fully developed and extensively used for non-metallic constituents than they are for metals or organic compounds. Suitable procedures are available for most of the common anions and for several of the nitrogen fractions of particular interest in biological systems. No satisfactory microgram procedure has yet been described for determining the sulfate ion.* Phosphate is more easily determined by certain spectrophotometric than by volumetric methods, but not more reliably or accurately. A few non-metallic constituents are not satisfactorily determined on the microgram scale by either volumetric or colorimetric methods, and the field for further development in this direction is an appealing one.

Because of its relative completeness, the system of analysis for the fractions of nitrogen is considered first in this chapter. For convenience the relatively few titrimetric procedures for determination of organic compounds or groups are also included with the inorganic non-metals.

NITROGEN COMPOUNDS

The analysis of nitrogen-containing compounds frequently resolves itself in the final stages to the determination of ammonia. This is true not only of ammonium salts but also of total nitro-

*Leithe (1) has described a volumetric microgram procedure for determining sulfate, the accuracy of which is subject to several uncertainties. Confirmation or further study of the procedure should be carried out.

gen, urea, and amides. For this reason, the determination of ammonia is basic to several methods and will be described first.

Ammonia and Ammonium Compounds

Ammonia is most easily determined in small amounts and with high accuracy by separating it as a gas, collecting it in acid, and determining the quantity absorbed by back-titration. For minute amounts, the most reliable procedures are modifications of the Conway and Byrne diffusion method (2). With some of the modifications it is possible to determine ammonia in quantities as small as 0.1 γ and to maintain an accuracy of about 1% on amounts between 1 and 50 γ. The method described here is that of Gibbs and Kirk (3). The equipment was described in Chapter 4, and the micro diffusion cell is shown in Figs. 67 and 68. These cells are constructed with an internal chamber which contains the absorbent, and an external chamber to contain the sample from which the ammonia is released by the action of alkali. The unit is sealed on the top, and the transfer in the closed vessel is accomplished by diffusion or isothermal distillation at somewhat raised temperature.

Principle of Method (3–5). Ammonia in a sample placed in the outside chamber of a small modified Conway-type diffusion cell is alkalinized by addition of saturated potassium carbonate, and the ammonia is collected in a small measured volume of standard sulfuric acid contained in the internal compartment. The excess acid is titrated back with standard alkali after a sufficient diffusion time has been allowed.

Reagents. 1. *Potassium carbonate solution*, saturated.

2. *Sulfuric acid solution*, approximately 0.02 to 0.05 N, containing methyl red indicator. This solution does not require accurate standardization but should be approximately known.

3. *Sodium hydroxide solution*, approximately 0.02 to 0.03 N, accurately standardized.

Procedure. Either a glass or plastic (e.g., Lucite) diffusion cell is employed. It is thoroughly rinsed with distilled water but should not be treated with chromic acid or detergent solution unless exceptionally dirty. Chromic acid should never be used with a plastic cell. Glass cells operate better if they have a very slight film of grease on them to prevent easy wetting by and

spreading of aqueous solutions, but they must be scrupulously free of acidic or basic materials.

The sample to be analyzed, which should be slightly acid (e.g., urine), is added with rinsing from a capillary pipet to one side of the exterior chamber. On the other side of the chamber is placed about 0.1 ml. of saturated potassium carbonate solution in such a way that the two drops do not merge. A carefully measured sample of sulfuric acid containing methyl red indicator is placed in the central compartment. It must be in sufficient quantity to be in excess of the ammonia of the sample but preferably not more than two or three times the equivalent of the ammonia. A ring of stopcock lubricant or a Vaseline-paraffin mixture is placed around the top rim of the diffusion cell, and a glass or plastic cover is placed on it so as not to seal the cell completely. The unit is warmed in an incubator or oven to a temperature of 35° to 50° C., and, after complete warming, the cell is entirely sealed. It is then rotated so as to merge the potassium carbonate solution with the sample; the cell is then placed in the incubator for about 2 hours.

At the end of this period, the cell is removed from the incubator and opened. A vibrating stirrer is inserted in the acid drop, along with the tip of a capillary buret filled with the standard alkali solution, and the excess acid is determined by titration in the usual manner. A control in which distilled water replaces the sample is determined simultaneously. The difference between the titration value of the control and that of the determination in terms of standard alkali is equivalent to the ammonia diffused. For this reason, no accurate normality of the acid need be determined. In general a series of control runs are made with a series of determinations to establish an accurate value for the control. Although the actual time of a single determination is considerable, by employing a number of cells the operator is able to run a large number of analyses in a working day. Nearly all the consumption of time is due to the incubation, and, by staggering the loading, incubation, and titration, the method may be adapted readily to routine use on a large scale.

Remarks

As shown by Conway and confirmed by the author and others, the success of diffusion analysis of ammonia depends on having shallow liquid layers, particularly in the alkalinized sample. A large surface-

to-volume ratio allows rapid transfer of ammonia gas to the acid absorbent. It is desirable that the gas chamber have the minimum dead space and short diffusion distance, but these factors rarely limit the diffusion time as compared with the liquid configuration. The design of the Conway cell achieves these conditions almost ideally, but other designs are not markedly inferior. The range of the method as determined by the originators was from 1.55 to 8.27 γ. The upper range was not explored completely, and experience with the method demonstrates its applicability to amounts even lower than 1 γ. The accuracy in general was better than 1%, though occasional analyses deviated by more than this amount.

Ammonia and Ammonia Compounds—Alternative Methods

Of the various procedures for determination of ammonia other than described above, that of Linderstrøm-Lang and Holter (6) particularly deserves discussion. Although the range described by these authors, 0.2 to 28 γ of ammonia, is somewhat more extended than that studied by Gibbs and Kirk, the absolute error was approximately the same, i.e., ±0.01 γ of ammonia nitrogen. The success of their method was highly dependent on using very small volumes throughout, since their apparatus did not have the advantage enjoyed by the Conway-type vessels of giving a thin layer with relatively large volumes.

Principle of Method. The sample containing ammonia is alkalinized in the bottom of a paraffined micro test tube, which contains a sealing drop of standard acid. The ammonia is diffused into the acid drop in a water bath at raised temperature. The excess acid is titrated with standard borate solution delivered from a capillary buret, with bromocresol purple as indicator.

Apparatus. *Diffusion tube.* This is a small test tube, 25 mm. long and 3.8 mm. in internal diameter. The clean tube is coated internally by immersing it in paraffin (b.p. 72° C.), heated to 100° C. in an oven. Each tube is removed separately, the molten paraffin is thrown out, and the tube is rotated in the hand until a smooth even coat has formed. The used tube is cleaned by boiling with dilute acid, boiling with two portions of distilled water, rinsing twice with acetone, boiling once with toluene, rinsing with a second portion of toluene, and drying in an oven.

Stirrer. Stirring is performed with a glass-coated iron fragment actuated by an external magnet, in the manner commonly

used in the Carlsberg Laboratory and considered briefly in Chapter 2.

Procedure. The sample, which should have a volume not greater than 14 λ is placed on the bottom of a well-paraffined tube, and a stirrer is inserted. The tube is turned horizontally, and a droplet of 2 *N* sodium hydroxide (about 7 λ) is placed on the side of the vessel about 5 mm. from the bottom with a pipet having a tip bent so as to contact the wall. This is performed by placing a moderately close-fitting glass cap containing a central hole over the tube while the latter is held horizontally in the left hand. The pipet containing the caustic and fitted with a soda lime tube and rubber tube leading to the mouth is carefully inserted through the hole in the cap, and the bent point is touched to the location desired for the droplet. By blowing gently and giving to the pipet a short careful jerk, the tip is disengaged from the delivered droplet. The pipet is then removed very carefully to avoid touching the side walls with any residual caustic. A water seal is placed about 5 mm. from the open end of the tube, using about 35 λ of carbon dioxide-free water placed with a curved tip pipet locating the drop so as to fill completely the bore of the tube.

The tube is turned vertically, allowing the droplet of caustic to enter and mix with the sample. Seven microliters of hydrochloric acid of the appropriate strength and containing bromocresol purple is added rapidly to the water seal. The solution in the bottom of the tube is mixed with the stirrer, and the tube is capped and half immersed in a water bath at 40° C. After a period of 2.5 to 5 hours diffusion, the tube is removed, and the excess acid is titrated directly in the seal with a standard borate solution of appropriate strength (i.e., ~0.01 *N* sodium borate) by means of a capillary buret. Stirring is performed with the interrupted magnetic stirrer. Appropriate controls are run as in the first method described.

UREA DETERMINATION

The problem of determining urea is identical with that of ammonia, if the urea is converted first to an ammonium salt. This may be accomplished by means of the enzyme urease.

Principle of Method (3)

The urea in a sample is treated with the enzyme urease in a modified Conway cell, after which the mixture is alkalinized with saturated potassium carbonate and diffused into standard acid as in the analysis of ammonia. Blood and other proteinaceous solutions do not require deproteinization, but they may be deproteinized if it is desired to obtain protein-free filtrate for analysis of other constituents.

Reagents

1. *Urease preparation.* This is most conveniently made with a commercial urease tablet or power, dissolved according to the manufacturer's instructions. If it is preferred to prepare the urease in the laboratory, it is made as follows. One gram of Permutit (according to Folin) is washed with 30 ml. of water acidified with 2 drops of glacial acetic acid, rinsed four to five times with distilled water, and stirred with 0.5 g. of jack bean meal. To the paste is added 30 ml. of 30% alcohol, after which it is stirred or shaken for 10 minutes and finally centrifuged to obtain a nearly clear extract. Finally there is added 1 ml. of buffer solution made from 9.6 g. of monosodium phosphate and 17.9 g. of disodium phosphate dissolved in 100 ml. of water. The urease preparation should be made fresh each day.

If deproteinization is required,

2. *Sodium tungstate solution,* 10%.

3. *Sulfuric acid,* 0.66 *N*, which has been so adjusted that equal volumes of reagents 2 and 3 precipitate all the tungstic acid but leave little excess acid.

Other reagents as described for ammonia analysis.

Procedure

The sample to be analyzed may be initially free of protein, deproteinized,* or used in the presence of protein, e.g., whole blood. It is added quantitatively with a capillary pipet to the external chamber of a micro Conway cell. Close to it, but not merging, is placed about 50 λ of urease solution. On the opposite side of the external chamber is placed 0.1 ml. of saturated potas-

* Cf. Chapter 4.

sium carbonate solution. Care must be taken to keep the three solutions apart. To the central chamber is added 25 to 50 λ, accurately measured, of standard 0.02 *N* sulfuric acid containing methyl red. The cell, covered but not sealed, is warmed in the incubator, sealed with a cover as described for ammonia determination, and the sample and urease drops merged by tipping the cell slightly, and tapping it if necessary. The cell is allowed to incubate at 37° C. for about ¾ to 1 hour, after which the digest is mixed with the potassium carbonate solution without opening the cell, and the determination is carried out exactly as described for ammonia determination. The range and accuracy of the method are similar to those for ammonia analysis. Control determinations are carried out in the same manner as the analysis with the same amount of urease preparation and with distilled water substituted for the sample. A small amount of ammonia may be obtained from the urease preparations at times, and it is therefore desirable that the enzyme preparation always be measured carefully and controls run whenever a new preparation is employed.

TOTAL NITROGEN DETERMINATION

The determination of total nitrogen differs from that of ammonia and urea in that a comparatively lengthy digestion is required, at the end of which there are present considerable quantities of strong acid. This necessitates dilution and neutralization with a corresponding increase in the volume and an increase in the difficulty of diffusion as a result. The determination is more complex technically and offers a less-favorable set of conditions for diffusion.

Various procedures for determining the ammonia of a Kjeldahl digest by diffusion have appeared in the literature. These include methods by Bentley and Kirk (7), Borsook and Dubnoff (5), Needham and Boell (8), Tompkins and Kirk (9), and Brüel, Holter, Linderstrøm-Lang, and Rozits (10). All the earlier methods had certain limitations of a technical nature or of speed and accuracy which were discussed by Tompkins and Kirk whose method is described below. This procedure is technically simple, relatively rapid, and capable of determining quantities of nitrogen down to about 0.5 γ with an error rarely exceeding 1%. Brüel et al. (10) have succeeded in determining quantities down to

0.1 γ with a limiting sensitivity of about 0.005 γ, which is approximately twice the sensitivity obtained by Tompkins and Kirk. The method undoubtedly represents by a small margin the most refined technique so far applied to the Kjeldahl analysis. It suffers from a degree of complexity in equipment and technique which limits its applicability strictly to research. It is therefore not competitive with the method of Tompkins and Kirk, with which as many as 30 analyses a day have been performed by one operator without extensive special training.

Principle of Method (9)

The small sample, which contains nitrogen in a form or forms which are susceptible to Kjeldahl analysis, is subjected to digestion by a modified Kjeldahl method in a combination digestion-diffusion vessel. Without removal, the digest is neutralized and the ammonia released is diffused into standard sulfuric acid, the excess of which is titrated back with standard alkali. The tested range of the method was 0.5 to 20 γ nitrogen. The standard error was of the order of 0.01 γ *N*, corresponding to about 0.5%. The recovery was usually within ±1% of theoretical.

Apparatus

The digestion-distillation vessels are shown in Chapter 4, Fig. 70. The first of these is a rubber-stoppered vessel of relatively simple construction, designed to operate at atmospheric pressure. The second is a glass-stoppered modification which allows the diffusion to be performed in vacuum, thus increasing the speed of diffusion markedly. Both vessels are characterized by three essential portions. The small bulbous end is the digestion vessel proper, which is used with the unit in the vertical position with the upper end unstoppered. The large flattened bulb is the portion used for the diffusion, taking advantage of the rather large flat surface to spread the liquid out and increase the diffusion rate with the vessel on its side. The third portion is the acid cup in the center which contains the receiver acid which collects the ammonia. It is supported on the stopper and is present only during the diffusion.

Reagents

1. *Digestion mixture.* A good grade of sulfuric acid is diluted with an equal volume of water, saturated with potassium sulfate,

and made 0.1% in copper selenite. Various modifications of this mixture undoubtedly can be used (11). Mercury, particularly, has been shown to be a superior catalyst for protein digestion and could be used if desired.

2. *Sodium hydroxide solution,* made by diluting saturated sodium hydroxide solution with an equal volume of water.

3. *Sodium hydroxide solution,* standard, about 0.02 to 0.025 *N*, accurately standardized.

4. *Sulfuric acid solution,* approximately 0.025 *N*, containing methyl red indicator. Accurate standardization of this reagent is not essential.

Digestion Procedure

The sample, in solution, and containing 0.5 to 20 γ of nitrogen in a form or forms susceptible to Kjeldahl determination, is transferred by capillary pipet to the bulb of either type of digestion-diffusion vessel. To it is added 0.1 ml. of digestion mixture from a pipet, with care to maintain the same amount of the mixture in every vessel of a series, so as to standardize the blank.

A sand bath is heated so that a thermometer inserted to the bottom of the sand reads about 300° C. The digestion-diffusion vessel is inserted into the sand just under the surface and at an angle of 10 to 20 degrees from the horizontal. In a short time the water is expelled from the sample, after which the vessel is placed upright and pushed deep into the sand where it is allowed to remain until digestion is complete.

If the digest clears slowly, a small sample (about 5 λ) of 10% hydrogen peroxide may be added. If this is done with the sample, an exactly equal quantity must be added to the blank since this material often contains a small amount of nitrogen-containing preservative. For complete digestion and conversion of all nitrogen to ammonia, it is usually necessary to allow digestion to proceed for about an hour after the solution appears completely clear (12). Some materials, notably proteins, require long digestion times to obtain complete recovery. Studies of the digestion of such materials in microgram quantities are very limited but the indication is clear that microgram digestions are at least as rapid as those performed on the milligram or decigram scale and probably considerably more rapid.

Diffusion Procedure—Atmospheric Pressure

The digest is cooled by removal from the sand bath and diluted with not more than 0.5 ml. of water. A sample of about 0.3 ml. of the strong sodium hydroxide solution is taken with a long capillary-tipped pipet, the end of which has been coated with Vaseline. This coated tip is inserted to the bottom of the diluted digest, and the caustic is delivered in a layer under the digest without mixing. On removal of the pipet tip, none of the digest is lost by adherence. The vessel is placed on a rack which holds it vertically without mixing the layers while the acid cup is prepared. This is accomplished by wiping a thin film of Vaseline on the top rim of the acid cup, and adding to it a carefully measured 30-λ sample of the standard sulfuric acid containing indicator. The cup may be turned in any position; the acid will not drop from the cup unless it is jarred sharply. The amount of rinsing water added must not be so large that the total volume exceeds 100 λ, or this will no longer be true.

The vessel is held by the small end and the large portion is brushed through a flame several times, taking care that the layers are not mixed. When the vessel is moderately warm, the acid cup is inserted and the stopper forced in tightly. On cooling, a slight vacuum is produced, and, if the warming has been sufficient, incubation will not create a positive pressure.

After the vessel is cool, it is tilted and rotated to mix the caustic and acid layers and finally to wet the entire interior with the alkalinized mixture, thus neutralizing any acid which has condensed on the walls. Care must be taken to avoid splashing any of the alkaline solution on the acid cup. The vessel is now placed in an incubator or oven at raised temperature, usually 37° to 50° C., where it is allowed to remain for about 3 hours. The rate of diffusion at 37° C. is shown in Fig. 79. Diffusion is speeded somewhat if the vessel is rocked either occasionally or continuously during the period of standing.

Finally, the absorption cup containing the standard acid is withdrawn, and the acid is titrated with standard caustic added from the capillary buret in the usual manner. The end point is taken as the appearance of a clear canary yellow color. Each series of determinations is accompanied by one or more controls in which distilled water is substituted for the sample. The titration values of the controls are used to establish a corrected

standardization and blank value for the acid in the cup. The titration values of the determinations are subtracted from those of the controls, the difference being equivalent to the ammonia diffused.

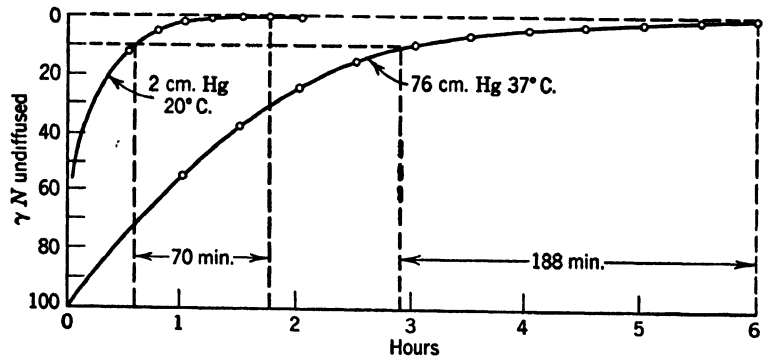


FIG. 79. Diffusion curve, ammonia analysis.

Diffusion Procedure—Vacuum

As shown in Fig. 79, diffusion in vacuum is decidedly more rapid than at atmospheric pressure. Amounts up to 10 γ of nitrogen may be quantitatively diffused in 70 minutes. A vessel similar to the second one illustrated in Fig. 70 must be used for this procedure. All operations with this vessel are identical with the description for ordinary pressures, except that, after all reagents are added, the ground joint is sealed with stopcock lubricant and turned to the open position. While the vessel is cooled in ice water, it is evacuated with a pump or water aspirator to about 2 cm. of mercury pressure and the stopcock rotated to close it. The diffusion is allowed to proceed at room temperature or somewhat below rather than at raised temperature, since with raised temperature evaporation of liquids is increased to a point at which the diffusion rate is diminished rather than increased.

Remarks

Some analysts prefer mixed indicators to methyl red for this titration. There is no reason why such mixtures should not be used if preferred. There must always be enough indicator to yield a clearly visible color change, even though it introduces a considerable drop error into the titration. It should be remembered that the drop error

can be standardized and will cancel out when run identically on the control determination. Satisfactory mixed indicators have been described by numerous authors. A suitable mixture is that of Groak (13) or of Tashiro (4), who employed 200 ml. of 0.1% alcoholic methyl red solution mixed with 150 ml. of 0.1% alcoholic methylene blue. This yields a color change from purple to green instead of red to yellow.

Although the diffusion time necessary with this method is considerable, it should be noted that it also requires little of the operator's time and attention. He can, then, with a sufficient number of vessels confine his activities to digestion and titration, thus making possible a large number of analyses in a working day.

TOTAL NITROGEN DETERMINATION—ALTERNATIVE METHOD

Of the various methods mentioned, it is believed that the only procedure which has any definite points of superiority over the method described above is that of Brüel, Holter, Linderstrøm-Lang, and Rozits (10). This method is slightly more sensitive, having a claimed accuracy of $\pm 0.005\ \gamma$ as compared with $0.01\ \gamma$ given by the method of Tompkins and Kirk. In addition, it has been applied to samples containing as little as $0.1\ \gamma$ of nitrogen. It is considerably more time-consuming and requires a higher order of manual skill than the method described above. It is less suitable for a large number of analyses but may be useful in a particular research application not covered by the simpler method.

Principle of Method

The sample is digested with sulfuric acid to which is added copper sulfate, potassium sulfate, selenium catalyst, and sucrose. The digest is transferred to a test-tube-type distillation or diffusion vessel in which distillation of ammonia is conducted between the alkalinized sample at 40° C. and an acidified drop which seals the upper portion of the tube. The excess acid is neutralized with standard disodium hydrogen phosphate solution, the excess of which is determined by back-titration with standard hydrochloric acid.

Apparatus

Digestion tubes. These are small test tubes, 40 mm. long, 1.8 mm. in internal diameter, and 2.4 mm. in external diameter, constructed with uniformly thick rounded bottoms.

Pipets. A variety of pipets are employed for different purposes. The sample is measured with any suitable pipet. Digestion mixture is added in two portions by means of two pipets, the Levy semiautomatic pipet shown in Fig. 10, constructed to deliver 4 λ, and the horizontal graduated pipet shown in Fig. 80, constructed to deliver 1 λ in a distance of 10 mm. The dimensions of the pipet used for the transfer of the digest to the distillation tube are particularly critical. If large, the tip tends to allow

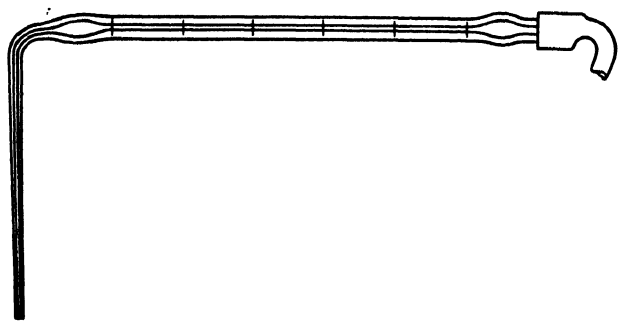


FIG. 80. Horizontal graduated pipet according to Brüel, et al.

digest to flow upward between its exterior and the tube wall. If too small, it is difficult to operate. A pipet of the construction and dimensions shown in Fig. 81 is suitable for this operation.

Sulfuric acid bath. A small bath shown in Fig. 82 is constructed by constricting an insulin ampoule as shown. The bottom is covered with 1 ml. of concentrated sulfuric acid containing 0.4 g. of potassium sulfate. The digestion tubes wet by the acid mixture adhere to the constriction because of surface tension. When not in use the bath is loosely stoppered by means of a glass bulb with a tapered extension which is placed in the mouth of the vessel with the tapered point inside.

Distillation vessels. The vessels in which the distillation is performed are also small test tubes, 25 mm. in length, 4 mm. in internal diameter, and 6 mm. in external diameter. The clean dry tubes are coated with paraffin before use by immersion in a bath of molten paraffin (m.p. 82° C.) heated to 150° to 200° C. Each tube is removed with forceps from the bath, the excess molten paraffin is removed by throwing or knocking it out, and the tube is rotated with a clean towel until the layer hardens. Excess

paraffin on the outside is wiped off. After each use the tubes are cleaned by rinsing with water, then with acetone, next with hot toluene, a second time with acetone, and finally by boiling twice with water. They are stored in a closed container.

Paraffin block. A block of clean paraffin holds rinse water and receives the diluted digest before it is transferred into the distillation vessel.

Water thermostat. A water bath, thermostatically controlled at 40° C., is used for heating the bottoms of the distillation tubes during the distillation.

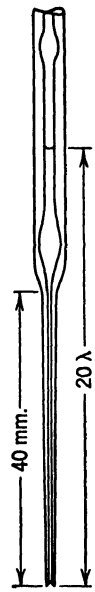


FIG. 81. Special transfer pipet according to Brüel, et al.

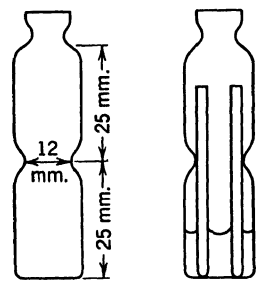


FIG. 82. Kjeldahl digestion bath according to Brüel, et al.

Reagents

1. *Digestion mixture.* The acid mixture used for the digestion is made in two parts which are added separately to the sample.

Solution A contains 1 g. of $CuSO_4 \cdot 5H_2O$, 10 g. of potassium sulfate, 0.2 g. of sucrose, and 5 ml. of concentrated sulfuric acid in 100 ml. of solution.

Solution B is prepared by boiling 1 ml. of concentrated sulfuric acid with 10 mg. of selenium until the mixture is clear.

2. *Sodium hydroxide solution,* 18 *N*, made by saturating a solution with sodium hydroxide at room temperature.

3. *Sulfuric acid,* standard 0.015 *N*.

4. *Indicator-alkali mixture,* made with 0.5 ml. of *M*/15 disodium phosphate, 2 ml. of aqueous bromocresol green containing

0.4 mg. of indicator per milliliter, and 2.5 ml. of freshly boiled water. The mixture must be prepared fresh each day.

5. *Hydrochloric acid,* standard 0.01 *N* solution, containing bromocresol green sufficient to match that in the indicator-alkali mixture.

Procedure

The sample, if liquid, is transferred by pipet to the bottom of a digestion tube; if solid it is placed with a needle. About 4 λ of digestion mixture A is added with a Levy semiautomatic pipet, taking care that the solution does not rise between the pipet tip and the sidewalls of the tube. Considerable care is necessary to adjust the pressure on the pipet correctly so that it discharges automatically when it touches the surfaces of a liquid. The digestion tube is now placed in a vacuum dessicator at about 150 mm. mercury pressure for about 24 hours. The pressure is then lowered to 0.1 mm. and the tube is allowed to remain another 24 hours to evaporate the excess water. The tubes are then placed in a diver clamp, and 1 λ of mixture B is added carefully from the graduated horizontal pipet. Here, also, great care must be taken to avoid creeping upward of liquid to form a seal in the tube.

The sulfuric acid bath is placed in a copper block and heated to 295° C., at which temperature the acid fumes freely but does not boil. The bath is lifted out and cooled for a half minute, after which the digestion tubes are inserted in an upright row around the inside of the constriction. The bath is replaced on the copper block, and for a time the tubes are observed for the formation of gases which cause rising of liquid and formation of a seal. If this happens, the tube is removed immediately and cooled until the gases dissipate. After one cooling, the phenomenon rarely appears again. Digestion usually requires 5 to 6 hours, after which the tubes are removed individually with a conical glass rod which is inserted in the top of each tube. The outside of the tube is rinsed with distilled water, and the tube is placed in a dessicator over phosphorous pentoxide until the distillation of ammonia is started.

The digest is transferred to a distillation tube by the following process: A drop of water (about 20 λ) is placed on a clean paraffin

block. A capillary transfer pipet is used to draw up about ⅓ of this drop, and the pipet tip is inserted carefully to the bottom of the digestion tube, well centered in the tube. The water is expelled into the digest, and the diluted material is sucked into the pipet. The contents are removed and delivered to the paraffin block. Another third of the water drop is taken up with the pipet and introduced into the digestion tube, this time rinsing down the walls. This liquid is removed and added to the diluted digest on the paraffin block. The remaining water is used as before to rinse the last of the digest from the digestion tube.

The combined digest and washings on the paraffin block are now drawn into the pipet which is clamped in a vertical position. The distillation tube is mounted on a simple rack-and-pinion manipulator and raised so as to avoid touching the tips. The diluted digest is placed in the bottom of the distillation tube, usually without rinsing the pipet. To the digest is added in a similar fashion 9 λ of 18 *N* sodium hydroxide, delivered directly into the drop. A water seal (45 λ) is added to the top of the distillation tube as quickly as possible, and to it is added 7 λ of 0.015 *N* sulfuric acid with a semiautomatic pipet. The vessel is covered with a rubber cap and then immersed half-way in the water thermostat at 40° C. After a distillation time of about 1.5 hours, the tube is ready for titration.

The excess acid in the water seal is neutralized with a measured excess of indicator-alkali mixture. By means of a semiautomatic pipet, 18 λ of this mixture is added. The excess disodium hydrogen phosphate is titrated by means of a capillary buret filled with 0.01 *N* hydrochloric acid to which has been added enough bromocresol green so that the indicator concentration at the end point is approximately equal to that in the sample. In order to determine the end point very accurately, a color standard is made by titrating a mixture of 45 λ of water, 7 λ of 0.015 *N* sulfuric acid and 18 λ of indicator-alkali mixture until its color matches that of a citrate buffer solution of *p*H 4.6 also containing the indicator.*

* The authors state that "the color of the indicator is a little different in the citrate buffer and we therefore prefer the titrated blank as a color standard in the titration." It is a little difficult to understand how the insertion of the second citrate buffer standard resolves this difficulty.

AMIDE NITROGEN DETERMINATION

Amide nitrogen is of interest to the biochemical analyst in connection with metabolic studies in which the amide is determined in physiological fluids or culture media. It is also of interest in protein chemistry since the protein molecule contains or yields on hydrolysis a fraction termed the amide nitrogen, at least a large portion of which is contributed by glutamine and asparagine bound in the molecule. Under the conditions of total hydrolysis of protein, it is probable that considerable nitrogen other than amide is split off to form ammonia. For this reason, the amide form of nitrogen in the molecule has been studied by a number of investigators (14–16). There has been no effort made, so far as is known, to develop or use any microgram procedure for this purpose.

Concerning amide nitrogen found in physiological fluids (and tissues), the analytical problem is different from that of the protein molecule. Here it is impossible to use the strong hydrolysis methods because of the presence of urea and possibly other easily split nitrogen compounds. Mild hydrolysis is very likely to fail to hydrolyze all the amide. In some fluids such as urine, the interference of ammonia is serious because it exists in much greater quantities than amide and is released by any procedure that releases the ammonia formed by hydrolysis of amide. Borsook and Dubnoff (5) outline a microgram procedure for determination of amide but provide no data as to its applicability, reliability, or the recoveries to be expected. Investigation of this method in the author's laboratory showed that it gave somewhat low results and considerable variability when tested with asparagine, glutamine, and acetamide. It also hydrolyzed ammonia from urea to a small but definite extent. As expected, the yield was better with the more easily hydrolyzed amides, e.g., acetamide, and poorest with asparagine which is known to be difficult to hydrolyze. Increasing the time of hydrolysis improved the yield and increased the hydrolysis of any urea present. Other procedures tested (15, 16), including alkaline hydrolysis, were not found to be superior, except that hydrolysis in the autoclave at 125° C. for 2 hours in 3 *N* sulfuric acid gave approximately complete recovery with pure amides but decomposed about 50% of any urea present. The procedure given below is a modification of that of Borsook and Dubnoff, which was found to yield approximately theoretical results.

Principle of Method

The sample containing microgram quantities of amide nitrogen is hydrolyzed in a boiling water bath in 3 *N* sulfuric acid for 5 hours. The resulting ammonia is separated by diffusion into standard acid, the excess of which is titrated with standard sodium hydroxide solution.

Procedure

The sample, containing from 1 to 20 γ of nitrogen as amide and in a volume not exceeding 50 λ, is measured into the digestion bulb of the Kirk and Tompkins type of Kjeldahl apparatus (Fig. 70). To this is added 200 λ of 3.5 *N* sulfuric acid, and the vessel is capped by a piece of tin foil. It is placed for 5 hours in a boiling water bath to hydrolyze the amide to ammonia. An appropriate quantity of standard sulfuric acid (40 to 50 λ of 0.02 *N*) is placed in the acid cup, which has been greased on the top rim with a little Vaseline, and laid aside. With a long pipet whose tip has been coated externally with paraffin is inserted 0.2 ml. of half-saturated sodium hydroxide solution to the bottom of the digestion bulb so that it is not mixed with the acid solution. The stopper carrying the acid cup is inserted after the vessel is warmed in a flame, and the hydrolyzate and caustic in the digestion cup are mixed and used to rinse the entire interior of the vessel. The ammonia is allowed to diffuse for about 5 hours at about 37°C., a period somewhat longer than usual because of the rather large volume of liquid. The cup is then withdrawn and the excess acid titrated with standard sodium hydroxide solution about 0.02 *N*; methyl red indicator is used, and the base is delivered in the standard manner from a capillary buret.

The method is entirely analogous to the diffusion method for total nitrogen described earlier, except for the difference in treating the sample which is hydrolyzed instead of decomposed by complete digestion. Blank determinations and calculations are carried out in a similar manner to those in the total nitrogen method.

Remarks

The recovery of amide nitrogen by the method given is expected to be close to theoretical. The danger of low recovery is greater with asparagine and other difficultly hydrolyzable amides than with gluta-

mine, acetamide, or other less-stable material. The indications are that it is applicable to proteins with good recovery. If a large amount of urea is present, it is desirable that it be removed or a correction made by determination of the ammonia formed from a control determination with comparable urea concentration. Presumably, the urea could be destroyed by urease and the ammonia removed by aeration from the solution alkalinized with borate prior to the hydrolysis. If ammonia is present in the sample as ammonium ion, it will be necessary to determine it independently and to subtract it from that found in the amide determination. Alternatively, it may be aerated after the solution is alkalinized prior to hydrolysis. In such operations, it must be remembered that continued exposure of amide to alkaline conditions also causes hydrolysis of the amide. The procedure should be as rapid as possible, and excessive alkalinity should be avoided.

AMINO NITROGEN DETERMINATION

Amino nitrogen is frequently determined by the formol titration which has been shown to be applicable also to determination of microgram quantities. There are various other titration procedures such as that of Linderstrøm-Lang and Holter (17) in which the amino nitrogen compound is titrated in alcohol-acetone solution. The alcohol titration method does not apply to all types of sample and in this respect is inferior to the more generally useful formol titration. A microgram method was first published by Borsook and Dubnoff (5) with use of a glass electrode. An indicator method was later described by Sisco, Cunningham, and Kirk (18) as well as a modification of the glass electrode method. These procedures are described in the following paragraphs.

It is well recognized that formol titration is to a very considerable extent empirical, yielding less than 100% recovery for some amino acids, notably lysine and to a lesser extent several other amino acids including histidine and phenylalanine. The empirical factor is due largely to the fact that the true end points for the individual amino acids do not lie at the same *p*H. In mixtures a single end point *p*H must be chosen, and, depending on its value, some amino acids will be overtitrated and others less than completely titrated. Various indicators changing in the alkaline range may be used. Of these phenolphthalein has given the best results. Thymolphthalein was not suitable on the microgram scale because of a poorly defined end point, as was also

true of several other indicators, most of which did not change at the correct *p*H. Any end point in the alkaline range is subject to considerable influence of carbon dioxide of the atmosphere which causes fading more rapidly with a small volume than with a large, because of the increased relative surface. The glass electrode avoids most of these difficulties when the *p*H is properly chosen and when a carbon dioxide-free atmosphere is maintained over the liquid. Another interference with the formol titration is due to the presence of ammonia which also combines chemically with formaldehyde with a loss of its basic properties.

Principle of Method

The solution containing amino nitrogen is adjusted to the faint pink color of phenolphthalein by means of sodium hydroxide. Formaldehyde is added in excess, and the solution is again adjusted to the pink color of phenolphthalein. The value of the latter titration corresponds reasonably well with the amount of amino nitrogen present. If the glass electrode is used, a carbon dioxide-free atmosphere is maintained, and the titration is performed to an arbitrary end point determined potentiometrically. If ammonia is present, it must be determined and subtracted from the apparent amino nitrogen content.

Apparatus

For the glass electrode procedure a convenient arrangement is shown in Fig. 28. This consists of a double-chamber vessel constructed of plastic (acrylic resin such as Lucite) which contains the glass electrode and reference electrode, a reference half-cell and appropriate openings for introduction of a standard calomel cell, a buret tip, and an inlet for carbon dioxide-free air. The plastic vessel is made in three portions as indicated, which telescope together.

Solutions

1. *Formaldehyde solution,* prepared from Merck reagent-grade formaldehyde, 36 to 38%, diluted with two volumes of redistilled water.

2. *Indicator solution.* Phenophthalein in 0.03% solution in 50% alcohol. This is diluted with an equal volume of water before use.

3. *Standard sodium hydroxide solution,* 0.02 *N*.

Procedure 1 (Indicator Method)

A sample containing from 1 to 20 γ of amino nitrogen is carefully measured with rinsing into a porcelain titration dish. To this is added a 10-λ sample of diluted phenolphthalein solution, and the sample is titrated as rapidly as possible with 0.02 *N* sodium hydroxide, using the capillary buret. When the solution becomes faintly but definitely tinged with pink, a volume of diluted formaldehyde equal to the volume of the sample is added immediately. The mixture is again titrated as rapidly as possible to the phenolphthalein end point. Fading of the color is disregarded since it represents the effect of carbon dioxide. A blank determination is run with distilled water in place of the sample and with all concentrations and volumes of reagents as specified above. This blank is subtracted from the titration value of the determination. In general it is possible to read the buret at the end of the first *p*H adjustment and again at the end of the titration and subtract these values to determine the value of standard sodium hydroxide solution added. If ammonia is present it should be separately determined and subtracted. It is advisable to make determinations with a known amount of ammonia under the same circumstances and to correct the ammonia value to the observed titer because ammonia is not usually determined stoichiometrically by the formol titration.

Procedure 2 (Electrometric Method)

The electrometric method may be run exactly as described above except that the sample is placed on the cup-type glass electrode and the *p*H is determined potentiometrically at all stages. Air which has been passed through a solution of sodium hydroxide to free it of carbon dioxide or, alternatively, a gas containing no carbon dioxide is blown through the gas inlet which is placed in such a manner as to impinge on the edge of the drop, giving a whirling motion to the drop which stirs it. The end point should be chosen between *p*H 8 and 9, depending on the experience and choice of the analyst. A *p*H value of 9 appears to be more nearly correct than the lower value. Borsook and Dubnoff specify preliminary adjustment to *p*H 7, addition of formaldehyde at *p*H 5, and titration of the mixture to *p*H 8. The experience of Sisco, Cunningham, and Kirk indicated that low

recoveries were found with most of the amino acids under these conditions. Variation in the exact conditions of *p*H may be made necessary by the type of sample being analyzed. This is due at least in part to the nature of the amino compounds being titrated. In any case, the electrometric titration is preferable to the color indicator method under the proper conditions. It would appear desirable for the operator to standardize his own conditions prior to determining unknown samples.

Miscellaneous Methods—Nitrogen Fractions

A number of other methods have been described for the determination of quantities of nitrogen in the microgram range, involving a variety of procedures different from those described here in some detail. Among these should be mentioned the hypobromite method of Levy and Palmer (19) who determined as little as 5 γ of ammonia nitrogen without distillation, using the hypobromite reaction with ammonia and iodometric determination of the excess reagent. Similar procedures for larger amounts have often been quite successful, as in the work of Rappaport et al. (20) and particularly Theorell (21), whose naphthyl red titration procedure was modified also by Krogh (22) for determination of ammonia in sea water and air in amounts down to fractional micrograms. Sobel et al. (23) have devised an aeration technique with which they determine total urea and amino acid nitrogen in amounts as low as 1 γ. The separated ammonia is collected in boric acid and titrated acidimetrically. Albanese and Irby (24) have adapted the copper method for amino nitrogen to use with 1 ml. of serum or plasma which places it in the microgram range.

CHLORIDE DETERMINATION

Titrimetric determination of chloride with color indicators has never been completely satisfactory for analysis of biological materials, even when comparatively large amounts of chloride are present. Various modifications of the Volhard and Mohr principles are often applied to blood analysis with reasonable success. On the microgram scale the errors are likely to be so large as to prohibit their use because of the increased importance of end-point and solubility errors. Other procedures using ab-

sorption indicators such as fluorescein and dichlorofluorescein (25, 26) are not suitable because the end points are not sufficiently distinct or because the titration cannot be performed except in almost neutral solution. Close to neutrality, silver may precipitate or coprecipitate various constituents of the physiological system other than chloride, and the careful adjustment of the slight allowable acidity is troublesome when the volumes are very small. Titrations with mercuric ion, such as that of Schales (27), have not been successfully adapted to the microgram scale, though they are often successful with larger amounts. The same considerations apply to the titrimetric method of Sendroy (28). Conway (4) was successful in analyzing chloride by oxidizing it to chlorine which was diffused into potassium iodide solution. Titration of the collected iodine was carried out with thiosulfate down to amounts as low as 7 γ of chloride. In the lower ranges, the volatility of iodine produced in the absorption liquid can introduce a very serious loss, particularly when the solution is spread in a thin layer as in the Conway cell. Titrimetric diffusion methods for quantities of chloride lower than this have not so far been developed, though a colorimetric modification for smaller amounts was described by Conway who made use of the color of starch iodine. Empirical calibration of the method in these low ranges was necessary.

Both Schwarz (29) and Linderstrøm-Lang, Palmer, and Holter (30) have described potentiometric methods which determined microgram quantities of chloride with satisfactory accuracy. The method of Schwarz was not adapted to biological materials, and the lower limit and accuracy of the latter method was not the greatest attainable. Wigglesworth (31) reported the determination of the chloride content of single mosquito larva by a procedure which was quite involved and probably contained relatively large errors. Moreover, the results were in arbitrary rather than absolute units and of undetermined reliability.

Cunningham, Kirk, and Brooks (32) adapted the electrode system of Clark (33) to the determination of amounts of chloride as low as 0.5 γ with an accuracy which was usually about $\pm 0.5\%$. Moreover, it was developed for general use in biological systems and could be applied directly to proteinaceous fluids without ashing or deproteinizing, which is believed to be the first such procedure to be developed.

Principle of Method (32)

The chloride is titrated with silver nitrate in a medium made fairly acid with sulfuric acid. The end point is determined potentiometrically by use of a silver-silver amalgam electrode pair operating as a bimetallic system. Protein need not be removed if the acidity of the medium is adjusted properly to avoid formation and coprecipitation of silver protein compounds.

Apparatus

The electrode system is shown in Fig. 29. The electrodes are made by fusing a ball about 2 mm. in diameter on the ends of each of two pieces of No. 20 silver wire. One electrode is immersed in dilute nitric acid until gas escapes freely, after which it is washed in distilled water. The other electrode is dipped repeatedly into a pool of clean mercury beneath a layer of dilute nitric acid until the wire is well amalgamated. The electrodes are placed in a small holder and connected to the leads of a potentiometer capable of reading to about 0.1 mv. This is attached to a lamp and scale galvanometer drawing about 1 μa per scale division. The electrodes are not used for at least an hour after preparation but may then be used continuously for several days before requiring cleaning and re-amalgamation.

Reagents

1. *Silver nitrate solution,* 0.01 *N*, prepared by dilution of 0.1 *N* silver nitrate solution which has been carefully standardized against sodium or potassium chloride solution. All silver nitrate solutions must be stored in dark bottles. This solution may be diluted further (e.g., 0.005 *N* to 0.001 *N*) for very small amounts of chloride.

2. *Sulfuric acid solution,* 1 *N*, made from redistilled sulfuric acid free of chloride.

Water used in making all solutions must be redistilled from an alkaline solution in an all-Pyrex glass still.

Procedure

The sample, which may contain from 0.5 to 30 γ of chloride, is measured with rinsing into a porcelain titration dish with a capillary pipet. Sufficient 1 *N* sulfuric acid is added to give a final concentration of about 0.3 to 0.4 *N*. The buret tip, stirrer, and

pair of electrodes are lowered until they just dip into the chloride solution. The titration is then carried out by addition of equal increments of silver nitrate solution from the buret. The potential is determined after each addition. The first small addition of silver produces a marked change in potential, after which there is little change until the titration is about 95% complete. Thereafter, small increments should be added, measuring the large changes of potential which occur after each addition. When the potential curve is plotted on a graph, a sharp peak will be observed at the end point. Thirty-second periods should be observed for equilibration after each addition because equilibrium is not established immediately, especially near the end point. Although the electrodes may not be at final equilibrium in this time, the position of the end point is not affected nor is the shape of the curve. The potential obtained from this electrode system is not necessarily reproducible, and different pairs of electrodes invariably give different absolute potentials with the same solution. They do, however, indicate accurately and sharply the position of the end point. Virtually any solution containing chloride may be analyzed by this procedure without ashing or deproteinization. If bromide or iodide are present, they will be titrated ahead of the chloride and will give separate end points.

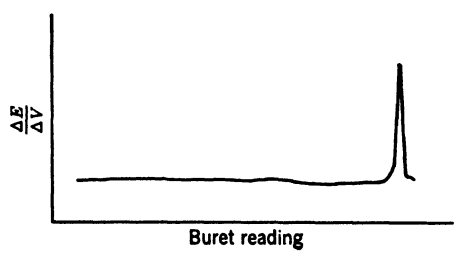


FIG. 83. Electrometric end point, chloride titration.

Remarks

Ordinarily, the strength of the silver nitrate used is 0.01 *N*. If the amounts of chloride are excessively small, the titration volume may be so small that it is not convenient to divide it into accurate increments. If this difficulty is encountered, more dilute silver nitrate may be used satisfactorily. The end point is of the type shown in Fig. 83, in which the end point is indicated by the sharp peak of the curve.

The titration in the presence of protein, as in the direct determination

of chloride in blood serum, was checked originally by comparing the results obtained with those of similar samples ashed in the presence of excess sodium bicarbonate in a platinum crucible. No significant differences were found in the results. Ashing without addition of sodium bicarbonate invariably led to serious loss of chloride.

IODINE DETERMINATION

The microgram determination of iodine in biological materials has always been troublesome because of the very low concentration of this element in all but a few such materials, the most notable exception being the thyroid gland. Most biological materials taken in rather large samples, such as blood serum, yield only fractional microgram quantities of iodine. This necessitates extensive destruction of organic matter, separation of the iodine, and its determination in traces. The latter operation like the digestion is difficult because of the relatively great volatility of iodine which causes significant loss when the volumes of solution are small. The large amounts of reagents usually employed because of the necessity of destroying large quantities of organic matter may lead to the introduction of considerable extra iodine as an impurity.

The great importance of the problem at a time before the principles of microgram analysis were understood even to a moderate extent led to tremendous efforts on the part of many investigators to solve the analytical problem, which in turn gave rise to a voluminous literature (34–40) describing almost every conceivable variation of technique, except perhaps the one which now seems most rational of all, viz., a technique based on the principles of reducing the size of apparatus and amounts of reagent to a scale comparable with the iodine to be determined. Even this logical step inherently fails to some extent when traces are analyzed because of the relatively large samples required. It remains interesting that at this writing no record has been found of a volumetric microgram procedure which utilizes completely the equipment and information available from microgram analytical research of recent years, though some start has been made in this direction. Even without this development, extremely sensitive but comparatively inaccurate methods exist in profusion for the analysis of biological iodine and its fractions, as well as trace iodine in inorganic systems. The most sensitive methods are

either colorimetric or catalytic in nature. The latter have been developed to estimate iodine to a lower limit of 10 to 20 mγ. Since the rate of the catalytic reaction is followed ordinarily by photometric methods, it will not be included in this chapter, nor will the colorimetric procedures. Digestion and distillation of iodine is not different whether volumetric, colorimetric, or catalytic methods are employed.

Digestion of Biological Material

Iodine is commonly determined in from 1 to 10 ml. of plasma or serum, and protein-bound iodine in the precipitated protein from a sample of similar size. It is also determined in smaller weights of thyroid gland, and at times in other tissues or fluids. In nearly all instances, the amount of iodine contained is less than 1 γ, ranging down to as little as 0.01 γ, which may be determined at present only by the sensitive catalytic procedures.

The destruction of organic matter is performed most advantageously by digestion with acid oxidizing agent, in contrast to many early techniques with dry combustion or alkaline fusion. Permanganic (41, 42), chromic (43), or chloric acids (44) destroy the organic matter and oxidize the iodine to iodic acid which is not volatile at the temperatures employed. The procedure for digestion given by Taurog and Chaikoff (45) is perhaps as useful as any available and is given below. Somewhat larger samples are desirable for volumetric determination. Even smaller samples may be analyzed by the catalytic method.

Digestion Procedure. A double-necked round-bottom flask of 300-ml. capacity fitted with standard-taper ground joints for use with a modified Chaney (46) distilling apparatus serves as digestion vessel. To it is added 25 ml. of purified 70% sulfuric acid and 2 ml. of 60% chromic acid, followed by the sample which should contain for volumetric determination at least 0.3 to 0.5 γ of iodine, and a tenth of this amount for the catalytic procedures. The sulfuric acid should be redistilled, with collection of the middle and final fractions only, or alternatively 2 liters of concentrated acid are boiled for 2 hours with 10 ml. of concentrated hydrochloric acid solution to eliminate trace of iodine impurity. All water is redistilled from potassium hydroxide in a glass apparatus. Silicon carbide chips are added to the digestion flask to prevent bumping, and the digestion is carried out until the flask

is approximately filled with fumes. The heat is removed and the flask allowed to cool, after which 15 ml. of redistilled water are added and the flask is reheated as before. The digest is then ready for the separation of iodine.

Other methods which have been found useful for the digestion are the permanganic acid digestion of Riggs and Man (41) who criticize the use of chromic acid on the ground that chromyl chloride may distill along with the iodine, a danger which seems to be eliminated by the heating during and after digestion in the above procedure; and the chloric acid method of Shahrokh (44). Numerous modifications of all basic procedures have been proposed.

Separation of Iodine

Nearly all procedures call for the separation of iodine from the digest, a step made desirable by the large amount of reagents in the latter, including oxidizing agents and excess strong acid. If digestion is not required, as in analysis of simple inorganic systems, the distillation step may also be omitted. Conway (4) states that iodine may be diffused from the solution into a collecting solution of potassium iodide but includes no reference or data on which may be judged the reliability of the procedure. If successful, such a method should be suited to small quantities in comparison with the involved and difficult distillation procedures which are the only ones for which reliable data are available.

The distillation apparatus described by Chaney has been slightly modified by several authors and widely used. The Riggs modification described by Talbot et al. (42) and with the flask capacity specified by Taurog and Chaikoff (45) is shown in Fig. 84. Its operation in separating iodine is as follows.

The digestion flask is allowed to cool, and 25 ml. of redistilled water is added. The flask is attached to the condenser unit, and the dropping funnel containing 3 ml. of 50% phosphorous acid is added to the other neck. The flask is heated until liquid starts to condense in the trap when there is added to the latter 1.2 ml. of 1% sodium hydroxide solution by way of the condenser. This with the distillate seals the trap, after which the phosphorous acid is added slowly from the dropping funnel, the acid being blown into the flask to prevent loss of iodine back through the stopcock. The condenser is turned so as to return the distillate to the flask,

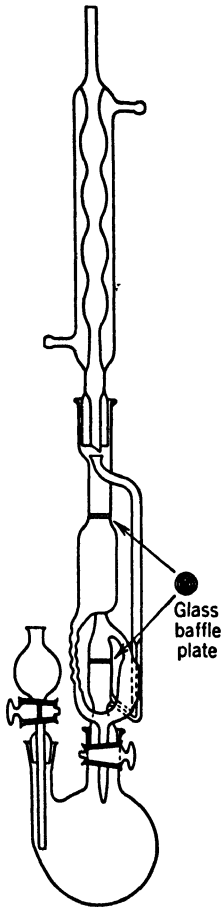


FIG. 84. Modified Chaney distillation apparatus for iodine according to Riggs. (Reproduced from Talbot, Butler, Salzman, and Rodriguez.)

and the distillation is carried on for about 7 minutes. The trapped iodine is then available for final determination by whatever procedure is to be used.

Determination of Separated Iodine (Volumetric)

In many instances, particularly when the sample is small, it is desirable to determine the separated iodine by means of the catalytic procedure* which is much more sensitive than the volumetric titration (47). Hahn (48) followed the catalysis of the reaction between ceric sulfate and arsenious acid by a potentiometric method which he found extremely sensitive but less accurate than the colorimetric method. The range of the procedure was up to a few hundredths of a microgram of iodine. Hahn and Adler (49) extended the study to analysis of salt mixtures, mineral waters, and food. It was not studied in its application to physiological fluids but should be applicable if the reliability of the potentiometric method is comparable with that of the photometric, as should be the case.

When the amount of iodine is as much as 0.5 γ, it may be determined by titration with at least as great accuracy as by other methods. Either the procedure of Groak, as modified by Riggs and Man, or the Kendall (50) titration may be used. Both involve the oxidation of the iodide ion and the determination of the latter as shown by the equation.

$$IO_3^- + 6H^+ + 5I^- \longrightarrow 3I_2 + 3H_2O$$

The iodine released is titrated with standard thiosulfate solution.

* Cf. Chapter 9, for the colorimetric method of measuring the catalytic rate of reaction.

The procedure of Riggs and Man is as follows: The solution from the distillation is evaporated if necessary to 6 or 7 ml. The flask containing it is placed in a water bath at about 70° to 80° C., and 0.2 *N* potassium permanganate solution is added, a drop at a time, until a permanent purple color is obtained. After 4 minutes, 10 drops of 8 *N* sulfuric acid is added, after which the color should persist. After 4 additional minutes of heating, the flask is removed from the water bath, and 0.75 *N* sodium nitrite solution is added, a drop at a time, with constant shaking until there is no further evidence of manganese dioxide and the solution is perfectly clear. An excess single drop of nitrite is added, and the flask walls are rinsed with the solution. To this is added 2 drops of 5 *M* urea solution to destroy the excess nitrite. The flask walls are again rinsed with the solution. The volume is then reduced further on the water bath to about 2 ml. The solution is chilled in ice water, and a drop of 1% stabilized starch solution made from arrowroot starch preserved with a little salicylic acid is added. Freshly prepared 0.2% potassium iodide solution (0.06 ml.) is then added, and the liberated iodine is titrated with 0.001 *N* sodium thiosulfate solution added from a microburet. This titration may be made advantageously with a capillary buret with somewhat stronger thiosulfate than prescribed. The flask is chilled again just before the end point and is titrated with great care to match a similar flask containing only 2 ml. of water and a drop of the same starch solution.

The greatest care must be taken in the preparation of reagents to ensure the essential absence of iodine. A blank must be run in exactly the same manner to correct for traces of iodine and other factors producing errors. A negative error of about 0.02 γ of iodine was found routinely, owing to the limited sensitivity of starch to minute amounts of iodine. The operator should compare the observed and theoretical titers of known iodine solutions to determine the correction.

The Kendall titration is equally applicable to the final determination of iodine (51, 52), though it has been criticized by Riggs and Man on the ground that there is a danger that the last traces of bromine will not be destroyed. The distillate is reduced in volume as described above to about 2 ml. and neutralized with a slight excess of acetic acid or sodium dihydrogen phosphate to produce a slightly acid buffered solution. A little bromine water

or a droplet of liquid bromine is added, and the solution is heated just under boiling until nearly all the bromine has been volatilized. A drop or more of dilute phenol solution is added to dispel the last of the faint bromine color, and the solution is shaken and warmed for a few minutes more, after which starch and potassium iodide solution are added as described and the resulting iodine is titrated with standard 0.001 *N* thiosulfate solution (stronger, if a capillary buret is used).

PHOSPHATE DETERMINATION

The volumetric determination of phosphate described by Lindner and Kirk (68) may be performed with microgram quantities of phosphorus as small as 0.5 γ. With a few micrograms of phosphate phosphorus, the procedure is ordinarily accurate to about 0.5% and is a very reliable method. It is as sensitive as any of the common colorimetric or spectrophotometric methods but is technically less rapid and somewhat more difficult to perform. Capillary cell spectrophotometry greatly increases the sensitivity. The procedure here described is modified from the milligram method of Pregl and adapted to manipulation of very minute quantities.

Principle of Method

The phosphate is precipitated in the form of ammonium phosphomolybdate. This is separated from the solution by filtration, washed, and dissolved in standard sodium hydroxide solution, neutralized with excess standard acid, the excess of which is titrated with standard sodium hydroxide solution.

Reagents

1. *Sulfate molybdate solution.* Five grams of ammonium sulfate are dissolved in 50 ml. of concentrated nitric acid (d. 1.36); 15 g. of ammonium molybdate are dissolved in 40 ml. of boiling water. The molybdate solution is cooled and poured with stirring into the nitric acid. The resulting solution is diluted with water to 100 ml. and allowed to stand for about 2 days, after which it is filtered through a sintered-glass filter into a glass-stoppered brown bottle.

2. *Nitric-sulfuric acid mixture.* To a mixture of 42 ml. of concentrated nitric acid with 58 ml. of water is added 3 ml. of concentrated sulfuric acid.

3. *50% ethyl alcohol solution.*

4. *Standard sodium hydroxide,* approximately 0.1 *N*. This solution is made from a carbonate-free concentrated sodium hydroxide solution which is diluted with carbon dioxide-free water and accurately standardized It should be used to fill a small glass-stoppered reagent flask from which samples are removed as needed. The content of this flask may be discarded frequently to avoid error due to absorption of carbon dioxide from the air.

5. *Hydrochloric acid solution, about* 0.1 *N*, made by diluting c.p. concentrated hydrochloric acid and accurately standardizing it. To the hydrochloric acid is added sufficient phenolphthalein to yield a satisfactory end point when titrated on the microgram scale.

If a standard phospate solution is required, twice recrystallized and dried potassium dihydrogen phosphate is weighed accurately and dissolved in redistilled water.

Procedure 1

If the phosphate exists in the form of inorganic salt, the analysis is conducted as follows: A sample containing from 0.5 to 10 γ of phosphorus is measured into a titrating dish in the usual manner with a capillary pipet which is rinsed. An equal volume of nitric-sulfuric acid mixture is added, followed by four volumes of molybdate reagent. The solution is mixed and allowed to remain at room temperature overnight to ensure complete precipitation. It should be covered with a small, rather tight dish to prevent evaporation. The precipitate should be yellow, containing no visible white molybdic acid. It is filtered with an asbestos-coated filter stick as described previously and washed with at least 20 drops of 50% alcohol. The asbestos pad and precipitate are transferred back into the original dish with several drops of redistilled water. A measured excess of 0.1 *N* sodium hydroxide is added, and the mixture is heated for about 5 minutes with stirring at just below boiling. An equivalent amount of the standard 0.1 *N* hydrochloric acid is added, followed by titration of the excess acid with the standard sodium hydroxide to the pink phenolphthalein end point. During the titration the solution is stirred and heated mildly and continuously to the end. The end point is not particularly sharp and tends to fade because of the

effect of carbon dioxide of the air. It should be reached as rapidly as possible, and the titration should be stopped immediately when the pink color is uniform throughout the solution. A blank is run on the reagents in the same manner, with distilled water instead of sample. The blank value is subtracted from the final buret reading. The total amount of sodium hydroxide solution in terms of microequivalents minus the total amount of hydrochloric acid in the same terms is a measure of the phosphate present. Since 28 moles of sodium hydroxide are used for each gram-atom of phosphorus, the number of microliters of 0.1 *N* sodium hydroxide multiplied by 0.1107 yields the number of micrograms of phosphorus present.

Procedure 2

Biological materials usually require preliminary destruction of organic matter prior to analysis. This is readily accomplished by ashing of the sample in the presence of a little sodium carbonate in a platinum dish in the micro muffle furnace. The temperature should remain at 450° C. or less until a white ash results. The alkaline ash is dissolved in about 50 λ of the nitric sulfuric mixture and transferred to a micro porcelain dish with a capillary pipet, using several water rinsings. The solution is then analyzed by Procedure 1 above. Acid digestion may also be used, but it is less convenient and more time-consuming than the ashing procedure.

REDUCING SUGAR DETERMINATION

Of the volumetric methods described for determination of small amounts of glucose in blood, or of reducing sugars in general in physiological material, the most satisfactory for reduction to the microgram scale have depended on oxidation of the sugar by ferricyanide. Titration of the ferrocyanide formed by means of ceric sulfate as originally proposed by Whitmoyer (53) has proved simple and reproducible. This procedure was adapted to small amounts of blood (0.1 ml. containing reducing sugar to the extent of about 0.1 mg.) by Miller and Van Slyke (54), Giragossintz, Davidson, and Kirk (55), and Vanossi and Ferramola (56). Heck, Brown, and Kirk (57) adapted the method to the determination of 1 to 12 γ of glucose in blood, with a reproducibility of 1% or better with nearly all samples. This corresponds approxi-

mately to the glucose in 1 to 12 λ of normal blood. Stern and Kirk (58) revised the procedure to reduce the blank and improve the reproducibility. The method, like all glucose methods except fermentation procedures, is empirical. Also in common with other chemical methods, it determines the amount of reducing substances that will include other reducing sugars if present, and certain other reducing compounds found in blood and physiological fluids. In this respect it is comparable with most or all other chemical methods, regardless of the scale of analysis.

Principle of Method

The oxidation of glucose or other reducing sugar is performed by heating the sample for 5 minutes with an excess of potassium ferricyanide alkalinized with sodium carbonate. After acidification with sulfuric acid, the ferrocyanide formed is titrated with standard ceric sulfate. The method is calibrated against known quantities of glucose, 1 ε of ceric sulfate being equivalent to approximately 39 γ of glucose, depending slightly on the exact technique and conditions. The choice of deproteinization reagents is important, as has been shown in various publications. The modified (55) copper tungstate method of Somoygi (59) is chosen because it has been shown to remove most of the reducing non-sugar components that cause high values and because it is readily used with microgram techniques.

Reagents

1. *Glucose standard solution,* made by dissolving 100 mg. of pure glucose and diluting to 1 l.

2. *Potassium ferricyanide solution,* 0.8%, containing 2% sodium carbonate. The reagent is stored in the cold and should be made fresh each week.

3. *Sulfuric acid solution,* 10% by volume. Redistilled sulfuric acid and redistilled water should be used.

4. *Standard ceric sulfate solution,* about 0.01 *N*; standardized against pure, dry potassium ferrocyanide made in fresh solution.* The ceric sulfate solution is stored in a clean all-glass container.

5. *Indicator solution,* 0.05% Setopaline C solution made and used as described previously;* or 0.0025 *M* ferrous *o*-phenanthroline solution made and used as described previously.*

* Cf. Chapter 5.

6. *Copper sulfate solution,* 10%, made by dissolving 10 g. of $CuSO_4 \cdot 5H_2O$ and diluting to 100 ml. with redistilled water.

7. *Sodium tungstate solution,* 10%, made by dissolving 10 g. sodium tungstate and 2 g. of sodium carbonate together and diluting to 100 ml. with redistilled water.

Apparatus

In addition to the usual titration equipment, a series of "sugar tubes" is prepared as follows. A series of small flared-top test tubes about 35 mm. in length is prepared from glass tubing of 3-mm. internal diameter.

A small water bath in which to heat the sugar tubes is made from a 100-ml. beaker fitted with a sheet-metal top containing holes for the sugar tubes, the latter being supported in the holes by the flare at the top.

Procedure

The sample containing from 1 to 12 γ of glucose (or other reducing sugar) and free of protein and significant amounts of other readily oxidized substances is measured by capillary transfer pipet into the bottom of a sugar tube. To the sample is added about 40 λ of ferricyanide-sodium carbonate solution, and the tube is inverted and twirled between the palms of the hands until thorough mixing is obtained. It need not be stoppered for this operation. If liquid adheres to the walls, the tube should be centrifuged briefly. It is inserted in boiling water in the special water bath for 5 minutes. The timing is not critical so long as the blanks are heated the same time as the sample.

After being heated, the tube is withdrawn from the bath and cooled in cold water. The content is removed with a long-tipped capillary pipet, paraffined on the outside, and placed in a porcelain titration dish. A drop of water is taken by the pipet from the tip of a wash bottle. This drop of water is used to rinse the pipet and then is transferred to the sugar tube to rinse it. The water is transferred to the dish, and the rinsing is repeated a second time. To the dish is added 50 λ of 10% sulfuric acid solution, and 5 λ of indicator, measured with a 5-λ pipet. The ferrocyanide is then titrated in the usual manner with 0.01 *N* ceric sulfate delivered at a reasonably constant rate from a capillary buret. All pipets for this method should be rinsed,

either with the reagent measured (quantitative) or distilled water (transfer), and employed wet. If they are dried, all air drawn through them must be filtered, because deposition of any dust causes erratic values. Blank determinations are necessary and should be run along with the analytical determination, distilled water substituting for sample. The blank titrations are subtracted from those of the analyses as a correction.

Physiological Fluids

Materials such as blood which contain protein and frequently non-sugar reducing substances should be deproteinized by a method tending to remove non-glucose reducing substances. The procedure for blood is as follows: The blood sample, usually 10 λ, is measured into a deproteinization tube (Fig. 52) of approximately 10 times the sample volume (e.g., 100 λ). To it are added the rinsings of the pipet, an equal volume of 10% copper sulfate which is mixed, followed by an equal volume of 10% sodium tungstate made with 2% sodium carbonate. The deproteinization tube is filled exactly to the mark with redistilled water, and the entire content of the tube is thoroughly mixed with the magnetic thread stirrer. The tube is then centrifuged to pack the protein precipitate, and aliquots of the supernatant solution are taken for analysis as described above. The exact dilution and the aliquot volume must be considered when the glucose content of the blood is calculated.

It is necessary that the operator calibrate the method in terms of pure glucose under his particular conditions of working, an operation which amounts to standardizing the ceric sulfate against glucose. It has been found that different operators vary only slightly in the factors they obtain, but the variation found may be significant and should be considered in the calculation. The factor is determined by running analyses of pure glucose solutions in the range of the unknowns and calculating the factor in terms of γ glucose/ϵ ceric sulfate. This value varies slightly in the region of 39 γ/ϵ.

Remarks

The analyst must realize that all chemical methods for glucose analysis are empirical to a considerable extent, i.e., the exact reaction for oxidation cannot be written and a theoretical factor established. Many methods show a variable factor depending on the amount of glucose

present. The method described shows no regular variation with the amount of glucose, so the factor is reasonably constant over the range. It may vary, however, with the exact conditions of the determination. Thus the temperature of boiling water varies with altitude; reagents are not of identical purity and strength, etc. For these reasons the method requires calibration by the operator, but, once determined, the factor values found should remain essentially constant. New batches of reagent or other significant changes in the conditions of the analysis make necessary a redetermination of the factor and the blank. The magnitude of the latter was shown by Stern and Kirk to depend strongly on the amount and age of ferricyanide and on its alkalinity. Large and erratic errors in both blank and determination were at times caused by dust or fumes deposited in the pipet during drying with an air stream.

DETERMINATION OF SUCROSE AND POLYSACCHARIDES

The determination of polysaccharides in general and sucrose in particular requires separation from or correction for reducing sugars and hydrolysis to monosaccharides which can be determined by the procedure given for reducing sugars. If the amount of reducing sugar is low, it may be corrected as a blank instead of being separated. Sucrose may be extracted from plant material by use of alcohol (60), and other polysaccharides are removable with various solvents, e.g., glycogen is commonly removed from tissues by strong caustic solution or by trichloroacetic acid extraction. The method will be given in detail here only for sucrose, but it should be understood that reducing sugars which are formed by hydrolysis of other polysaccharides can be determined in an analogous fashion.

Principle of Method (58, 61)

The polysaccharide in solution is hydrolyzed with dilute hydrochloric acid, and the resulting reducing sugar is determined exactly as described above. Sucrose is extracted from plant material with alcohol in a special extractor.

Reagents

1. *Hydrochloric acid,* 5% solution made by diluting one part of concentrated acid with five parts of water.

2. *Sodium hydroxide-sodium carbonate solution.* A solution of sodium hydroxide is made isonormal with the hydrochloric

acid above by titration and adjustment. Sodium carbonate is added to it to bring it to a concentration of 10% in this constituent.

Hydrolysis Procedure

The sample containing sucrose in quantities of a few micrograms is placed in a micro test tube, as described for glucose, and an equal volume of hydrochloric acid is added. The mixture is heated in a water bath at 69° C. for 10 minutes. At the end of this time the tubes are cooled and the contents neutralized by adding an equivalent volume of the sodium hydroxide-sodium carbonate reagent to that of the hydrochloric acid originally present. The neutralized material is then treated with alkaline ferricyanide and determined exactly as described for glucose. The equivalence factor should be determined by analysis of known sucrose standard solutions.

Extraction of Sucrose

An extractor shown in Fig. 60 is used for removal of a few micrograms of sucrose from small amounts of plant or other materials containing this constituent. The weighed sample of solid plant material is placed in the small conical extraction cup, which may be made of glass or of platinum. In the bottom of the extraction cup is placed a small wad of cotton to retain the solid material. In the detachable bulb is placed about 200 λ of 80% ethanol, and the entire extractor is heated by means of a loose resistance wire wrapped around both the bulb and chamber, the temperature of which is controlled by a variable voltage transformer. The proper adjustment of heat allows a uniform and smooth refluxing of the alcohol through the sample being extracted. The method of heating prevents significant hold-up on the walls and keeps most of the solvent condensed. From 5 to 24 hours of extraction was generally used by the authors of the method, but the exact time depends on the circumstances. Six hours gives complete extraction with onion root tips.

After extraction, the alcohol is evaporated to about 10 λ in place in the bulb. To accomplish this, the condenser water is halted, the cup removed, and a vacuum line attached to the extractor. The solution is transferred with washing to a micro sugar tube.

The extract from plant materials requires clarification with

lead which is carried out as follows: To the evaporated extracts in the tube is added 5 λ of saturated neutral lead acetate, and the sides of the vessel are rinsed with approximately the same volume of water. Ten microliters of saturated disodium phosphate is then added, well mixed, and allowed to stand for 5 or 10 minutes. The precipitate is centrifuged 5 to 10 minutes in the air centrifuge at high speed. The material is then filtered by one of the procedures which has been described in an earlier chapter or with the filter shown in Fig. 85. This consists of a capillary tube with a slightly enlarged bore at the top, over which is placed a piece of filter paper cut with a paper punch. The material to be filtered is delivered by a capillary pipet, the tip of which barely touches the paper at the center while suction is applied. The suction draws the solution through almost instantaneously, collecting the solid material on the surface. The filter can be washed with a little water, and excellent recovery of the solution is obtained. The clarified liquid is then subjected to hydrolysis and determination of the hydrolyzed reducing sugars. Aliquots of the original may be used when the sugar content is sufficiently high. The results are corrected by application of a blank determination, as with glucose, and are subjected to arbitrary standardization against known quantities of the carbohydrate. The results are of the same order of accuracy as described for glucose determination and are applicable in the same general range.

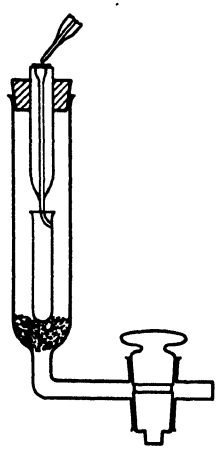


FIG. 85. Filter apparatus for collection of filtrate. (½ actual size.)

LACTIC ACID DETERMINATION

Aside from certain colorimetric methods for the determination of lactic acid (62), only one titrimetric method is known to have been developed for the determination of microgram quantities of this material, though Winnick (63) adapted the ceric sulfate oxidation of Gordon and Quastel (64) for application with the Conway cell to determine fractional milligram quantities of lactic

acid. The method of McCready, Mitchell, and Kirk (65) is a microgram modification of the titrimetric methods of Friedmann, Cotonio, and Shaffer (66) and Friedmann and Graeser (67). The method was tested in the range 1 to 25 γ of lactic acid and was found to yield average recoveries of better than 98% with a variation which was usually less than ±2%, an occasional value being greater. This is not less accurate than the method from which it was modified, even though it involved a reduction in scale of two to three orders of magnitude.

Principle of Method

The lactic acid in the sample is oxidized in a boiling solution by the slow addition of potassium permanganate to the sample containing manganous sulfate, and acidified with phosphoric acid. The oxidation produces acetaldehyde which is aerated and distilled past a reflux condenser which returns most of the water and certain interfering volatile products to the boiling solution. The acetaldehyde is collected in a sodium bisulfite solution. The excess bisulfite is titrated to a starch-iodine end point with iodine solution. The acetaldehyde-bisulfite compound is then dissociated by addition of sodium bicarbonate solution, and the liberated bisulfite is titrated with standard iodine solution to the same starch-iodine end point. Physiological solutions require deproteinization and removal of sugars which interfere. This is accomplished by a zinc hydroxide precipitation of protein followed by precipitation of sugars with copper sulfate solution and calcium hydroxide suspension, the two precipitates being removed simultaneously.

Apparatus

The distillation apparatus is shown in Fig. 86. The reaction vessel *A* is equipped with a capillary inlet from a funnel *C* through which reagents are added, an air inlet attached through a ball-and-socket ground joint to a source of air pressure, an outlet to the atmosphere, and a rubber bulb *B*. The capacity of the bulb *A* is about 8 ml. The outlet from the reaction chamber *A* carries a small reflux condenser and a delivery tube to the titration chamber *E*. A small bulb *d* serves as a trap to prevent sucking back of reagent from *E* during distillation.

Reagents

1. *Potassium permanganate solution,* approximately 0.04 *M.*

2. *Catalyst solution,* prepared by dissolving 10 g. of manganous sulfate and 2.5 ml. of 85% *o*-phosphoric acid and diluting it to 100 ml.

3. *Sodium bicarbonate solution,* saturated at room temperature.

4. *Sodium bisulfite solution,* about 0.1 *M,* made fresh each week.

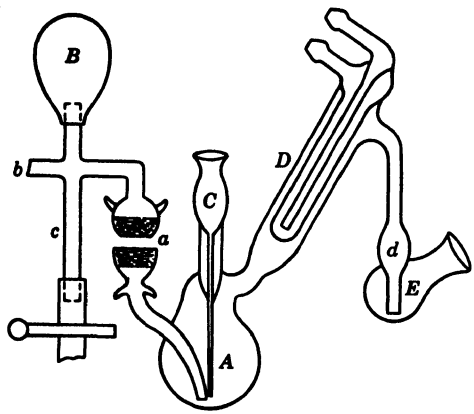


FIG. 86. Lactic acid apparatus. (½ actual size.)

5. *Iodine-potassium iodine solution,* approximately 0.05 *M* in iodine, made with the smallest practical amount of potassium iodide to dissolve the iodine. No standardization is necessary.

6. *Iodine-potassium iodide solution,* standard, approximately 0.01 *N.* This is restandardized daily by titrating a measured quantity of standard thiosulfate solution with the capillary buret in the receiver of the distillation apparatus so as to duplicate the end-point appearance.

7. *Sodium thiosulfate solution,* standard, approximately 0.01 *N.* This is standardized against a standard potassium iodate solution, with a 10-ml. buret.

8. *Potassium iodate solution,* standard, 0.0100 *N,* made by dissolving the required quantity of pure iodate, accurately weighed and made to volume.

9. *Starch indicator solution,* 0.5%, made fresh every second day.

10. *Standard lithium lactate solution,* made by dissolving

0.0375 g. of pure dry salt and diluting to 100 ml. This will be equivalent to 0.35 γ of lactic acid per 1 λ of solution.

11. *Zinc sulfate solution,* containing 10 g. of $ZnSO_4 \cdot 5H_2O$ in 100 ml.

12. *Sodium hydroxide solution,* 0.50 *N*.

13. *Copper sulfate solution,* containing 10 g. of $CuSO_4 \cdot 5H_2O$ in 100 ml.

14. *Calcium hydroxide suspension,* made by shaking 5 g. of pure calcium hydroxide with 100 ml. of distilled water.

Procedure

A sample, such as blood filtrate freed of sugars or of zinc lactate solution, is added with the capillary pipet to the distillation apparatus, followed by the addition of 100 λ of catalyst solution. The solutions are rinsed into the bulb with 2 or 3 ml. of distilled water, to fill the bulb about half full. A little distilled water is added to the receiver just to cover the end of the delivery tube. Low-pressure compressed air which has been washed through strong sodium hydroxide solution is then admitted through tube *b* to give an aeration rate of about 1 bubble per second in the receiver. The pressure of the air may be controlled nicely with a pressure regulator of the type described by Pregl for carbon and hydrogen combustion. The distillation bulb is then heated with a small flame from the burner shown in Fig. 53 to give a boiling rate such that only the lower inch of the reflux condenser is used in condensing steam. When the boiling and aeration are adjusted satisfactorily, about 50 λ of sodium bisulfite solution is added to the receiver. Then, about 90 λ of 0.04 *M* potassium permanganate is run into the capillary cup *C* and added to the solution over a period of about 20 seconds. The distillation and aeration are continued for about 10 minutes, after which the flame is removed and the aeration continued to prevent sucking back of the receiver solution.

To the contents of the receiver is added 50 λ of 0.5% starch solution to serve as indicator. With a pipet, approximately 0.05 *M* iodine solution is added carefully until a definite blue color is produced throughout the solution. Aeration is then stopped, and the rubber bulb is used to stir the receiver solution by pumping it up and down in the delivery tube and trap. With a capillary pipet, 0.01 *N* sodium thiosulfate is added carefully until the

blue color is just dissipated, the solution being stirred continuously. The tip of the capillary buret charged with 0.01 *N* iodine solution is then inserted, and the solution is again titrated to a definite, faint blue. During this and the subsequent titration, good illumination from above and a non-reflecting white background are essential. Unglazed paper or cotton is suitable for a proper background. After the first end point is obtained, which represents the exact destruction of the excess bisulfite, the buret is read. To the solution is then added 100 λ of saturated sodium bicarbonate solution to decompose the aldehyde-bisulfite complex. The bisulfite released is then titrated to the same end point as at first. The difference in the buret readings gives the lactic acid in terms of standard iodine solution, one mole of lactic acid requiring two equivalents of iodine to oxidize the bisulfite corresponding to the acetaldehyde formed in the oxidation.

A blank determination must be made for each set of reagents. The blank titration is to be subtracted from that of the determination. Blanks found by the authors were about 0.6 λ of 0.01 *N* iodine solution.

Blood Analysis. Blood contains protein and glucose, both of which interfere with the lactic acid determination. These constituents must be removed before the analysis can be carried out. A suitable sample for analysis is 100 λ of whole blood or serum.

The sample is measured into a 2-ml. calibrated deproteinization tube. The pipet is rinsed with two fillings of water which are added to the tube and mixed. With the same pipet is added one volume of 10% zinc sulfate solution, which is mixed, and then a pipet full of 0.50 *N* sodium hydroxide solution. A final mixing completes the precipitation of protein. Without separation of the latter, a volume of 10% copper sulfate equal to that of the blood is added and mixed, followed by one volume of freshly shaken 5% calcium hydroxide suspension. The entire mixture is stirred thoroughly, diluted to the mark with distilled water, and stirred again. The precipitated protein and glucose are removed together by centrifuging. Aliquots of the supernatant are taken for analysis. The size of sample depends on the lactic acid content. Fresh normal blood may require a sample of 1 ml., whereas bloods containing higher lactic acid content are reduced in size. Between 5 and 20 γ of lactic acid is a convenient range for analysis. The determination is made as described above.

Alternative Procedures. Aside from colorimetric determinations, it appears that no other method so far described operates in a range as low as that described above. However, certain other methods for larger quantities may well be subject to refinement which would place them in the full microgram range. This appears to be especially true of the diffusion method of Winnick (63), also described by Conway (4). In this method, 1 ml. of blood is used. The protein and glucose are precipitated and removed essentially as described above. The clear supernatant solution is placed in the outer chamber of a Conway diffusion unit. An appropriate amount of sodium bisulfite (1 to 1.5 ml. in the method as described) is placed in the inner chamber. To the sample is added a saturated solution of ceric sulfate in 2 *N* sulfuric acid, and the unit is immediately sealed. The unit is allowed to stand for 5 hours at room temperature or 2 hours at 50° C., after which the titration is performed essentially as described above. It appears that the small glass Conway-type diffusion unit would be suitable for determining microgram quantities of lactic acid by this method and the reagents and sample size might well be adjusted down to the required range. No experimental verification of this suggestion has been noted at this writing.

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CHAPTER 8

Volumetric Gas Methods

Few truly gasometric methods for application in microgram chemical analysis have been developed. Both gas analysis and respirometry have been reduced to the microgram scale for a considerable time. Of all the gasometric techniques in biochemical analysis, those based on the Van Slyke and Neill apparatus (1) and the Warburg apparatus (2) are the best known and most widely employed. Neither of the above general procedures can be considered to apply in the microgram range, and few modifications of them to this scale have been developed, though an attractive field for such modifications exists.

The gasometric determination of amino nitrogen on the microgram scale was described by Sandkuhle, Cunningham, and Kirk (3). It provided a clear demonstration that gasometric chemical analysis of microgram quantities is entirely practical and indicated that further developments along similar lines may well be made. The method is described below.

GASOMETRIC AMINO NITROGEN DETERMINATION

Principle of Method

The sample containing primary amino nitrogen is reacted with nitrous acid in a closed container from which all air has been removed. The gases generated consisting of nitrogen from the reaction

$$HONO + RNH_2 = ROH + N_2 + H_2O$$

and nitrous oxide, along with a little carbon dioxide introduced with the sample to lower the blank, are then exposed to alkaline permanganate solution. The latter absorbs all the gases except the nitrogen, which is transferred to a calibrated capillary tube where the volume is measured at atmospheric pressure. The reactions are identical with those described by Van Slyke (4).

Apparatus

The apparatus consists of the simple gas reactor shown in Fig. 87. The reaction chamber *RC*, with a capacity of about 6 ml., carries an outlet at the top through a three-way stopcock S_2 to two filling funnels R_1 and R_2, with capacities of approximately 5 ml. and 0.5 ml., respectively. To the bottom of the

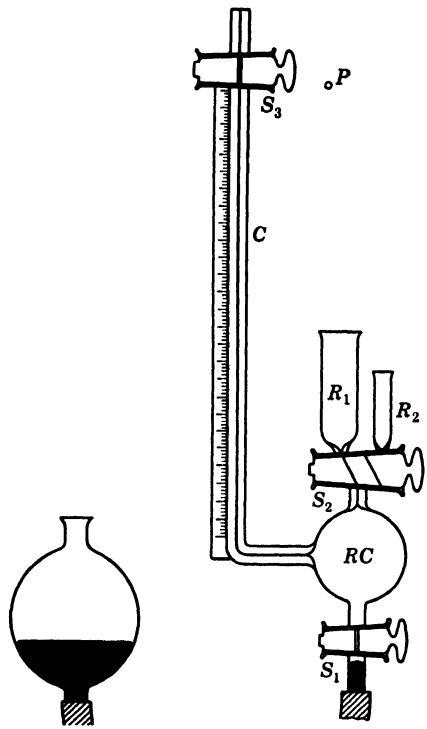


FIG. 87. Gasometric amino nitrogen apparatus. (½ actual size.)

reaction chamber is attached a two-way stopcock S_1 leading to a mercury-leveling bulb. From the side of the reaction chamber a capillary leads to a calibrated capillary *C* terminating in a stopcock S_3. The entire apparatus is mounted on a back which is suspended at a point *P*, allowing the entire apparatus to be shaken and removed from the suspension so that it may be turned in any position desired. Shaking may be performed by hand or by a small motor.

Reagents

1. *Sodium nitrite solution,* 800 g. dissolved to make 1 l. of solution.
2. *Glacial acetic acid.*
3. *Alkaline permanganate solution,* made by saturating a 10% sodium hydroxide solution with potassium permanganate.

Procedure

The capillary C is first filled with water which is locked in place by closing the stopcock S_3. With stopcock S_2 opened to connect the reaction chamber with R_2, mercury is raised until it just enters R_2, and the stopcock is closed. About 150 λ of sodium nitrite solution is placed in R_2 and drawn down until nearly all of it is in the reaction chamber. About 70 λ of glacial acetic acid is then placed in R_2 and drawn almost completely into the reaction chamber. The remainder of the acid is rinsed into the chamber with about 0.4 ml. of distilled water which has been treated by bubbling carbon dioxide through it for 10 or 15 minutes to remove nearly all the dissolved air. With S_2 closed, the leveling bulb is lowered for about 15 seconds, and the evolved gas is expelled through R_1. This is repeated to remove air from the chamber.

The sample, which may contain 0.5 to 20 γ of amino nitrogen, is measured into the small filling funnel R_2, and the sample and rinsings of the pipet are drawn carefully into the reaction chamber. Several 75-λ portions of distilled water saturated with carbon dioxide are added to rinse all the sample into the reaction chamber, care being taken that no air is admitted during the rinsing.

The apparatus is shaken by hand or by motor at an even, unhurried rate for 5 minutes, the mercury in the chamber giving effective stirring without vigorous shaking. At the end of this agitation the mercury is lowered just below the stopcock S_1 which is then closed. Slightly more than 5 ml. of alkaline permanganate reagent is then added to funnel R_1, taking care that no air is entrapped. This funnel is then connected with the reaction chamber by opening stopcock S_2, and the permanganate is sucked in until most of the excess gas is absorbed. The apparatus is then detached from its support and shaken in a horizontal position for 4 to 5 minutes to complete the absorption of the last traces of nitrous oxide and carbon dioxide. The pressure is

equalized with the atmosphere by opening S_2 to connect with R_1 which still contains some permanganate. By rotation of the apparatus, the remaining bubble of nitrogen is trapped at the opening of the side capillary. The leveling bulb is raised slightly, and S_1 is opened; S_3 is then opened slightly to move the gas bubble into the capillary for measurement. S_1 is then closed, with S_3 left open, and the apparatus is laid in a horizontal position, which places the gas under exactly atmospheric pressure. The volume of gas is determined from the scale on the capillary in comparison with a previous calibration of the capillary. The original apparatus employed two sizes of capillary, the lower half of 1-mm. diameter, the upper of 0.5 mm. The portion used is determined by the amount of gas to be measured.

A blank determination to correct for air dissolved in the reagents and sample is run and subtracted from the volume determined. A new blank should be determined when new carbon dioxide-saturated water is used. Calculation is performed as in the original Van Slyke method. The corrected gas volume is converted to standard conditions of temperature and pressure and converted to amino nitrogen by calculation or by use of standard tables.

Remarks

The recoveries found by the authors of the method were not significantly different from those of the original Van Slyke method with most of the amino acids tested, though amounts ranging from 0.5 to 25 γ of nitrogen were determined. Eight representative amino acids analyzed gave recoveries ranging from 96.0 to 104.6%, with standard errors usually less than 2%. Lysine, which is recognized as yielding inaccurate results with the nitrous acid procedure, gave recoveries of 72 to 76% at room temperature and 88% at 37° C. The recovery claimed by Van Slyke for this amino acid was 85.2%.

MICROGRAM GAS ANALYSIS METHODS

Numerous techniques for micro gas analysis have been published, some of them quite convenient and yielding a reasonable accuracy (5). Since 1 λ of the common gases weighs from 1 to 2 γ, it is apparent that, unless the gas analysis methods are applicable to microliter volumes, they do not fall strictly in the microgram range. Few procedures have been developed in this range since most of the micromethods involve samples of the

order of 0.05 to 0.1 ml. of gas and are accurate to a few tenths of a per cent, 0.1% of a 0.1-ml. sample corresponding to 0.1 λ. The technique of Scholander and coworkers is notable in connection with true microgram gas analysis since it combines comparative simplicity with a reasonable accuracy and considerable ease of operation. It has been developed almost entirely for those gases involved in respiration, viz., carbon dioxide and oxygen. It is not unlikely that the technique may eventually be extended to a more general system of gas analysis. Clearly, microgram gas analysis for the respiratory gases will be useful where, for example, an organism has respired in a small and relatively enclosed space such as a capillary tube. As will be seen later, the technique can be modified to measure blood gases of the same order of magnitude and is applicable already to biological studies in a much broader sense than as gas analysis alone.

The development of these ultramicro gas analysis methods rests on a specialized capillary buret in which a microplunger is operated by a micrometer. A piano-wire plunger, 0.304 mm. in diameter, used as a microspindle, allows the buret to deliver volumes with a measurable sensitivity of 0.0007 λ. The technical details of transferring, storing, and sampling of gas volumes which may be smaller than 1 λ require a high degree of constructional and manipulative perfection. For example, all operations must be viewed through a low-power stereoscopic microscope, and it is desirable to make use of a limited amount of manipulative equipment. Because of these requirements, the description of the apparatus and technique is most critical and requires rather detailed treatment.

Principle of Method (6)

The sample of gas is taken by means of a special pipet. The sample is sealed with mercury and stored over mercury in a glass transfer cup. The gas is drawn from the cup into the special capillary buret, its volume is measured, and it is transferred into a series of vials which contain absorption fluids for carbon dioxide and oxygen. The volume remaining after absorption measures the nitrogen and other inert gases present.

Apparatus

The micrometer buret shown in Fig. 88 is the central piece of apparatus. A micrometer screw *A*, obtained by removal of the

anvil and bow from a micrometer, has the spindle cut off at the point of attachment of the threads *B*. The microspindle *D* made from No. 3 gage stainless steel piano wire, 0.012 in. in diameter, is attached through a holder *C*, which is press-fitted into a hole drilled in the end of the micrometer spindle. The microspindle must be carefully aligned at the center of the holder *C* by means of a jig; it is then soldered in place. Its tip is ground to a blunt point. When the spindle is screwed back into the spindle bearing, the microspindle should retract almost to the end of the bearing.

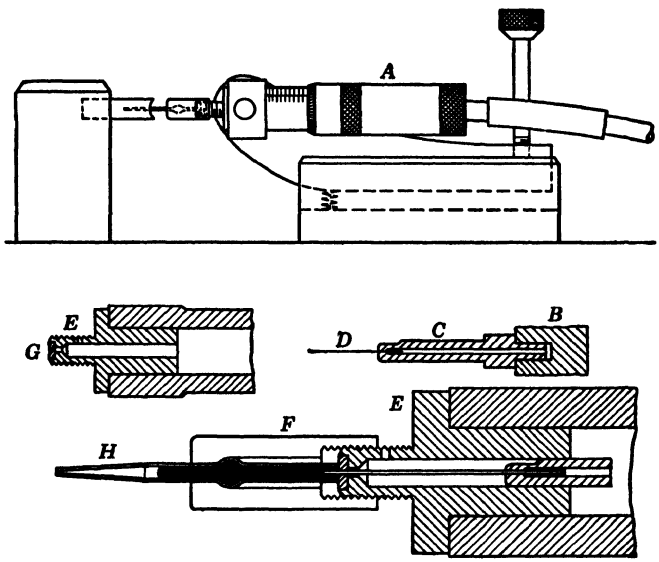


FIG. 88. Ultramicro gas analysis apparatus according to Scholander and Evans.

Into the end of the spindle bearing is press-fitted a steel plug *E* threaded to carry a plastic needle yoke *F* and a recess for a disk *G* constructed of fiber through which the microspindle passes. Other materials than fiber might well be substituted, e.g., Teflon. An exit port for entrapped air behind the disk gasket is obtained either by drilling a hole through the side of *E* or by having a loose fit of the plunger holder *C* in the steel plug *E*. The hole in the fiber gasket is drilled with a No. 10 pivot drill. The plastic yoke *F* is constructed from Lucite and is threaded to fit over the steel plug *E*. The plastic yoke *F* encloses and holds a glass

capillary tube, termed by the authors a buret needle *H*, whose internal diameter is considerably greater than the outer diameter of the microspindle. The tube is equipped with a shoulder at the center, a ground end which is pressed against the fiber disk, and a fine, long point. The fiber yoke is screwed up sufficiently tightly to hold the ground piece of the tube firmly against the fiber bearing. The entire apparatus is operated on a steel block with two adjustments for tilting or rotation. The steel block is equipped with a rubber base to prevent it from slipping when placed on a glass plate surface during analysis.

The buret is assembled as follows: The micrometer spindle is screwed all the way out, and the fiber washer is pushed from its socket. The spindle is then advanced until the microspindle projects about 5 mm. from the end. It is greased with light grease from a syringe and, while it is twisted back and forth, the fiber bearing is slipped on it, pushed back, and pressed into position. The microspindle is then withdrawn until it projects only 2 mm. and is covered with a drop of light grease. The buret needle is filled with mercury from a syringe by holding the base of the tube against the flat opening of the syringe needle. When the needle is full, the tip is closed with the finger, and the base end is covered completely with a drop of light grease, care being taken not to trap any air bubble. The drops of grease are joined together, the needle being fitted over the microspindle in such a way as to prevent air from entering the buret. The yoke is then slipped on and tightened gently. If the assembly is carried out correctly, the movement of mercury in and out of the tip will be completely smooth as the micrometer is rotated. If it advances in a jerky manner, there is either a leak in the system or an entrapped air bubble. The amount of mercury may be adjusted until the micrometer reading is essentially zero when the tube is just full of mercury.

Construction of the Buret Tube. Buret tubes, or needles, are constructed in a jig in order to obtain accurate dimensions. Figure 89 shows the arrangement of the burner and jig. *A* is a micro gas flame shielded from drafts by a piece of glass tubing pressed over a piece of split rubber tubing which surrounds the tip of the burner. The steel jig which is mounted on a hinge *C* is shown in lateral view at *B*. It consists of a metal plate with a central opening drilled to receive two metal tubes *D*, tapered at

their inner ends. One tube on the right is drilled throughout its length; the other on the left is drilled to a depth of 8 mm. with a drill slightly larger than the capillary tube used to make the buret tube. The metal tubes are held in place by set screws. The mounting on hinge *C* is so arranged that a capillary tube passing through the drilled holes in the plugs *D* will come directly

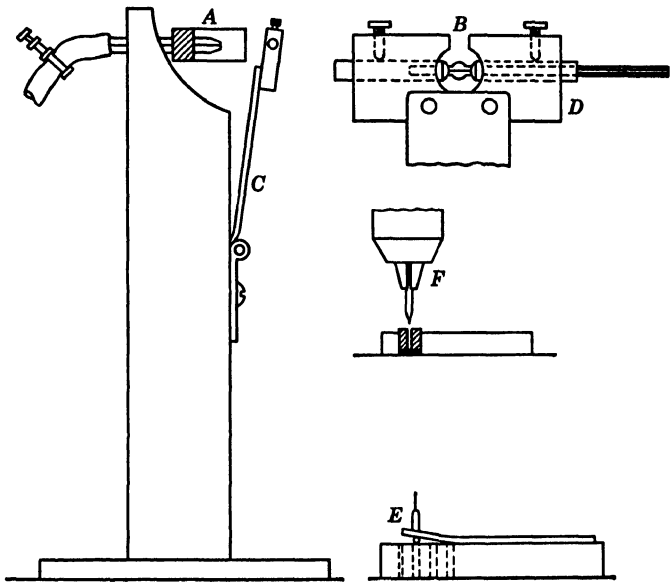


FIG. 89. Burner and jig assembly for Scholander and Evans gas apparatus.

before the flame of the microburner *A*. The capillary is turned rapidly while it is pushed toward the flame and is slightly pushed together as it melts to form the shoulder. A tip is pulled, free hand, about 1 cm. from the shoulder by moving the tubing into the flame until it becomes plastic, removing it from the flame, and pulling the ends apart to give a constriction about 1 cm. long. Tubing is cut by means of a Carboloy tool. The tubing is ground by being placed in another jig holder *E*, which holds the tube exactly vertically. This jig is constructed from a block of Bakelite with holes of various sizes drilled through it so that the tube can be placed in the hole that fits it most tightly. A thin sheet of perforated plastic provides pressure by forcing downward on

the shoulder. Fine-grade emery paper on the glass plate is used to grind the tube flat with light pressure. The tube is cleaned with nitric acid, water, and acetone and dried before it is installed in the buret. The base is fire-polished in order to eliminate any sharp corners.

Gas Transfer Pipet. The pipet for transferring minute volumes of gases must be arranged to seal the sample and allow fine manipulation in filling and emptying. This is achieved by

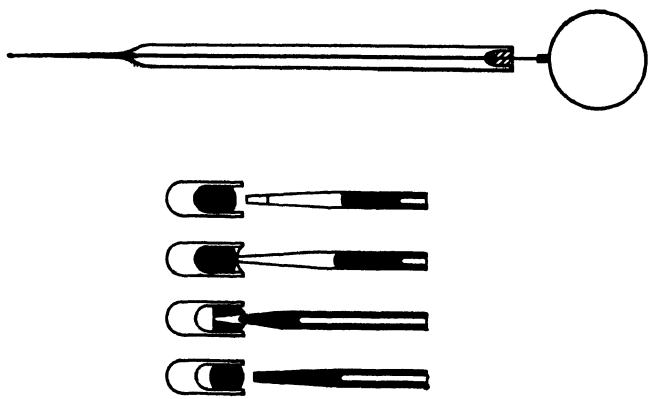


FIG. 90. Design and use of Scholander and Evans gas pipet.

the design shown in Fig. 90. A piece of No. 3 piano wire is formed to a loop about 1 in. in diameter on one end. The free end is ground to a blunt point. This is placed in a thin capillary tubing selected for a snug fit. The upper end of the pipet is blown out to give a cup which is filled with grease. At the tip, a thin-walled bubble is blown and drawn to form a fine, long tip with thin walls. The capillary portion of the pipet is filled with mercury, and the grease chamber is filled from a syringe with medium Nevastane or similar grease. The wire is inserted through the grease into the capillary and pushed downward to a distance of about 0.5 cm. from the tip constriction. If the wire fits the capillary properly, the mercury will be retained in any position at the tip of the pipet. With this arrangement, the smallest amounts of gases may be manipulated.

Gas and Reagent Vessels. Small transfer cups made of thin-walled glass tubing, 2 to 2.5 mm. in internal diameter and fused

together at the end to form a rod, are used for holding gas samples. The inside bottom of the cup must be smoothly rounded. The shank is placed in a well of heavy grease in which it can move easily. Plasticene can probably be substituted for the grease to advantage. The height of the cup is adjusted to correspond with that of the buret tip. A wooden or plastic block with a row of five horizontal holes for holding the vials of mercury and reagents is also provided. These vials are made from 7-mm. tubing, 25 mm. in length. The vial containing mercury is slightly fused together at the opening, the others being coated with Clarite in toluene around the edges to avoid loss of fluid. All tubes are held horizontally at the same height as the buret.

Reagents

The reagents must be composed so as to have good wetting properties for glass and as low solubility as possible for gases. For this reason, somewhat unusual acids and bases are employed. The transfer of very small volumes of gas is technically difficult and requires careful control of wetting, capillarity, and gas solubility.

1. *Acid solution,* made by dissolving 8.5 g. of sodium citrate in 12 ml. of water and adding 0.3 g. of citric acid.

2. *Alkaline solution for carbon dioxide absorption,* made by dissolving 8.5 g. of sodium citrate in 15 ml. of water to which is added 0.5 g. of sodium hydroxide.

3. *Oxygen-absorbing solution,* made in three parts as follows: (*a*) Solution A—6 g. of potassium hydroxide is dissolved in 100 ml. of water, the solution being stored in a wide-mouth vessel. (*b*) Powder A—20 g. of fresh sodium hydrosulfite, $Na_2S_2O_4$, is added to 0.1 g. of sodium anthraquinone-β-sulfonate, the two being ground together in a mortar and stored in a stoppered vessel. (*c*) Solution B—5 ml. of solution A is placed in a 5.5-ml. vial to which is added 0.6 g. of powder A. The vial is closed immediately with the finger, the powder is dissolved anaerobically under a hot-water faucet, and the vial cooled rapidly under cold water. Immediately the lower three-fourths of the solution is drawn off with the least possible air contact into a 5-ml. syringe fitted with a short No. 20 gage needle with a square-cut tip. The solution is equilibrated by drawing in a bubble of nitrogen into the syringe.

4. *Mercury.* Well-cleaned mercury is stored in a 1-ml. tuberculin syringe furnished with a short No. 20 gage steel needle with a straight-cut end.

5. *Grease.* Nevastane in light, medium, and heavy grades made by the Keystone Lubricating Company, Philadelphia, may be employed. This also is stored in a 1-ml. tuberculin syringe with the same needle arrangement as described above.

All reagents are stored and delivered from 5-ml. syringes provided with the same type of needle, and the acid solution, alkaline solution, and oxygen absorber are placed in a vessel of mercury to prevent drying and crystallization in the needle tip. An additional syringe of distilled water is also provided. Immediately before analysis, the five vials set horizontally in the block are filled in succession with mercury, acid, alkaline carbon dioxide absorber, oxygen absorber, and water plus 1 drop of acid solution.

Procedure

The analysis depends almost entirely on quantitative transfer of gases between holders and absorbing solutions and the measurement of volumes in the different stages. In general, the gas to be analyzed is contained under a sealing liquid in a small tube or similar vessel. If the conditions are such that the gas is accessible to the micrometer buret, the tip of the latter may be inserted through the liquid seal and used to remove the gas bubble which is to be analyzed. This may frequently not be possible, in which case the gas transfer pipet and cup are employed. The cup is filled completely with mercury and examined under magnification to make certain that no air is trapped. The transfer pipet is likewise filled exactly to the tip with mercury, and the gas bubble to be analyzed is withdrawn into the transfer pipet from its initial position. A little of the sealing liquid retains the bubble in the pipet. The latter is brought to the filled cup, forced through the mercury, and expelled as shown in Fig. 91. Movement of the wire plunger is used to transfer the gas in or out of the pipet. The cup containing the gas is placed in front of the buret tip, and the gas is transferred to the buret, following a similar procedure. The buret tip which is exactly full of mercury is forced through the mercury seal. When the last of the gas is just inside the buret tip, a drop of sealing acid is added to the open end of the cup, and the buret is withdrawn. Capillary

forces draw acid into the buret, sealing the gas in place on removal of the tip. The analysis is carried out by inserting the gas bubble into the appropriate vial containing the reagent and manipulating it, as illustrated in Fig. 91. The buret tip is inserted well inside the reagent, and the micrometer is adjusted to place the leading gas meniscus accurately at the buret tip at which point the micrometer is read to yield the total gas volume. The gas is next transferred to the caustic vial as shown at *G*, and the bubble is forced partly out and in a number of times by the micrometer so as to yield good exposure of the droplet to the

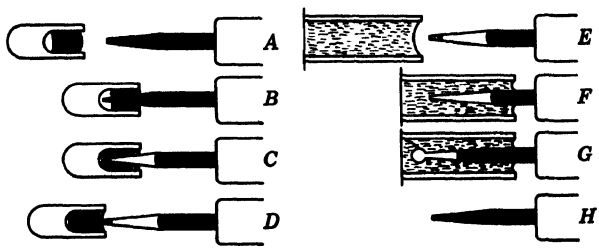


FIG. 91. Use of Scholander and Evans gas apparatus.

sodium hydroxide. In a few seconds, the meniscus is adjusted again exactly at the tip, and the second volume reading is taken, the difference of which from the first volume corresponds with the loss of carbon dioxide absorbed. Oxygen may then be determined by placing the oxygen-absorbing liquid over the buret tip and again extruding the gas several times for oxygen absorption as described. When this is complete, the meniscus is again located at the tip, and the dilute acid of the final wash vial is placed over the needle. This washes away the residue of oxygen absorbent and allows the final reading to be taken, after the buret is moved back into the stronger acid solution used originally. The shrinkage in volume corresponds to the oxygen absorbed. The remaining gas is the nitrogen contained in the original droplet. After the buret tip is rinsed several times with the acid and the outside and inside of the tip are dried with a suction tube held close to it, the apparatus is ready for the next determination.

Remarks

The accuracy of the procedure described naturally varies with the size of sample. In general, the errors with the larger samples (0.35 λ) were

found not to exceed 0.2 volume per cent, and with the smaller (0.07 λ) not to exceed ±0.5 volume per cent. The operation is rapid, requiring only 4 to 6 minutes when the equipment and solutions are properly arranged. The construction requirements are somewhat rigid with respect to various critical points. The microspindle must fit very well; there must be no air pockets or entrapped air bubbles; and the glass portions have to be made carefully in order to fit the plastic and metal portions. It is probable that the substitution of greases other than that specified, e.g., silicone, may be allowable or possibly favorable. Gasket materials other than fiber, e.g., Teflon, may also improve the operation. Apparently none of these substitutes has been tried.

The extension of this basic technique to analysis of small volumes of other gases is primarily a question of finding suitable liquid absorbents. It seems likely that there are several such developments which could be made rather easily. There may be a question as to the necessity or desirability of analyzing other gases in so minute samples, since it is usually the small biological system only which makes necessary gas analysis on this scale of operation. It is possible, however, that such needs do exist in various other instances.

BLOOD GAS ANALYSIS

Scholander and coworkers have published an extensive series of blood gas analysis methods which are correlated with the gas analysis methods referred to or described above. These include determination of carbon monoxide (7), carbon dioxide (8), oxygen (9), and nitrogen (10) in amounts of blood ranging from 1 λ up to 120 λ. The smallest-scale analyses are described below, except for the earlier methods developed for samples ranging from 40 to 120 λ which are scarcely in the true microgram range. The accuracy obtainable by these methods is naturally somewhat less with the small quantities than with the larger but is sufficient for the purposes of most such analyses, and the methods are applicable to bloods from non-mammalian as well as mammalian species. The methods are considerably simpler than most earlier studies which also were devoted to analyses of small samples, such as those of Donal (11). Scholander, Flemister, and Irving (12) adapted the earlier work of Scholander and Roughton (8) to the determination of carbon dioxide and oxygen in 12-λ samples of blood. This was the first of their methods which was found equally ap-

plicable to the lower forms of life and to mammalian species. The smallest-scale technique of this group, in the field of blood analysis, was that of Scholander and Irving (13) who adapted the gas analysis method previously described to the analysis of oxygen, carbon dioxide, and nitrogen in fractional microliter samples of blood by means of a modification of the micrometer gas buret described in the preceding section. This method is described below.

Principle of Method

The blood is sampled by means of a fine glass capillary tube and transferred to a modified microsyringe buret of 1-λ capacity which has an accuracy of about 1 in 3000. The buret is equipped with a special detachable extraction tube containing dried acid ferricyanide which serves as reagent for the extraction. The sample contained in the needle is subjected to vacuum extraction in a centrifuge, and the gas evolved is then analyzed as described previously.

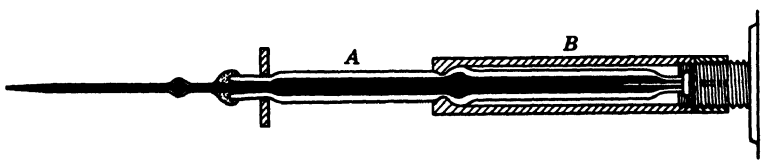


FIG. 92. Blood gas apparatus according to Scholander and Irving.

Apparatus

The micrometer buret previously illustrated in Fig. 88 is modified by removing the plastic yoke and needle and installing instead the device shown in Fig. 92. It consists of an extraction tube fitted to the micrometer buret with a steel yoke and a standard buret needle attached with wax to the extraction tube *A*. The extraction tube is made from a 1.5-mm.-bore glass tube prepared in the jig previously described in which an enlargement is made to allow attachment by the steel yoke *B* to the micrometer assembly by drawing the tube tightly against the fiber gasket on the original buret. The neck must be quite round, to allow a snug fit of the buret needle which is attached with wax. The steel yoke *B* is threaded to the micrometer and adapted to fit the shoulder of the extraction tube, substituting for the plastic yoke

which also fits the micrometer. It is made by drilling a steel rod and filing the sides away, finally splitting the threaded end.

Auxiliary apparatus required for efficient operation includes a small oven for melting wax, made from a piece of vertical brass tubing with air vents and a side tube, placed over an electric light bulb which furnishes enough heat to keep a small beaker of beeswax just above the melting point; a small angle centrifuge equipped with a braking unit made by cutting a 12-cm. hole in a small board and lining it with felt so that it may be pressed firmly on the cone of the centrifuge to stop it rapidly; an oil vacuum pump of conventional type attached to a small test tube just large enough to contain the reaction chamber and buret needle assembly; fine wire for cleaning the reaction chamber and needle; and a fine glass rod broken to form a sharp edge for cutting off the wax seals.

Reagents

The reagents required for gas analysis described previously are required. In addition the following must be available.

1. *Acid ferricyanide solution.* To 5 ml. of concentrated lactic acid is added 15 ml. of water, and the mixture is placed in a mortar. Two grams of potassium ferricyanide is added and dissolved in the mortar. The solution is placed in a wide-mouth 50-ml. vial.

2. *Urea solution,* made by dissolving 40 g. of urea in 100 ml. of water. Part of this is stored in a wide-mouth 50-ml. vial.

Other similar vials are charged with water, carbon tetrachloride, and acetone.

Procedure

The extraction tube must be cleaned and charged with acid ferricyanide. The cleaning is accomplished by means of urea solution and a small wire. Wax is removed by carbon tetrachloride and acetone. The tube is attached to a suction line, and water is drawn through, followed by the acid ferricyanide reagent, followed by warm air from the side tube of the wax oven, to dry the reagent as a layer on the entire interior of the tube. The buret needle is cleaned the same way and inserted in the reaction tube where it is sealed carefully with the molten beeswax which is warmed just enough to allow it to flow around and seal the inside angle of the needle end with the reaction tube.

The entire tube assembly is filled with mercury by applying a superficial wax seal to the micrometer end and inserting the assembly into the test tube attached to the vacuum pump and containing mercury in excess of the capacity of the tubes. The test tube is completely evacuated with the open tip of the needle out of the mercury. After the open end is covered with mercury by rotation of the tube, air is slowly admitted to the test tube, forcing the mercury to fill the reaction tube and needle completely. The tube assembly is withdrawn, being handled by the rubber collar, and the tip is closed with the finger. The wax seal is pushed off by the finger nail, and the upper end is filled with a little more mercury if necessary. A drop of grease is applied over the end of the reaction tube and also over the microspindle which just protrudes from the fiber gasket. The tube is attached firmly with the steel yoke, allowing no entrapment of air. The needle tip is immersed in mercury, and the micrometer is adjusted to zero. A little time is allowed for the enclosed mercury to reach temperature equilibrium before the mercury covering the tip is removed.

The blood to be analyzed is sampled by means of a fine capillary just large enough in diameter to slip over the end of the buret needle. Fresh capillary blood is used and allowed to flow directly into the sampling capillary. The end of the capillary is placed around the buret needle tip which is filled exactly to the end with mercury and with a "0" reading on the micrometer. The sample of blood (e.g., 10 mm. on the micrometer) is drawn into the buret needle, as measured by the micrometer reading, and the tip is sealed with successive applications of molten wax until a firm pear-shaped seal is obtained. The tube assembly is removed from the micrometer assembly and filled with mercury until a small drop protrudes. It is then fitted into a small centrifuge tube with the open end down and covered with mercury in the tube, as shown in Fig. 93. The rubber yoke prevents the tube assembly from being forced lower in the centrifuge tube. The latter is fitted into a wooden adapter as shown, and the assembly is whirled in an angle centrifuge up to full speed. This subjects the sample to vacuum and allows the blood to dissolve and react with the acid ferricyanide. The centrifuge is stopped with the brake as rapidly as possible, producing a differential flow rate of mercury and aqueous solution in the tube assembly which causes the mercury to flow to the upper tip end of the tube,

enclose the gas phase, and effectively seal it from the aqueous phase. The inversion of phases is checked under the dissecting microscope without breaking of the mercury seal at the open end of the tube.

The buret needle is then detached from the extraction chamber by being pulled straight out, and the open end is filled with mercury if necessary. By means of the plastic yoke used in the gas analysis method, the needle is attached to the micrometer assembly; it is sealed with grease to avoid all entrapped air as

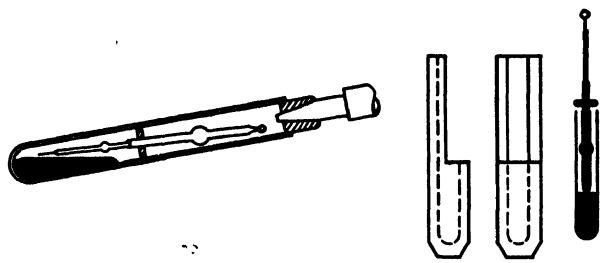


FIG. 93. Centrifugal vacuum extraction equipment according to Scholander and Irving.

usual. The positive pressure produced is relieved by screwing the micrometer back about 1½ to 2 turns. The tip is surrounded by the acid in the reagent block as described previously, and the wax tip is cut with the sharp glass rod, exerting pressure obliquely toward the tip to prevent breakage. When the seal is broken, the acid meniscus is drawn into the tip. It is immediately pushed out with the micrometer until it is even with the tip, and the micrometer is read. The gases are then analyzed exactly as described in the previous section for carbon dioxide and oxygen, and the residual gas is measured as nitrogen.

Correction Factors

Because of the small size of the apparatus and sample, a correction for the effect of capillarity must be determined for each buret needle. As with all vacuum extractions, a further correction for incomplete extraction must be determined.

The capillarity correction is readily determined by drawing into the needle exactly 10 mm. of air, the tip then being covered with the acid reagent, and the meniscus readjusted to the tip. The

amount of this adjustment is the correction and is added as a factor to the total micrometer movement in taking the sample.

The incomplete extraction factor is different for carbon dioxide and oxygen because of the different rates of reabsorption. A sample of blood of known composition is extracted as described, and the gas is expelled. It is then re-extracted, and the amount of each gas obtained on analysis of the second extracted phase is calculated and added as a factor, allowing the correction to be applied subsequently to all analyses.

The calculation of results is accomplished by the formula

$$V = \frac{B - W}{760} \times F_t \times F_i \times F_c$$

where V = gas volume factor under standard conditions in terms of micrometer divisions.

B = barometric pressure.

W = water vapor tension of blood (approximately $T° - 5$, i.e., at 25° C., $W = 20$).

F_t = factor for temperature, obtained from Table 12.

F_i = factor for incomplete extraction.

F_c = factor for capillarity.

TABLE 12. TEMPERATURE CORRECTION FACTOR, F_t*

° C.	F_t	° C.	F_t	° C.	F_t
15	0 9455	20	0.9286	25	0 9124
16	0.9421	21	0 9255	26	0 9092
17	0 9382	22	0 9221	27	0 9060
18	0.9353	23	0.9188	28	0 9029
19	0.9320	24	0 9156	29	0.8998

* Scholander and Irving (13) adapted from Peters and Van Slyke, Table 27 (14).

The accuracy of the method is stated to be approximately ±1%, depending on the sample size, for carbon dioxide and oxygen. The accuracy for nitrogen is less because of the smaller amounts involved. The time of analysis for an experienced operator is from 15 to 20 minutes.

RESPIRATORY GAS MEASUREMENTS

A number of very satisfactory microrespirometers have been described (15). Most of them require appreciable amounts of

tissue or rather large numbers of single cells. A few have been designed so as to measure single-cell respiration, even in short periods. In the latter category, the most sensitive procedures are those based on the Cartesian diver as described by Linderstrøm-Lang (16) and Holter (17) and the method of Cunningham and Kirk (18) which has a sensitivity slightly less than the maximum obtained from the Cartesian diver. It was found very adequate for single-cell respiratory measurements and is in several respects simpler than the diver.

In most instances, somewhat larger amounts of biological materials are available, e.g., a number of single cells or a small piece of tissue which might require a respirometer with a sensitivity of about 0.1 λ per hour. Such systems include suspensions of bacteria or protozoa, tissue cultures, tissue slices, developing seeds, or pieces of plant tissue such as a single anther. For respiratory measurements with the above systems, the differential microrespirometer has proved very simple and dependable. A design for such an instrument was described by Cunningham and Kirk (19). It involved two chambers in a metal block, the chambers being connected through a small capillary containing an index droplet of purified kerosene. The construction of the instrument from metal simplified the problem of temperature control, and indeed, except with the smallest capillaries, temperature control was ordinarily not necessary.

When the sensitivity of the measurement is increased by means of a fine capillary, it becomes desirable to employ a relatively insensitive thermostat with these instruments. The metal system transmits heat rapidly and approximately equally to both chambers, thus canceling the temperature effect and producing no movement of the index droplet with moderate temperature changes. Since a sealed system is used, the effect of barometric pressure is also eliminated.

This respirometer was modified by Barth and Kirk (20) to improve its ease of construction and later by Stern and Kirk (21), who increased the range of application and added certain refinements.

Since this instrument is one of the simplest in construction and operation in the range indicated, it will be described in some detail first.

DIFFERENTIAL CAPILLARY MICRORESPIROMETER MEASUREMENTS

Principle of Method

Two chambers connected with a capillary contain, respectively, the sample of material whose respiration is being measured and a blank containing everything present in the first except the tissue or cells. The oxygen consumption of the biological material is indicated by the movement of a droplet of kerosene in the capillary, caused by consumption of oxygen and absorption of the carbon dioxide liberated in a droplet of sodium hydroxide. Depending primarily on the size of the capillary, the sensitivity of the instrument can be varied within rather wide limits. The movement of the index droplet is independent of barometric changes and relatively independent of temperature change, provided the change of temperature is symmetrical with respect to the two chambers. Arrangements can be made to allow mixing of reagents within the chambers without opening them and for the control of the gas phase present at the beginning of respiratory measurements.

Apparatus

A design of the instrument slightly modified from that of Stern and Kirk is shown in Fig. 94. It consists of a block of Duralumin or brass as shown, with two chambers of equal size drilled in the top. These are usually made 3/8 in. in diameter or smaller and are variable in depth, depending on the size of the tissue being measured. The depth of the chamber may be varied by filling with paraffin to any desired extent in order to reduce its size. This makes the response of the respirometer more rapid and is desirable for smaller amounts of biological materials.

The chambers should usually be lined with a thin coat of lacquer in order to prevent reaction of the material with any caustic which may be spilled. Each chamber has access to the exterior air through the small needle valves shown. Each of these consists of a small steel needle which seals a hole in a Teflon gasket. The needle valves allow the chamber to be opened, thus permitting adjustment of the indicator droplet, equalizing the pressure, and changing the type of gas mixture used in the chambers.

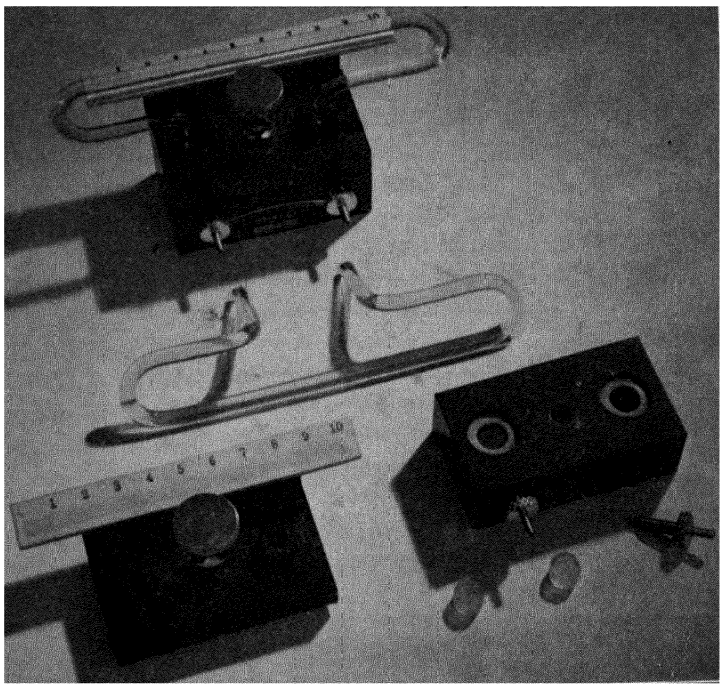


FIG. 94. Differential capillary microrespirometer. (*Courtesy Microchemical Specialties Co.*)

A top plate of brass or Duralumin seals the chambers through Teflon or rubber gaskets, as shown. The design is such that a rubber gasket can be expanded to seal simultaneously the ends of the capillary which pass through the top plate over each chamber. The plate is secured to the main block by means of a single bolt at the center. Capillaries of widely variable diameters can be used, depending on the sensitivity desired. With plant tissues, capillaries as small as 0.3 mm. are convenient because of the rather low rate of respiration. Animal tissues may make desirable capillaries up to 1 mm. in diameter. In the construction of the capillary it is important that all bends be made carefully to avoid constriction which will trap kerosene and prevent proper functioning of the instrument.

Reagents

1. *Kerosene* for the index droplet is carefully purified by being shaken occasionally over a period of several days with concentrated sulfuric acid, separated and neutralized with a little sodium hydroxide solution, dried by standing for some time over anhydrous sodium sulfate, and finally distilled, retaining only the middle fraction. The material should be stored over sodium hydroxide pellets.

2. *1% sodium hydroxide solution,* is used for absorbing carbon dioxide.

Procedure

Depending on the type of sample, the respirometer is charged in a variety of ways. If the organism whose respiration is being measured is in aqueous suspension, a sample of the latter is placed in a small cup which may be made from glass or, more easily, a suitable plastic, such as Lucite or Teflon. With chambers of this type, a small piece of filter paper is dipped in 1% sodium hydroxide solution and placed over the chamber to absorb carbon dioxide. Some biological materials such as the anther of a plant may be placed across the rim of a cup which contains the absorbing caustic.

These two methods can be varied to adapt the respirometer to any common form of biological material.

The chamber is loaded with the biological substance and the accompanying caustic, and a comparable system lacking respiring cells is placed in the other chamber. The top plate carrying the capillary which already contains a droplet of kerosene is put in place and tightened. The needle valves to the chambers are left open during the placing of the top plate. The index droplet is shifted to the desired position on the scale by tilting the respirometer and closing the needle valves when the droplet is in place. If the required sensitivity is high, it may be necessary to place the respirometer in an air thermostat or in a shallow water bath. With the larger capillaries, it is usually possible to operate on a laboratory bench without temperature control.

It is important that no light bulbs or other hot objects operate in the immediate vicinity of the respirometer because of the absorption of radiant energy in the capillary tube and unsymmetrical heating of the blocks themselves. A fluorescent light is

considered an improvement over the original methods of Kalmus (22) and Howland and Bernstein (23) who first measured oxygen consumption of single cells of *Paramecium caudatum.*

The design used by these authors suffered from the failure to compromise between a capillary large enough for a favorable rate of gas exchange and small enough to obtain a high sensitivity of the instrument. In other words, if the capillary was large enough to prevent oxygen starvation of the organism and allow rapid transfer of gas between the medium and absorbent, it was too large to obtain accurate measurements of the movement of a liquid meniscus. The essential change introduced by Cunningham and Kirk was in the use of a capillary of about 0.5-mm. diameter as respiration chamber, the tube being drawn to a capillary of about 0.08 mm. in the region in which meniscus movement was measured. It was shown that the factors involved in gas diffusion, surface tension, and absorption of gases were favorable with dimensions of this order. It was possible to add to the culture one, two, or three organisms and obtain a corresponding increase in the oxygen consumption, a check which could not be obtained with the Howland and Bernstein method of uniform-diameter capillaries small enough to have the requisite sensitivity.

The difficulties to be overcome in the design were primarily those caused by changes of temperature and barometric pressure. With the final apparatus design no serious problems of temperature control remain. It was also shown that the barometer rarely changed significantly during the limited period of operation. This matter, however, requires confirmation with each measurement, particularly when periods of longer than 1 hour are involved.

Principle of Method

The cell whose respiration is to be measured is placed in its medium in the end of an 0.5-mm. capillary tube and sealed in place with a cover slip. Close to, but not touching, the drop of culture medium is the meniscus of the absorption caustic which is about 0.5 *N*. The caustic extends into the upper fine portion of the capillary which is about 0.08 mm. in diameter. As carbon dioxide is produced it is almost immediately absorbed by the caustic, and the oxygen which is consumed in the gas bubble next to the culture medium causes a slight movement of the caustic meniscus which is magnified greatly in the movement at the upper

end in the finer capillary. This movement is measured by means of a microscope which is equipped with an ocular micrometer.

Apparatus

The equipment is shown in Fig. 95. The essential portion of the apparatus is a capillary tube of 0.5-mm. internal diameter drawn to a much finer straight capillary of about 0.08-mm. diameter and several inches long. The fine capillary is jacketed with a somewhat larger tube to avoid fragility and to allow passage

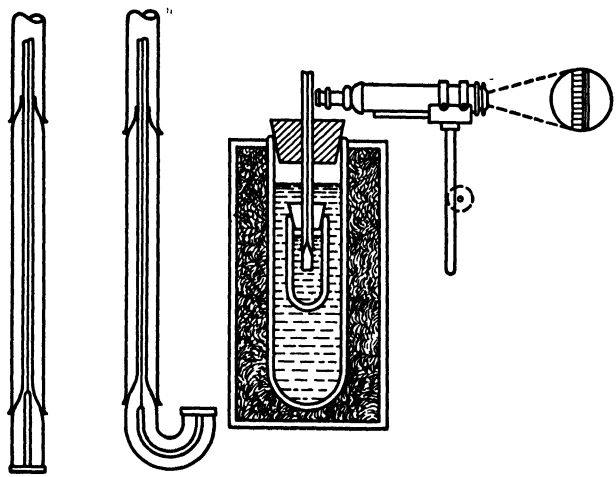


FIG. 95. Capillary respirometer according to Cunningham and Kirk.

through a rubber stopper. The top of the tube is drawn down to an approximate fit, as shown in the figure, and sealed with a little paraffin. On top of it is placed some more of the same tubing which is sealed to form a cup somewhat higher than the top of the tube. The assembly, with the respiration chamber downward, is mounted in a rubber stopper and placed in a small flask of the Dewar type containing water which covers the respiration chamber. The Dewar flask and stopper are immersed in water contained in a larger Dewar flask, also closed with a rubber stopper, through which is passed the fine jacketed capillary tube. The outer vacuum flask is buried in a box containing insulating material such as asbestos or magnesia powder. It was

shown by the authors that this method of insulating almost completely prevents fluctuation of temperature within the respirometer, the actual temperature of which is the mean temperature of the room. The effect in smoothing the temperature curve is shown in Fig. 96.

A horizontal microscope equipped with a calibrated ocular micrometer determines the meniscus position and distance of movement. A microscope cover slip seals the respiration chamber to which it is attached with a little Vaseline.

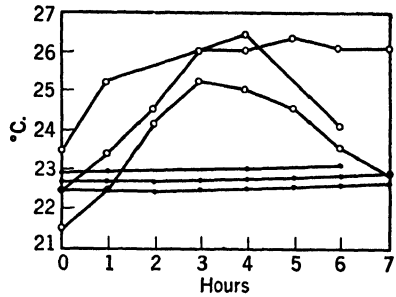


FIG. 96. Curves showing changes in room temperature and the degree of constancy produced by double vacuum flasks.

Procedure

The respirometer tube is cleaned by drawing chromic acid mixture through it, followed by distilled water and finally 0.1 *M* sodium bicarbonate. The bicarbonate is washed out with distilled water and the tube dried by drawing clean, dry air through it. The tube should be allowed to stand overnight to dissipate any effects of heating due to mixing of the cleaning solution with distilled water.

The capillary is charged by being placed in a vertical position with the cup end upward and adding 0.5 *N* sodium hydroxide to the cup until the top of the capillary is covered. The caustic flows downward in the small capillary; when it reaches 4 mm. of the lower end of the tube, the remaining caustic is poured immediately from the cup.

A small drop of liquid medium containing the cell is placed on the ground chamber end of the capillary, and a microscope is used to observe the cell. If it is free swimming, such as *Paramecium*, it will shortly move inside the tube; otherwise, it must be put in by means of a fine needle. When the cell is inside the tube, the remaining water or medium is wiped off, and the end of the capillary is sealed with a cover slip coated with Vaseline. The tube is placed inside the water of the inner Dewar flask, and the cup is again filled with hydroxide. After it stands for

about an hour to reach temperature equilibrium, the caustic is removed with a fine pipet from the cup which is then plugged with a wad of cotton. After the cup stands another hour, the microscope is focused on the meniscus, with magnification of about 100 diameters and with an ocular micrometer in the eyepiece. The rate of fall of the meniscus is observed in relation to the micrometer over a period of measurement. During all measurements, periodic readings of the thermometer and barometer are made; if they vary by significant amounts, the results are discarded.

Capillary tubes may be calibrated in a variety of ways, including direct microscopic measurement of the bore of the capillary at the end. This is relatively inaccurate because the area varies as a square power of the diameter. It is desirable to place a thread of mercury in the capillary, measure its length, expel it, and weigh the droplet on the microchemical balance to obtain the volume per unit length. The sensitivity of the measurement can be made as high as 5×10^{-5} λ without unusual difficulty.

Remarks

So many variable factors exist in measurements with a capillary respirometer, even when the instrument is of extreme simplicity, that corrections and calibrations of several of the more important ones are mandatory if the results are to have an absolute meaning. When it is remembered that the instrument described is capable of achieving a sensitivity of 5×10^{-5} λ, it will be realized that such small changes in volume may be readily affected by variables in the system. In so far as possible, these are controlled by maintaining the system constant. Temperature may be controlled with considerable precision as described. Variations in temperature would affect (*a*) the expansion of the gas phase, which with an 0.5-λ bubble gives a rate of about 4×10^{-5} λ per 0.02° C. per hr.; (*b*) expansion of the liquid phase for the same rate of temperature change, which can amount to 1 to 6×10^{-5} λ; (*c*) decrease in solubility of the gas phase, which can amount to about 10^{-4} λ; and (*d*) increase in vapor pressure of the liquid phase, which can amount to a volume change of 2×10^{-5} λ.

Other factors which affect the accuracy of the measurements are (*a*) barometric pressure changes which must be constant to about 0.1 mm. mercury per hour; (*b*) height of manometer liquid, and changes in it; (*c*) dilution of manometer liquid due to transfer of water; (*d*) change in volume of the gas phase due to change in partial pressure of the gases concerned; (*e*) variations in diameter of the capillary; and

(*f*) changes in surface tension by impurities. The corrections for these factors may be calculated with reasonable accuracy (18), and many of the factors can be maintained constant enough to be negligible in their effects.

The study of the respiration of individual *Paramecium* cells by this method revealed a correlation of respiratory rate with the size of the cell and showed no dropping off of rate with the time of measurement. Variations in the rate of oxygen consumption of different cells measured varied as much as 3.5 times; a study of the cell size and volume showed that surface area could vary as much as 7.5 times and volume as much as 13.

THE CARTESIAN DIVER MICRORESPIROMETER

Notable among the instruments for ultramicromeasurements which involve changes in the composition or quantity of the gas phase is the technique of the Cartesian diver which was first published by Linderstrøm-Lang (24) and Linderstrøm-Lang and Glick (25). The Cartesian diver has proved in practice to be an exceptionally versatile instrument, applicable not only to respirometry with small systems but also to enzyme studies in which there is a change in the volume of a gas phase and even to ultramicroweighings as discussed in Chapter 4. Among the applications of the method are those of Boell, Needham, et al. (26–28) and others (29, 30). The Cartesian diver is adaptable to measurements of respiration of systems containing a single cell and can readily be adapted to considerably larger systems. It has been used to study many microbiological systems (31) and will undoubtedly find numerous interesting applications in the future. The technique is characterized not only by its versatility but by its inherent high sensitivity. It is somewhat more complex in operation than some of its alternatives, and in common with all very delicate techniques it suffers from several sources of error which must be brought under reasonable control or for which appropriate corrections must be made. The most extensive discussion of the theory and errors was given by Linderstrøm-Lang (16) and of the detailed technique of operation by Holter (17). Anfinsen and Claff (32) have recently extended the technique to duplicate on the ultramicroscale many of those operations previously conducted only by means of the Warburg apparatus. As mentioned, Zeuthen found it useful for ultramicroweighings.

Principle

A small inverted bell-shaped or straight capillary tube which serves as the Cartesian diver contains a liquid phase in which some reaction may take place, in equilibrium with a gas phase whose volume is affected by the reaction. The gas phase is sealed in place by one or more liquid seals which are forced to move up or down the capillary, depending chiefly on the internal and external pressures applied. The diver is immersed in an appropriate liquid, to the surface of which is applied a controlled variable air pressure which is measured by a manometer. Because of the gas phase present in the diver, the over-all density of that device is rapidly altered by changes in the atmospheric pressure over the immersion liquid since the gas enclosed in the diver is free to expand or contract, depending on the pressure applied. Because a very minute change in the volume of the enclosed gas causes a significant shift of the over-all density, the diver alternately sinks or floats with very small pressure changes without or volume changes within it. When it is suspended in the control position of the immersion fluid, i.e., intermediate between the bottom and top of the fluid, it is at its position of maximum sensitivity.

A reference point can be used to locate accurately the elevation of the diver, and any internal change in the reacting system which alters the amount of gas present will require a comparatively great variation in the atmospheric pressure over the immersion liquid to balance it in this sensitive position. For example, if the diver contains a respiring cell, a bubble of air, a sodium hydroxide solution seal, and an oil seal to separate the caustic from the immersion liquid, oxygen will be consumed by the organism, reducing the volume of gas, whereas the carbon dioxide produced will be absorbed by the caustic, thus causing the diver to become heavier. A reduction in the pressure of the gas phase over the immersion liquid will cause the air bubble to re-expand until the diver is again raised in the liquid to its equilibrium point.

As given by Linderstrøm-Lang (16), the equilibrium pressure P of the diver is made up of the summation of several pressures: (*a*) the pressure of the atmosphere on the external side of the manometer used to measure the equilibrium pressure; (*b*) the hydrostatic pressure difference read on the manometer; (*c*) the hydrostatic pressure of the immersion medium column above the

equilibrium mark; and (*d*) the pressure originating in capillary forces of the diver's neck. In order for the change in equilibrium pressure to equal that of the manometer reading, all pressures other than the hydrostatic pressure of the manometer must be constant. The total gas volume V of the diver at the equilibrium pressure may be calculated from the compound density expression given by Linderstrøm-Lang, which must involve all the phases—liquid, solid, and gas—which are present in the diver. The overall density of the latter is equated to that of the immersion liquid:

$$\frac{g_D + V_{oil}\phi_{oil} + V_w\phi_w}{V + V_{oil} + V_w + (g_D/\phi_{gl})} = \phi_M$$

where g_D is the weight of the glass diver in milligrams.

V_{oil} is the volume of the oil phase in the diver.

V_w is the volume of reaction mixture.

V is the total gas volume at equilibrium pressure and temperature of the system.

ϕ_M, ϕ_w, ϕ_{oil}, and ϕ_{gl} are the densities of the immersion medium, the reaction mixture, oil, and glass, respectively.

In order to interpret the results of diver measurements, the connection must be established between the equilibrium pressure which is measured with the manometer and the quantity of gas in the diver which is the phase undergoing volume or pressure change. The theory and error calculations are complex and are to be found in detail in the publication of Linderstrøm-Lang (16).

Apparatus

The diver is operated in an immersion liquid contained in an elongated bulb attached to a manometer and an arrangement for adjusting pressure. A suitable form of this apparatus is shown schematically in Fig. 97, as given by Holter (17). The pressure over the immersion liquid is adjusted by altering the compression on a large piece of rubber tubing with screw clamps *C* and *B* which force more Brodie solution into manometer *D* or allow it to escape, thereby changing the pressure in the entire system. *E* is the bulb containing the diver immersed in its appropriate liquid. As many divers can be placed in the apparatus as there are stems to accommodate them. The standard types of diver are shown in Fig. 98. They may be made in a large variety of

sizes and shapes but usually are kept to a total volume of about 10 λ. Their construction is carried out as shown in Fig. 99 by blowing a small bulb on the end of a thin-walled glass capillary and attaching to it a piece of glass rod which produces stability by lowering the center of gravity. The internal diameter of the

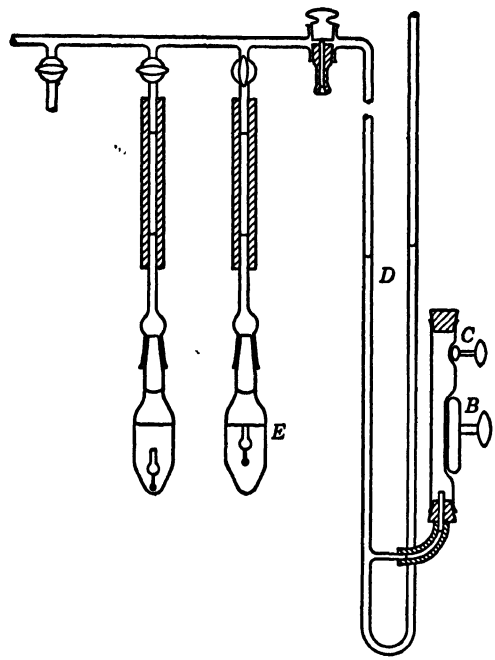


FIG. 97. Vessels and control apparatus for Cartesian diver according to Holter.

capillary is usually somewhat less than 1 mm., with a wall thickness of approximately 0.1 mm. Although fundamentally simple, the operation of making the diver requires considerable practice in that it involves working always in the flame and with constant rotation. The solid glass tail of the diver adjusts the glass phase so as to obtain a proper ratio of mass to volume. Many variations are possible, not only of size but of shape. The two types recommended by Linderstrøm-Lang are shown in Fig. 100. These are equipped with a widened flat bottom and with a small glass stopper which diminishes transfer of gases through the liquid

seals. The stopper may be made short and held in the bottom sealing drop, or a longer stopper may be held in the top liquid

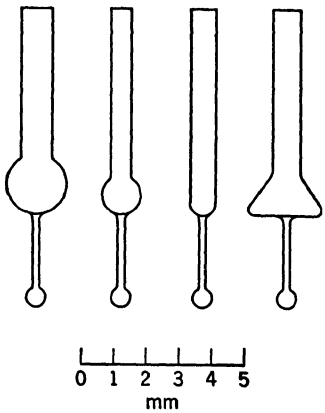


FIG. 98. Standard types of Cartesian divers.

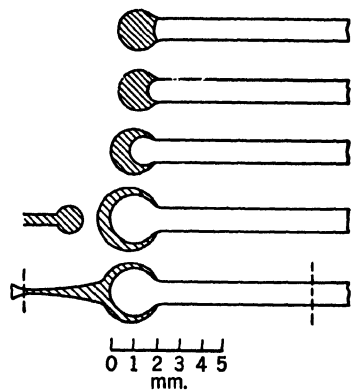


FIG. 99. Construction of Cartesian divers according to Holter.

which is usually the immersion liquid itself. Variations of the construction of divers are described in other publications (33).

Thermostat. The originators of the diver technique kept a constant temperature room of 21° C. Since this would normally be impractical for investigators employing the technique, the apparatus is ordinarily kept at constant temperature in a water thermostat. A suitable design which accommodates eight divers may have dimensions approximating those described by Holter, namely 58 by 30 by 30 cm., equipped with plate-glass walls and regulated with a precision of 0.01°. Such a thermostat must be equipped not only with the diver immersion vessels and the thermostat control equipment but with appropriate lighting arrangements and a large bottle filled with air which communicates with the outside branch of the manometer, replacing the atmosphere and allowing the diver to operate independently of barometric pressure changes. The recommended lighting comprises a number of small lamps illuminating

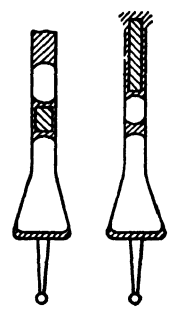


FIG. 100. Cartesian divers fitted with plugs according to Linderstrøm-Lang.

a ground-glass plate which forms a background for all flotation vessels. Fluorescent lighting appears to be more suitable. The plate and lights are placed back of the glass wall of the thermostat so that there is a minimum heating effect by the lighting system. Uniformity of illumination is necessary rather than high intensity. In special instances, a high-intensity source has been actually immersed in the thermostat in order that the diver position may be read with a microscope. With large divers, it is usually possible to adjust their height with only the naked eye. In all instances the top of the diver is adjusted to be even with a circular mark placed on the flotation vessel. Obviously, the size of the vessel must be such as to accommodate the particular size of diver with which the work is being performed. When the reactions are slow or with especially small divers, it is helpful to employ a low-power horizontal microscope for reading the diver position. Some magnification is usually desirable, even with larger divers, but ordinary magnifying glasses or stereoscopic loupes may be satisfactory.

Diver Holders and Manipulation. The precision necessary in filling the diver requires mechanical manipulation and specialized equipment for holding the diver and measuring liquids throughout. For holding the diver, a simple support shown in Fig. 101 made from a piece of small-bore pressure tubing is useful. The ball on the bottom of the diver must fit the tubing securely to prevent rotation of the diver. More useful is a clamp arrangement mounted on a rack and pinion, the jaws of which are shown in Fig. 102. The diver is held exactly vertically by the neck in a special spring clamp having a movable tongue actuated by a light spring which provides the light but constant pressure necessary to grasp the diver firmly and yet not break or distort it. The entire unit moves vertically on a rack-and-pinion mount not shown, similar to a microscope. Liquids are inserted in the diver from pipets also mounted firmly in the vertical position immediately above the diver. The diver is raised by the desired amount over the tip of the pipet by means of the rack-and-pinion gear. Because the pipet tip may not be completely axial, it may be necessary to adjust the capillary position by movement of the entire pipet. This is accomplished readily by a ball-and-socket attachment from a photographer's tripod, as shown in Fig. 103. Three spring clips grasp the pipet. For convenience, the pipet

clamps are usually mounted on a metal rail which carries all the pipets employed in loading the diver adjusted to the correct

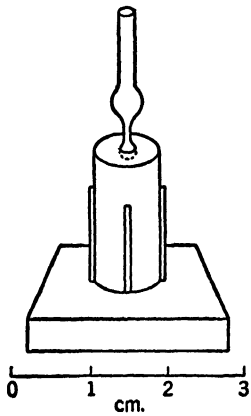


FIG. 101. Cartesian diver holder constructed from pressure tubing according to Holter.

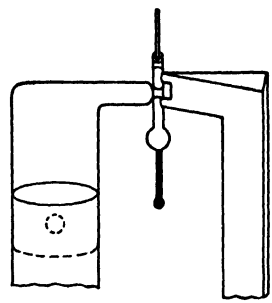


FIG. 102. Cartesian diver holder constructed with metal swivel jaws according to Holter.

height and with the tips exactly vertical. With this arrangement, the rapid loading of the diver is facilitated.

Pipets. Because of the extraordinarily small volumes, 0.2 to 2 λ, which must be measured with rather high accuracy, the pipets must be constructed in a special manner. It is most convenient to draw them from thermometer capillary having a bore of 0.2 to 0.3 mm. The capillary is enlarged, and the wall is thinned by pulling and blowing a heated section; the thinned region is drawn down in a microflame to a tip having an inside diameter of about 0.07 to 0.12 mm. and having an outside diameter of about 0.15 to 0.25 mm., depending on the size of the diver to be made. Some practice is necessary in forming a satisfactory pipet. The tip should be cut so as to be strictly cylindrical for about 10 mm. or even slightly enlarged at the

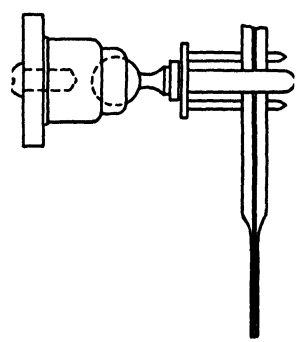


FIG. 103. Ball-and-socket pipet holder for loading Cartesian diver according to Holter.

very end. These pipets are controlled by the mouth and require some practice in operation. They must be very clean to prevent irregular flow of liquid when pressure is applied. Pipets with these diameters are used for aqueous liquids. When paraffin oil

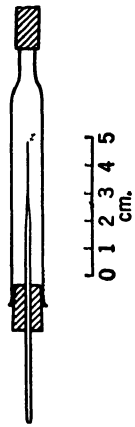


FIG. 104. Braking pipet according to Holter.



FIG. 105. Ball-tipped pipet according to Holter.

or other viscous liquid is to be measured, the diameter of the opening must not be less than 0.15 to 0.18 mm. and the glass thickness about 0.04 mm.

Another type of pipet termed the "braking" pipet serves chiefly for transfer of cells and tissue fragments along with small amounts of liquid. Its tip must be correspondingly large and the flow rate controlled by means of an extremely fine capillary at the top, as shown in Fig. 104. Even under considerable pressure or vacuum, the rate of flow from the pipet must be slow, e.g., a 1- to 5-mm. movement of meniscus per second.

Still another pipet which is at times valuable is the ball-tipped pipet shown in Fig. 105. When a drop is placed inside a diver, there is no tendency for the drop to creep up between the pipet tip and the wall, and this pipet may, therefore, be employed to place a droplet in the diver with great precision. The ball is blown on the end of a fine capillary by means of a compressed gas cylinder with a pressure of 0.5 to 2 atm. inside the sealed tube. On cautious application of a small flame, the ball will

be blown and perforated at its hottest spot. This pipet is chiefly useful with divers having necks of less than 0.7-mm. diameter.

With the exception of the braking pipet, delivery is read between marks. The braking type is calibrated for complete discharge and, therefore, has only one mark. Owing to the occasional difficulty of marking this pipet satisfactorily, an arbitrary filling under a cross-hair microscope and measurement of the length of liquid column with an ocular micrometer may be necessary. This occurs only when the pipet tip is so wide that the mark is below the diver mouth during loading. With narrow pipets, the mark can be applied in the usual fashion. All pipets are calibrated by iodometric titration of 1 *N* potassium iodate solution or acidimetric titration with 1 *M* potassium hydroxide. The pipet used for paraffin oil may be calibrated with the oil itself, by weighing the oil delivered by the pipet on a microbalance.

Platinum Loop. Because rings of beeswax must at times be applied to the inside of divers to prevent merging of liquid droplets, a special platinum loop arrangement for melting and applying the wax, as shown in Fig. 106, is desirable. A platinum wire *B* is heated electrically with a variable voltage transformer. To it is attached a loop *A* made from 0.2-mm. platinum wire which heats by conduction from the main wire.

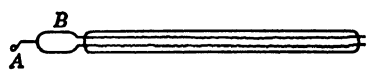


FIG. 106. Platinum wire loop for placing wax in Cartesian diver.

Liquid Media. Several liquids are used in operating the diver. The character of the flotation medium is critical inasmuch as it may dissolve other media in contact with it and particularly gases carried in the diver. It is desirable that it have as great a density as possible to minimize the difficulties of adjusting the diver's weight. The characteristics of the medium, according to Holter, should also include "low viscosity, chemical stability, transparency, biological innocuity, good ability to moisten glass surfaces and a low, well-reproducible surface tension." Media that have been employed include saturated ammonium sulfate solution (24), 11 *N* lithium chloride solution (26), saturated cadmium chloride solution (34), and a strong solution of sodium nitrate and sodium chloride (17). The latter has been most

widely adopted because it meets the stated requirements quite well. Strong salt solutions dissolve carbon dioxide less than most other liquids and are stable and unlikely to affect biological systems except when mixed with them in appreciable quantity. The preparation of this medium is carried out by dissolving 27.2 g. of sodium nitrate, 13.7 g. of sodium chloride and 0.2 g. of sodium taurocholate in 59.1 ml. of distilled water containing three drops of 0.1 *N* hydrochloric acid. To an aliquot of the solution is added a little bromocresol purple and 0.01 *N* hydrochloric acid until the color is the same as that of the dye in a salt-free solution at *p*H 5.8 to 6.0. The correct amount of 0.1 *N* hydrochloric acid calculated from that used by the aliquot is added to the main portion of the solution. The medium is carefully filtered by being passed through filter paper several times until it is perfectly clear. The density of the final medium must be checked at the temperature used with an accuracy of 0.1% since an error in this value is the greatest source of error in the final calculation of gas volume in the diver. The solution must be refiltered periodically and its density redetermined because of the accumulation of dust and evaporation of the solvent.

Other media or solutions that must be employed ordinarily include clear mineral oil having a viscosity of about 20 Engler degrees at 20° C. and a density of about 0.87. Highly refined oil of the type suitable for pharmaceutical purposes is best. Carbon dioxide is usually absorbed in dilute sodium hydroxide solution which may be of the order of 0.01 *N* and may contain 0.1% sodium taurocholate which reduces surface tension and allows efficient wetting of the walls. Other media include the manometer fluid and the solution which surrounds or contains the biological or chemical system in which the diver reaction takes place. The nature of this solution is determined by the type of measurement and the type of system whose activity is being measured. A suitable manometer fluid is Brodie's solution made from 23 g. of sodium chloride and 5 g. of sodium taurocholate dissolved in 500 ml. of water and preserved with thymol.

Calibration

A diver to be calibrated must have its total gas volume determined by being weighed when empty and then when filled with water to an accuracy of 0.1 mg. When the materials to be placed

in the diver and their amounts have been determined, the weight of the loaded diver is then calculated by the formula

$$g_D = \frac{v_T\phi_M - v_{oil}\phi_{oil} - v_W\phi_W - v_M\phi_M}{1 - \frac{\phi_M}{\phi_{gl}}}$$

where g_D is the desired weight of the diver.

v_T is the total volume found by weighing.

v_W is the volume of water (or the dilute aqueous solution) in the bottom and neck of the charged diver (the aqueous charge).

v_{oil} is the volume of the paraffin oil seal in the neck of the diver.

v_M is the volume of the mouth seal.

ϕ_M, ϕ_{oil}, ϕ_W, and ϕ_{gl} are the densities of the medium, the paraffin oil, the aqueous charge, and the glass of the diver.

In order to apply the formula, the density of the oil, the immersion fluid, the aqueous solution in the bottom and neck of the diver, and the glass must be known. All liquid densities are obtained on a Mohr-Westphal balance or by pycnometric weighings. The aqueous solutions can ordinarily be assumed to have a density of 1. The glass density is determined by weighing 2 to 5 g. of the glass with the Mohr-Westphal balance, or it may be conveniently ascertained by exact flotation of a small fragment in a mixture of organic solvents, such as bromobenzene and bromoform, the density of the mixture then being determined pycnometrically. The same procedure can be employed for measuring the density of the glass after constructing the diver, the latter being filled with the flotation liquid and adjusted as in the case of a fragment. This is necessary only if bubbles are present in the glass as a result of faulty construction.

When the weight of the loaded diver has been determined by calculation from the densities and desired volumes of each of the constituents, the weight may then be altered to a more suitable value by fusing on or removing from the tail a small amount of glass. The final weight of the charged diver should be very close to the weight of liquid which is displaced by it in the immersion vessel. It is recommended by the authors of the method that a diver that is too heavy be lightened by cutting off a relatively

large amount of glass and then adding weight by fusing on small glass threads rather than by attempting the removal of small amounts of glass. Frequently, the construction of the diver is not sufficiently exact, and excessive wall thickness may make the vessel too heavy for practical application. Lightening of the weight by removal of glass from the diver tail will then raise the center of gravity too high with resulting instability.

After the diver is adjusted to the proper weight to yield a practical equilibrium pressure, it is loaded and tested. Correct calculations should give an equilibrium pressure that does not deviate more than 20 cm. from the barometric pressure. Final adjustment may be made by altering the length of the mouth seal. It is more convenient to use the diver constant V, as defined by Linderstrøm-Lang to be equal to the total gas volume of the diver floating in its equilibrium position at pressure P and temperature $t°$. This quantity may be determined by subtracting the volume of all liquids added from the total internal volume of the diver and correcting the gas volumes for pressure and temperature or more easily by calculating the volume from the formula

$$V = \frac{g_D + v_{oil}\phi_{oil} + v_W\phi_W - v_{oil}\phi_M - v_W\phi_M - g_D\phi_M/\phi_{gl}}{\phi_M}$$

which is a variation of the diver equation previously given. Linderstrøm-Lang lists the errors in individual values in the formula, necessary to cause an error of 1% in the determination of V as follows: v_{oil}, 33%; v_W, 50%; ϕ_{gl}, 1%; ϕ_M, 0.5%; and ϕ_{oil} and ϕ_W, 12%.

Holter points out further that, if the immersion medium always has the same density ϕ_M, the quantity $g_D - g_D\phi_M/\phi_{gl}$ is a constant that is characteristic for each diver and may be determined for every diver. Also, the paraffin oil is normally the same throughout all experiments, so the values $v_{oil}\phi_{oil} - v_{oil}\phi_M$ may be calculated and plotted as a function of v_{oil}. The same system may be applied to the aqueous solutions since they are expected to deviate negligibly from a density of 1 in most biological work. The two curves having been determined, calculation of V is made by reading from the curves the values of $v_W - v_W\phi_M$ and $v_{oil}\phi_{oil} - v_{oil}\phi_M$, adding them to $g_D - g_D\phi_M/\phi_{gl}$, and dividing by the value of ϕ_M which is also known for the series. Measure-

ments with the diver not only give the change in pressure or volume at equilibrium as gases are produced or consumed inside the diver vessel but also include certain factors for which corrections must be made, such as the barometric pressure on the open end of the manometer (e.g., the thermostated air bottle), the hydrostatic pressure of the medium in the flotation vessel above the level of the diver, and the capillary pressure at the boundary between the medium and gas phase in the diver neck. Because all these factors are constant and do not affect the change in equilibrium pressure, they may be included as a part of the calibration. A correction is required for the change in pressure in the air bottle as the manometer level is altered. This correction is calculated by the formula

$$P = p\left(1 + 1000\frac{A}{V'_g}\right)$$

where A = the cross-sectional area of the bore of the manometer tube.
V'_g = the volume of the air bottle.
1000 = the barometric pressure in centimeters of Brodie solution.

Since it is the change in volume of the gas in the diver that is important, the relationship of the change of equilibrium pressure to ΔV is the significant relationship desired. Where there is no solubility of gas in the diver charge, the expression $\Delta V = V\,\Delta P/P_0$ applies. Since this is not always a completely valid assumption, the calculations must be corrected by means of the appropriate factors.

Procedure

Before actual measurements may be started, a number of preliminary operations must be carried out. If it is assumed that all glass parts of the apparatus are available and that the thermostat is adjusted and operating, it is necessary first to charge the flotation vessel with medium and, while the open end is closed with the finger, to shake the solution vigorously until it is entirely filled with air bubbles. The vessel is placed in the thermostat and withdrawn and reshaken at least twice at intervals of about 5 minutes. No measurements should be taken with the solution until at least 1 hour after saturation of the flotation

medium with air. Attachment of these vessels must always be made while the stopcocks are open to the exterior to prevent any increase in pressure of the gas which will occur while a lubricated ground-glass joint is inserted. The air bottle in the thermostat is open to the outside air to equalize its pressure with the barometric pressure existing at that time. During the final equilibration of the apparatus, the diver may be loaded.

Filling the Cartesian diver requires precision operation with manipulators if it is to be successful, particularly with the smaller sizes of diver. Pipets to insert media should be specially made as discussed earlier, and the positioning of the pipet tip with respect to the diver is critical in placing the droplet at exactly the correct point. With the larger divers the operations are considerably simplified. The diver and pipet are mounted in an appropriate diver support or clamp and positioned so that the tip of the pipet can be inserted directly down the center of the diver bore without touching the sides. The rack-and-pinion manipulators specifically developed for this purpose by the authors of the method may be replaced by other designs of manipulator which take account of the peculiar nature of the problem. In any case, the mounting of diver and pipets must be such that manipulations can be performed smoothly and without realignment during operations. In general, the materials to be loaded consist of the media forming one or more liquid seals, and fragments of tissue or other biological system which are to be placed at the bottom of the diver without touching the walls. The relationship of the amount of liquid and the size and construction of the diver must be such that at no time is a layer of liquid thicker than 0.5 mm. The biological material is usually added by means of the "braking pipet" (Fig. 104). If the amount is so large that it cannot be introduced by means of a pipet, the entire diver should be filled with the medium used in the bottom drop after which the object is led into the diver's mouth, allowed to sink to the bottom, and the excess solution removed by means of a pipet. Both the outside and inside of the diver must be dried, the inside being dried best by means of filter paper which carries no loose fibers. The amount of liquid placed in the diver under these circumstances is determined by weighing after the final seal has been placed in the neck.

These operations require the use of very fine tips which can

be inserted clear to the bottom of the diver without touching the sides. It is for this reason that specially drawn types of pipets are necessary. After the reacting medium is in place, the next solution to be added will ordinarily be sodium hydroxide for absorbing carbon dioxide, e.g., when the respiratory behavior of small biological systems is measured. By means of an appropriate straight-tipped pipet, less than 0.25 mm. in outside diameter, the caustic seal must be placed at a sufficient distance from the original biological medium so that there is no danger of the two creeping together. With small divers particularly, this may occur, and, when difficulty is encountered from this source, a wax ring should be placed above the position of the bottom drop, usually in the neck of the diver to prevent any creeping of either solution. This operation may be performed with a small electrically heated platinum loop (Fig. 106) carried in a manipulator. A good grade of beeswax is melted on the loop which is then inserted to the proper position; the wax is melted and drawn around the stem until a complete ring is formed. In general, even with the larger divers, it is preferable to use a manipulator and magnification in placing the ring.

Following the caustic seal, which should be at least 0.5 mm. in length, a final oil seal is usually placed near the top of the diver neck. At times a series of liquid seals are desired, but for most measurements, such as that of respiratory quotient, a caustic seal followed by an oil seal and a mouth seal are used. The oil seal is introduced in the same manner as the caustic. The oil is simple to place since it moistens the glass surface easily and flows into position better than water solutions. The purpose of the oil seal is to minimize diffusion of gases from the diver to the immersion liquid or the reverse, a function which it fulfils only partially. No better sealing liquid has so far been found, although the ideal would be one in which all gases present, particularly carbon dioxide, would be completely insoluble.

The mouth seal is the last one placed. It consists of the fluid in which the diver is immersed. It obstructs loss of gas from the diver and adjusts the initial equilibrium pressure of the diver to the desired value. The mouth seal must have a considerable length, usually several millimeters, in order to achieve the second purpose. The length in excess of this minimum value

depends on the size of the gas volume enclosed in the diver and, in general, may be obtained by considering that increasing its length by 0.1 mm. changes the equilibrium pressure by 5 to 10 cm. This makes necessary accurate adjustment of the length of seal to approximately 0.1 mm. In general, the length of mouth seal is a constant for a particular diver and, once determined, can be placed repeatedly to the same length. A microscope equipped with a micrometer screw is advantageous in determining the length of the seal as accurately as needed. The placing of the mouth seal by direct pipet measurement of the solution is not practical. A drop of the liquid is placed in the neck of the diver, and a very finely drawn pipet tip, with an internal diameter of 0.1 mm., and an external diameter of 0.15 mm., and with a finely drawn braking tip, is pushed through this droplet into the entrapped air space below, about 1 mm. below the position determined for the lower meniscus of the mouth seal. Suction is applied to the braking pipet to remove air while more flotation medium is placed with a fine pipet tip in the mouth of the diver until the meniscus is brought down to the correct position. At the final equilibrium point, the lower meniscus of the mouth seal will be accurately placed with respect to the mouth of the diver and the neck will be filled to the rim. Salt solution that is caught in the tip of the pipet is immediately removed since the liquid is almost saturated with salt which will precipitate and rapidly plug the pipet.

Variations of the technique described are necessary in special applications of the diver and because of differences in the size and shape of the diver vessel or the conditions of measurement. Preparatory testing and adjustment of the system is, therefore, necessary, and, once the diver is adjusted and calibrated, it may be used as long as possible, essentially in the same state. At times, biological samples are placed in the seal for special reasons which necessitate a change in the method of loading. The investigator must experiment with his system to obtain the correct positions before starting serial measurements.

Another variation which is often serviceable is to employ glass stoppers to diminish the diffusion rate of gases. The stopper consists of a short piece of glass rod with a diameter of 50 to 80 μ less than the inside diameter of the diver neck and with a length that is usually 2 to 3 mm. The weight of the solid rod

may be disadvantageous, in which case a fine capillary with sealed ends may be substituted, although this is considerably more difficult. The glass stoppers are placed in the oil seal by means of fine forceps, or they may be placed in the mouth seal with a special type of elongated forceps shown in Fig. 107 after the diver is introduced into the flotation vessel. The forceps are made from a piece of thin metal tubing, 15 cm. long and 2 to 5 mm. in outside diameter, enclosing a metal pin 1½ mm. in diameter, which is about 2 cm. longer than the tubing and is terminated by a knob. The actual tips of the forceps consist of two narrow

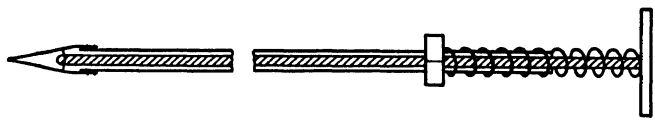


FIG. 107. Forceps for placing Cartesian diver according to Holter.

strips of spring metal, 2 mm. wide and 2 cm. long, soldered to the outside of the tube. Pressure on the top of the rod forces it down inside the tube, spreading the points. When the pressure is released, a spring forces the rod backward, allowing the tips to close on the glass stopper.

Having saturated the immersion fluid with air and loaded the diver, the latter is now ready for introduction into the fluid in the immersion vessel. This should be done as rapidly as possible after the mouth seal is placed. The diver may be dropped into the immersion vessel or lowered with a wire loop made of fine stainless steel wire. Often air bubbles are deposited on the outside of the diver. They may be removed by means of the wire loop. If the mouth seal has been correctly placed, there should be no air bubble formed in the mouth. The immersion vessel is connected to the main apparatus by means of the ground joint, leaving the apparatus open to the exterior air. The flotation vessel is then connected to the manometer, and the diver is made to float by lowering pressure to ascertain if it is adjusted approximately correctly. The equilibrium pressure is adjusted, which is simply done if the diver is correctly loaded, particularly with respect to the length of mouth seal. Inspection of the exterior of the diver with a magnifying glass for any air bubbles still present should be made.

After all flotation vessels are loaded, each one is connected in turn to the manometer and adjusted to the pressure at which it is to be kept between measurements. This must be the exact equilibrium pressure or a somewhat greater pressure which causes the diver to sink. The usual practice is to apply enough excess pressure to force the diver to the bottom of the vessel between measurements. This is usually 3 to 5 cm. of Brodie solution above the equilibrium pressure. As measurements are made, the pressure is allowed to diminish just sufficiently to bring the diver to its equilibrium pressure, after which the manometer is read. If the diver sticks to the bottom, it should not be freed by lowering the pressure further but rather by a slow change in pressure accompanied by a slight tapping on the neck of the flotation vessel. Fine adjustment of pressure is necessary to adjust the diver position accurately with respect to the reference mark on the immersion vessel. The latter is usually observed with a magnifying lens or, in very small divers, with a low-power microscope. Repetition of readings to assure equilibrium is carried out by altering the pressure very slightly after reading and restoring it to see that the equilibrium pressure obtained on the second reading agrees with the first. In general, the pressure readings are reproduced to 0.5 to 0.10 mm.; usually 0.5 mm. is sufficiently accurate.

A further point requiring attention is that the shapes of the menisci in the neck of the diver are normal. A typical half-spherical shape indicates the degree of moistening of the glass, and a distorted meniscus is always an indication that the diver is not in its equilibrium position because of the surface tension effects which resist free change of volume of the gas phase inside the diver. Should such a distortion be observed, the diver is allowed to sink and be restored several times to its equilibrium position in the effort to obtain normal menisci.

Variations of Technique

The discussion of the Cartesian diver microrespirometer which has been given was adapted directly from that of Holter and of Linderstrøm-Lang. The technique is clearly designed to produce the highest possible accuracy from this instrument and involves many details that are complex and may often be troublesome. It is equally clear that much biological experimentation

need not be carried out at the absolute maximum accuracy which is obtainable. For this reason, a considerable degree of simplification can unquestionably be introduced into the operation of the diver. As an illustration of this, if the immersion fluid is made with a higher specific gravity than that used by the originators of the method, the construction of the diver itself becomes simpler since the wall thickness is less critical. The use of cadmium or zinc chloride solutions (34) serves to increase the specific gravity of the medium to about 1.5, as compared with approximately 1.3 for the Holter mixture.

Claff (33) has reduced the making of divers to a comparatively routine operation by means of a jig illustrated in Fig. 108. He points out the advantage of this procedure over free-hand construction in that it produces more uniform divers which may be more readily adapted to different shapes and with a great deal less experience and skill in glass blowing. The jig consists of a stationary plate attached to a handle and a similar removable plate, both made of aluminum or brass. Two sets of paired holes are drilled through both plates, one for making divers with relatively thick tails, the other for thinner tails. The first set has paired holes of the same size in the plates, both being made with a No. 77 drill, 0.018 in. in diameter. The second set uses the same drill for the removable plate and has a set of different-sized holes in the stationary plate to accommodate the size of capillaries employed.

Thick-walled Pyrex capillary tubing, varying in outside diameter from 0.030 to 0.060 in. is selected, depending on the size of diver desired. The capillary is drawn essentially as described by Holter to the corresponding diameter. It is inserted through a paired set of holes so as to extend about 20 mm. from the left side. With the jig held in the left hand and the forefinger pushing lightly against the protruding capillary, a flame is used to melt the glass in the middle of the jig. At this point, the protruding capillary on the left is prevented from turning by being held with the left forefinger while the glass on the right-hand side is given three or four complete revolutions. This seals off the capillary, after which the pressure of the left forefinger is released and the entire capillary is turned freely in the flame, forming a solid glass rod 3 or 4 mm. long.

The thickness of the tail can be adjusted by collecting more

or less molten glass in the center. The tails may be thinned by heating and pulling them at the appropriate temperature. The capillaries are sealed off some distance from the solid rod portion, after which this blank is moved to one of the lower sets of

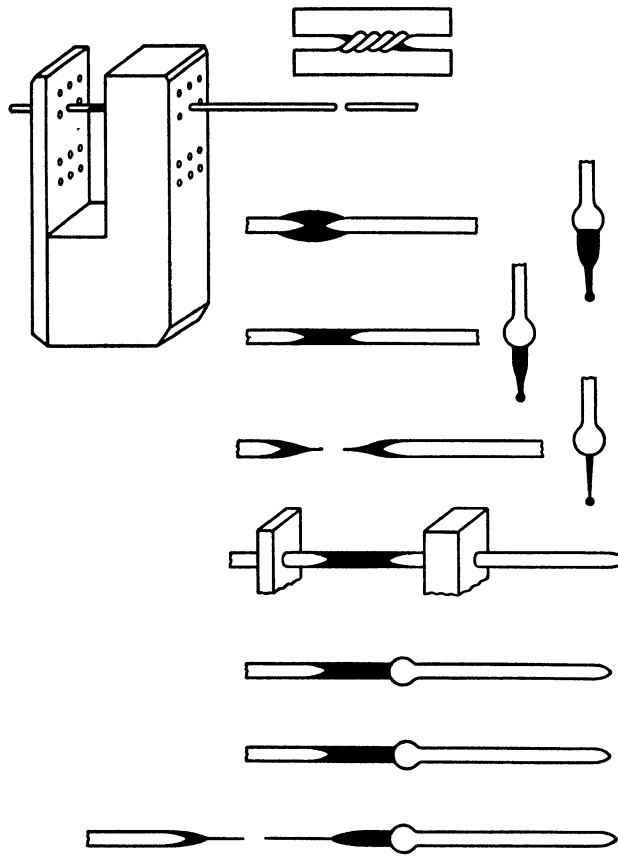


FIG. 108. Jig construction of Cartesian diver according to Claff.

holes so that the thin portion of the tail fits into one of the very small holes. A small bulb ("bubble") may then be placed in the blank by applying heat at the point of junction of the capillary with the rod, moving the jig a little as the glass swells to form a bubble. The blank is then withdrawn and twirled until it is cool. With divers having larger tails, the blank may be blown

into its final form without being moved to a smaller set of holes. After the lower part of the diver is formed, the tube is cut to the correct length, usually 8 to 10 mm. from the top of the bubble.

The reproduction of divers for uniformity can be achieved readily by measurement of an amount of mercury whose volume is that desired for the diver (34). This mercury is placed successively in capillary tubes which are cut off in the right position to give divers of a predetermined total capacity rather than making trial divers and calibrating them individually. This method may also be applied to the Claff technique by opening the sealed end of the capillary, placing the correct amount of mercury to obtain the desired length, and cutting the tube accordingly.

The adjustment of mouth seals can be more simply done by empirical means than by the rather elaborate calculation detailed above. If the amounts of liquid to be used in a diver, except that in the mouth seal, are placed immediately in their proper location in the diver and the latter with a trial mouth seal is introduced in the immersion liquid, a trial of the equilibrium pressure can be made rapidly and the amount of air within the diver changed by the insertion of a pipet as described above. When the air adjustment is correct, the position can be noted and reproduced later. This avoids some of the intricate calculations of the more detailed procedure.

Considerable simplification in the operation of the thermostat can be obtained by using a thermostat which is constant only to about 0.1° and enclosing the immersion vessel inside another vessel containing liquid which serves to smooth the variations in temperature of the main bath of water. Since this need only slow down the transfer of heat for the lag period of the thermostat itself, a smoothing from a constancy of 0.1 to 0.01° is not particularly difficult. Even a third vessel or a clear vacuum bottle could be used in a similar manner. The density variations, which are quite critical for the highest accuracy of the device, may be approximated with sufficient accuracy for many biological experiments. For these reasons, a technique which in its entirety and adapted to the highest obtainable accuracy may seem prohibitively difficult or time-consuming to the inexperienced investigator can be modified fairly easily to yield somewhat less accurate but entirely serviceable results for many of the purposes of the

biologist. Experience with the method bears out the fact that, for work within the range of the usual biological variation, the Cartesian diver technique is actually not very complicated.

It is assumed in the somewhat extended discussion given that the Cartesian diver serves principally for the easy determination of oxygen consumption of a biological system and for several enzymic and other reactions which produce a change in the volume of gas (31). The technique described does not allow for mixing of reagents which is essential if the apparatus is to be employed in the manner of the Warburg apparatus. Anfinsen and Claff (32) adapted the technique so that the Cartesian diver is capable of carrying out the essential operations of Warburg apparatus on about one thousandth the scale of magnitude. The principle used by them is to paraffin the interior of the neck of the diver with a thin coat throughout most of its length. Droplets to be merged are placed on the sides of the neck in order, as shown in Fig. 109, and above this a seal of liquid to close off the neck. On the application of extra pressure to the diver immersion fluid, the seal is forced downward until it merges with the drops in order of their position in the tube. Thus, one can add to the liquid in the seal one or more accurately measured droplets of reagent. As the pressure on the immersion fluid is gradually released until equilibrium pressure is restored, the original condition of the diver with respect to original gas contained in it is restored.

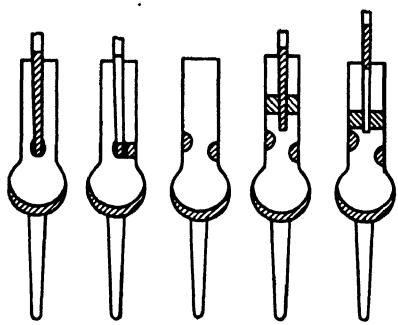


FIG. 109. Mixing of reagents in the Cartesian diver. (*Anfinsen and Claff.*)

The most important detail of technique involved in this type of operation of the diver, aside from the greater amount of wax coating, is the addition to the diver holder of a mechanical stage, which allows lateral movement of the diver during the filling operation. This is necessary in order to place droplets on the side of the capillary wall in accurately positioned locations. This operation requires a well-constructed pipet tip with sufficient

rigidity to prevent movements through capillary attraction. All operations are carried out under a horizontal binocular microscope in order to place the drops precisely. By means of sodium carbonate solution and excess acid, the evolution of carbon dioxide can be employed also for calibrating the diver and studying its behavior. Likewise, the carbon dioxide produced by an organism and absorbed in a caustic droplet can be subsequently released and measured by the change in equilibrium pressure. The authors also tested the device by inhibition of succinic oxidase by the antimalarial drug, 2-hydroxy-3(2-methyloctyl)-naphthoquinone-1,4. With this or a similar modification of the Cartesian diver technique, it is now possible to carry out virtually all the experimentation on extremely minute biological systems that was previously possible only with the Warburg apparatus which required very much greater amounts of reacting material.

A further improvement in the technique of mixing solutions in the diver, and therefore obtaining results comparable to those of the Warburg technique, was developed by Claff and Tahmisian (35). The diver vessel itself is made in the form of an hourglass with two bulbs, one above the other, and a relatively long neck. Construction is performed in the jig as described except for the blowing of the extra bulb. In the bottom bulb (or expansion chamber) may be placed the caustic for absorption of carbon dioxide. In the upper bulb (or reaction chamber) is placed the biological system, e.g., a suspension of cells, an enzyme substrate, or a sample of standard bicarbonate solution (for calibration). In the neck immediately above the upper bulb is placed the solution which is intended to exert an effect on that in the reaction chamber, e.g., a respiration inhibitor, an enzyme solution, or, for calibration, a drop of acid to release carbon dioxide.

For mixing the drops an improved method is used. Instead of altering the manometer pressure, the line is equipped with a three-way stopcock, to one arm of which is attached a sphygmomanometer bulb. When the diver chamber is connected to this bulb, pressure changes are readily produced without disturbing the manometer system. The interior of the diver is coated with a thin layer of Clarite dissolved in toluene, or another non-wetting surface such as lanolin. A single application of Clarite is sufficient to maintain the surface for a long period. The technique

was applied to preliminary studies of *Arbacia* eggs, *Paramecia*, and *Chaos chaos*.

It is apparent that, with the development of simpler and more reproducible methods of making and operating the Cartesian diver, this technique is rapidly assuming a position of major importance in biological research on the smallest systems.

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CHAPTER 9

Spectrophotometric Methods

Many colorimetric analyses and particularly those performed by means of a spectrophotometer have limiting sensitivities that lie in the range of microgram analysis. Thus it is not difficult to determine one or a few micrograms of many constituents for which sensitive color reactions exist. Numerous methods capable of yielding this sensitivity are given in standard textbooks, of which those by Sandell (1), Snell and Snell (2), and Yoe (3) are perhaps best known. Nearly all analytical biochemistry books include numbers of such methods. Biochemical and clinical methods of this type are found in Peters and Van Slyke (4), Hawk, Oser, and Summerson (5), Gradwohl (6), and others. It must be remembered that these standard methods are almost without exception at the very lower limits of their sensitivity when used to determine microgram quantities. For this reason such methods employed in this region are at the maximum of difficulty of operation and the minimum of accuracy and reliability.

Adaptation of the equipment as described in Chapter 3 makes it possible to diminish greatly the amount of material that can be accurately analyzed and in fact increases the dependability of the procedures with microgram quantities to a point approximately equivalent to that of the less sensitive standard methods. Submicrogram colorimetric procedures are accordingly of greater interest to the ultramicrochemist because it is in this direction that future progress toward smaller-scale analysis will be directed. A serious limitation to the discussion of submicrogram procedure is that very few such methods have actually been developed in detail and described. In several instances, the procedures, even though they have not been employed or described, are sufficiently simple and reliable so that some easy adaptation of the common methods can be made to utilize submicrogram colorimetric technique. Consequently it is most important that the principles of

application of such adaptation be completely understood. Indications of suitable methods for such adaptations are included for a number of important constituents.

SUBMICROGRAM METHODS

The requirements for adapting a method to the submicrogram range with as great an increase in sensitivity as possible are:

1. The color must be developed in a small liquid volume only slightly in excess of the capacity of the cell which is to be used.
2. The cell must be designed to have as long a light path as possible.
3. All the liquid in the cell must be included in the transmitted beam. By this means virtually all the colored substance actually absorbs light, thus producing the maximum effect for a given amount of total material determined.

Probably the smallest practical cells for absorption spectrophotometry are those of Lowry and Bessey (7) which operate with as little as 50 λ. Calculation indicates that it would be practical to operate horizontal capillary cells of 1-mm. diameter and 5-cm. length, though no such cells have as yet been described. They would also contain about 50 λ but would yield five times the sensitivity of the Lowry and Bessey cell with the same volume. The latter type of cell has several important advantages and certain disadvantages for sensitivity and general application. The advantages may be summarized as follows: (*a*) The cell fits the standard Beckman cell holder with which the spectrophotometer is equipped. (*b*) It is quite simple to fill and empty. (*c*) It requires little attention as to alignment. Its disadvantages are: (*a*) With any volume (50 to 200 λ), its sensitivity is limited by the 1-cm. light path and does not increase as the volume added is increased. (*b*) A metal mask is required to prevent transmission of light through the glass or quartz from which it is manufactured. (*c*) At maximum sensitivity, i.e., with the color developed in 50-λ volume, the technique of adding reagents and developing color is difficult. In fact, the full sensitivity of the cell has rarely been utilized, probably for this reason.

Compared with the above-discussed cells, the smallest cells described by Kirk, Rosenfels, and Hanahan (8) also have several advantages and disadvantages, the advantages of which are: (*a*) The sensitivity is capable of being increased five times

because of the longer light path if all other factors are identical. (*b*) Since the volume required is under 200 λ, to which the colored solution is made, it is easier to add reagents and to develop color. (*c*) The cells mask themselves because of their construction from opaque material. The disadvantages are: (*a*) A special type of cell holder is necessary, which can, however, serve for several types of cell, including horizontal macrocells up to 50-ml. capacity. (*b*) The cells require careful alignment and occasionally require realignment because of slight movements of the light beam of the spectrophotometer. (*c*) Difficulties in filling the cells have arisen, particularly when the filling ports were not drilled correctly.

The larger cells described by the above authors (8), which have a 4-mm. internal diameter, hold a little less than 1 ml. of solution, which is in a range of lower sensitivity than the Lowry and Bessey cell and intermediate between standard operation and submicrogram analysis. Manipulation offers no special difficulties on this scale, and the same cell holder employed with the smaller cells is also suitable here. In general, amounts ranging in the tenths and hundredths of micrograms may be analyzed by means of this equipment.

The sensitivity of the Beckman spectrophotometer is such as to allow use of cells of smaller diameters than the 2-mm. cells described, probably at least down to 1-mm. diameter. This corresponds with a fourfold increase in sensitivity over the 2-mm. cells and an increase fivefold greater than the maximum possible with the Lowry and Bessey type of cell. The manipulative difficulties in developing color would be identical with those of the latter type of cell. Filling and alignment may be more difficult. The development of this size cell, and indeed smaller ones, is to be expected as a natural extension of techniques already in use.

The advantages of submicrogram apparatus in spectrophotometry are obvious when the available amounts of material are small or when it is more convenient to obtain a small than a large sample. It is unfortunate that few detailed methods are available for discussion. It is evident, however, that most colorimetric methods may be adapted to operations in the submicrogram range, because the fundamental techniques are available. The advantages of applying such methods to systems such

as small animals, insects, small organs, or tissues of plants or animals, to samples containing high levels of radioactivity, to finger-blood analysis in the pediatrics and other clinical laboratories, and to a host of other similar analytical problems are too evident to require detailed discussion. The treatment of such methods in this chapter must be limited to a few specific illustrations of this type of adaptation and some suggestions for further similar developments.

Alignment and Adjustment of Capillary Cells

The light beam of the Beckman spectrophotometer is slightly divergent rather than exactly parallel as it emerges in the absorption cell chamber. Also, it occasionally emerges at an angle to the axis of the instrument, thus failing to fall squarely both on the absorption cell and on the phototube. The instrument must be checked to ascertain that the beam is directed parallel with the axis of the instrument and falls squarely on the phototube when there are no cells in place. If it fails to do so, an adjustment of the concave mirror in the light source housing will usually correct it. This is accomplished with the capillary cell holder in place, which moves the phototube approximately 10 cm. from the light source and makes very apparent a slight divergence from axial illumination. It is always advisable and usually necessary to restrict the vertical height of the light beam from its normal height of 9 mm. to about 6 mm. or less so that none of the beam can pass over or under the face of the absorption cell and affect the phototube. This is very simply done by inserting a mask cut from cardboard or light sheet metal in front of the light source, ahead of the capillary cells.

After the beam is checked or adjusted and the simple mask is inserted, it is next necessary to adjust the position of the cell holders with respect to the light beam. Since the latter is slightly divergent and not of uniform intensity throughout its cross-sectional area, some care is necessary that this preliminary adjustment be carried out properly. The cell holder is provided with both lateral and vertical adjustments, and the cells are designed in such a way that they cannot be moved in the axis of the light beam. The latter precaution is desirable because the emergent beam is not exactly parallel, and axial movement in the light beam would allow a variable amount of light to be

intercepted by the cell window. The only adjustments necessary then are the lateral adjustment to place the capillary in the exact center of the beam and a vertical adjustment to allow both cells in the instrument to intercept the same amount of light from a somewhat uneven source.

The lateral adjustment is made by placing two matched capillary cells (2 mm. in diameter and empty or filled with identical liquid) in the holder, putting the lid in place, and adjusting from the exterior until the maximum transmission reading is obtained with one cell. The other cell is then slid into the light beam and also adjusted to give the maximum transmission reading with the instrument operating. It is unimportant what wavelength of light is chosen, and the slit width and sensitivity are adjusted to prevent excessive response from the instrument.

The vertical adjustment is somewhat more difficult to make, since the adjusting screws are on the inside of the holder. With the shutter to the phototube closed, the lid is removed, and the height of the two cells is checked to ascertain that both cells are at about the same height in the light beam and approximately centered before the mask. The lid is replaced, the shutter opened, and a transmission reading taken with one of the cells. The other cell is pushed into place, and a transmission reading is taken. If this disagrees with the reading obtained with the first cell, the shutter is closed, the lid opened, and a vertical adjustment made. When the transmission readings of the two cells are identical on transposition of the cells, the adjustment is complete. In order to avoid disturbing the adjustment, it is desirable that the switch on the light source box should not be touched but left turned on at all times. A switch installed in the line to the outside battery terminals and not attached to the instrument should be used instead. This may be a double-pole double-throw switch, the center terminals of which are connected to the battery, one outside set to the instrument, and the other outside set to the battery charger. Thus, at the termination of a series of measurements, the switch is thrown to the opposite position which puts the battery on charge. Opening the switch turns off everything. This precaution of not manipulating the switch provided stems from the instability of the housing of the light source which contains not only the light but the focusing mirror for the beam which then displaces the emergent beam enough to cause

incorrect readings. Once the instrument and cells are adjusted, it is a worth-while precaution to avoid all contact with the light-source housing.

When the 2-mm.-diameter cells have been positioned correctly, it should not be necessary to make any changes to accommodate 4-mm. cells, though this point may be checked. The 4-mm. cells, being wider than the 2.6-mm. light beam, should have the light pass accurately through the center of the bore, a point which is assured if the 2-mm. cells employed to adjust the holder were actually placed at the exact center of the beam. Larger cells, such as those holding 7 ml. or more of solution naturally require no further adjustment.

Adaptation of Methods to 1-Ml. Volume

The 4-mm. capillary cell, 5 cm. in length and similar to that shown in Fig. 37, contains about 0.6 ml. when filled, and it is convenient to make the volume of colored solution up to 1 ml. This will allow 0.4 ml. for rinsing, excess solution in the filling ports, and waste. Clearly, the requirements of the adaptation are that all volumes of reagents and diluents be small enough to add up to a total volume not in excess of 1 ml.

The sample volume is rarely in excess of 0.1 ml. and may usually be less because of the high sensitivity obtained with the long light path.

When the color may be developed by the addition of reagents alone, the procedure is very simple. The sample is measured by pipet into an accurate 1-ml. volumetric flask (stem size not in excess of 4-mm. internal diameter), and the pipet is rinsed as usual. The reagents are added in order, in amounts scaled down proportionately to the sample size as specified in the method being adapted. The volume is brought exactly to the mark, mixed, and transferred to the cell by means of a transfer pipet equipped with a syringe control. The cell is held just slightly tilted, so that the filling port is at the lower end. The pipet is pressed firmly against the port, and the liquid is slowly expelled into it. As soon as the proper amount has been transferred, the cell is inspected against a good light to ascertain if there are air bubbles in the bore. If there are, the pipet is employed to partially withdraw the liquid which is forced back and forth once or twice with the syringe to sweep out any such

bubbles. When the cell is held correctly and the liquid added at the proper rate, there is rarely any difficulty from this source. The cell is then placed in the instrument, along with a matched cell carrying the blank, and the measurement is made in the usual fashion.

When additional operations are necessary for the development of color, e.g., heating, extraction, or refluxing, extensions of technique are essential. A 1-ml. flask may be heated satisfactorily, but its contents may not be boiled because of steam blocks which form in the neck and cause loss of liquid. A boiling water bath satisfactorily heats most solutions without difficulty. Actual boiling of the contents would require a tube or other wide vessel and subsequent transfer to the volumetric flask.

Extraction would impose only the condition that the total volume of extract be not greater than 1 ml. Satisfactory techniques for extraction in this range have been given previously.* The original volume of sample and previously added reagents would not usually be critical in this instance.

Adaptation of Methods to 200-λ Volume

When the amount of the constituent determined is in the truly millimicrogram range, cells of smaller capacity than 0.6 ml. are usually necessary. The horizontal capillary cell, 2 mm. in diameter and 5 cm. in length, containing about 160 λ, is suitable, as is also the Lowry and Bessey cell used at maximum capacity. The color is conveniently developed in a volumetric flask as shown in Figs. 25 and 26, having a capacity of 0.2 ml. and a stem diameter not greater than 2 mm.

In the development of color, the volumes (and concentrations) of reagents must be such that, when they are added to that of the sample and rinsings, the volume will not exceed 0.2 ml. It is clearly better to add a very small volume of a rather concentrated reagent instead of the quite dilute solutions often employed in colorimetry. The size of sample rarely exceeds 50 λ, and one of 10 to 20 λ is usually sufficient when working in this range. Smaller samples of high enough concentration are clearly usable, and, in fact, the actual concentrations that will give sufficient color are often surprisingly small, making possible the analysis in many instances of actual trace quantities in small

* Cf. Chapter 4.

volumes. When the extreme dilutions so often employed in conventional colorimetric and spectrophotometric methods are considered, it is seen that a dilution from even less than 10 to 200 λ is less than that often employed in more conventional procedures.

Rinsing of the sampling pipet is especially important in measuring small-sample volumes because the volume of rinsings must be kept minimal without sacrificing the quantitative transfer of the sample. The pipet is rinsed twice, each time with a small drop of liquid much less in volume than the content of the pipet. This liquid is drawn up to the mark and expelled. Two rinsings with a small volume are almost as efficient as two rinsings with total pipet volumes and much better than one rinsing with twice the small volume. The error from sample which is not removed is of the order of a few tenths of 1% at most. Since air blocks form readily in the stems of the small volumetric flasks, it is necessary to throw the solution to the bottom by giving the flask a quick jerk. Before the volume is made up to the mark, it is well to centrifuge the flask for a few seconds in a hand or slow-speed electric centrifuge to drive the liquid from the walls to which some of it adheres into the main liquid body. The error caused by neglecting this operation may be considerably greater than most of the other manipulative errors.

If the liquid must be heated to develop color, a hot-water bath is used. The content of the flask can never be boiled in place, and boiling it in the open is dangerous because of possible spattering and because of the rapid rate at which small open volumes diminish through evaporation when they are heated. Extraction of these small volumes is possible by means and equipment already described.* Use of solvent volumes up to 200 λ is possible, and the original phase extracted may be considerably larger than this.

Filling of the capillary cells at times causes some difficulty, as was discussed earlier. In general, the newer designs give little trouble from this source. If the cell tends to trap air bubbles, it is ordinarily effective to pump the liquid back and forth in the bore a few times with a pipet and syringe control, which usually displaces any air bubbles.

If the 1-cm. cell of Lowry and Bessey is adopted for the

* Cf. Chapter 4.

evaluation of the light absorption, the greater part of the 200-λ volume which may be placed in the cell is not in the light beam and therefore does not add to the sensitivity. It causes no difficulty with the reading and may be added merely as a matter of convenience, rather than adding only the 50-λ portion that absorbs light and wasting the remainder.

Alternative techniques which avoid the employment of a volumetric flask during color development are not always satisfactory with volumes as large as 200 λ. A calibrated tube with an internal diameter of 4 mm., for example, although somewhat more convenient to manipulate, gives a probable error in the volume determination which is four times that of the 2-mm. stem used with the flask. The magnitude of the error is of the order of 1 to 3%, as compared with a few tenths of 1% with the flask. If the analyst does not require more accurate results than this, it may save considerable time to use tubes, but it must be appreciated that the volume error is only one of several errors that are present in any colorimetric method and that all errors can at times become additive and make the total error much larger than anticipated.

Another alternative method of making up the volume of colored solution which is satisfactory from the standpoint of accuracy of dilution is similar to that described for 50-λ volumes in the next section. The color is developed in a micro test tube in a volume considerably smaller than 200 λ. The solution is removed carefully into a 200-λ pipet, the tube is rinsed, and the rinsings are added to the pipet content until the volume is exact. Mixing is performed by alternately expelling the solution to a paraffin surface and drawing it up in the pipet. This procedure utilizes several extra operations but may be preferred by some as a means of avoiding entrapment of air bubbles in a small volumetric flask.

Adaptation of Methods to 50-λ Volumes

If Lowry and Bessey cells are operated at minimum capacity or if a 5-cm. capillary cell of 1-mm. diameter is used, the lowest total volume to which a solution may be made is of the order of 50 λ. With either alternative, greater difficulties exist in adding reagents and rinsings than with larger volumes. It is, however, possible when the sample volume is not in excess of a few micro-

liters to perform the color development operations in a fashion quite similar to that employed in the larger cells. Ordinarily the sample should be measured with a self-filling or Levy-type pipet since the volumes are usually less than 5 λ. Although it is theoretically simple to construct a 50-λ volumetric flask, such a container to be accurate would require a stem so small as to preclude easy introduction of pipet tips into it. For this reason, a 50-λ pipet is more practical for the final adjustment of volume, and mixing is performed outside the volumetric apparatus.

A suitable technique for addition and mixing of reagents for color development is that so successfully employed by Brüel, Holter, Linderstrøm-Lang, and Rozīts (9). A clean paraffin or Teflon block is used for all operations. To the surface are added in separate droplets the various reagents required for color development. One droplet should consist of water in an amount which makes the total volume slightly less than 50 λ. The sample, measured with the self-filling pipet, is discharged into this droplet which is employed to rinse the pipet. With a 50-λ pipet, the sample and reagents are collected and mixed in the proper order. Mixing is accomplished by filling and emptying the pipet on the paraffin block. After the addition and mixing of the last reagent, the pipet is filled to the mark by sucking into it a little more water taken from a separate water droplet on the paraffin surface. A final mixing after adjustment to volume is then made by expelling the entire mixture to a fresh paraffin surface and re-collecting it a few times. The pipet may then be employed to fill the 1-mm. capillary cell in the ordinary way, or the contents may be expelled into the Lowry and Bessey cell.

The addition of other techniques necessary for color development to that of mixing reagents and making to 50-λ volume, although not impossible, is certainly expected to be difficult. Heating would have to be performed with the greatest care and probably in a closed vessel. Extraction probably could be carried out by proper manipulation but would entail considerable difficulty. Little has been done along the line of developing proper techniques for such operations when the volumes are of this order of magnitude and at the same time must be recovered quantitatively and made to a definite small volume. The methods should receive more experimental attention, because practical methods are probably capable of development and will

be necessary before the advantages of instrumental characteristics can be fully realized. It now appears likely that spectrophotometry will ultimately be used for quantitative analyses in a much smaller range than any described here, because all necessary instrument characteristics can be achieved.

APPLICATION TO SPECIFIC METHODS

Capillary cells and microcells for the spectrophotometer have been applied to various determinations in several laboratories. Publications of the exact experimental procedures are for the most part lacking, and some of those that are available do not appear to represent the best analytical practice for the particular determinations discussed. For this reason, there will be included here the outlines of certain carefully chosen methods, most of which are incompletely tested in practice but which serve to illustrate the method of applying the technique to various spectrophotometric determinations of a variety of substances. In some of the procedures, more detailed study is necessary to assure smooth and dependable operation. All of them should be applicable to submicrogram analysis when properly performed. Most of the procedures have been used at various times in one or more laboratories.

Determination of Iron

Iron, reduced to the ferrous state, is readily determined in very dilute solution (0.01 to 1 part per million in a 5-cm. cell) with *o*-phenanthroline (10) or with α,α'-bipyridyl (11), with either of which an intense red complex is formed. The color is indefinitely stable, independent of *p*H change within a wide range, and subject to the minimum of interference; it obeys the Beer-Lambert law accurately. Its adaptation to very small volumes depends then merely on employing small enough volumes of reagent to maintain the desired high concentration of the colored complex.

The iron, in acid solution, is treated with a strong excess of 10% hydroxylamine hydrochloride solution in iron-free water to reduce it. The necessary volume of this reagent will not exceed the volume of sample and may be considerably smaller in view of the low concentration of iron being determined.* To the

* Only very low concentrations of iron may be measured in 5-cm. cells. If the concentration exceeds the limits stated, it is necessary to employ shorter cells or to increase the dilution.

reduced solution is added an excess of 0.1% *o*-phenanthroline solution in water redistilled from glass. In 200-λ volume, the minimum volume of reagent would be 12 λ per γ of iron, which in the submicrogram range makes the addition of a suitable excess well within the necessary small range of volume. The solution is neutralized to the turning point of Congo red or nitrazine paper (a minute fragment of paper) with concentrated or somewhat dilute ammonium hydroxide solution free of iron. Excess sodium acetate may be substituted for ammonium hydroxide. The acetate buffer produced gives a *p*H range in which the color development is satisfactory. Dilution to final volume must be performed with water which is free of iron, best obtained by redistillation from glass.

The absorption of the solution at 508 mμ is measured in the capillary cell with the spectrophotometer.

If biological materials are analyzed, a preliminary destruction of organic matter is usually necessary for the greatest accuracy. The destruction is readily performed by digestion of the sample in a small Kjeldahl-type flask of less than 1-ml. bulb capacity, with nitric and perchloric acid, or with nitric acid and 30% hydrogen peroxide. The perchloric acid or peroxide is added to the hot nitric acid digest in small amounts until the solution is clear. The excess acid must be largely removed by heat so that the sample can be transferred to the color-developing flask with a very small amount of rinse water. An alternative method of destroying organic material is dry ashing in a micro platinum crucible at a temperature not in excess of 500° C. to prevent formation of acid-insoluble ferric oxide. A little iron-free sulfuric acid, which may be prepared by distillation of concentrated acid in glass and dilution with 5 parts of iron-free water, is used to transfer the ash to the reaction vessel. Dry ashing is most favorable when the iron content of organic material is very low, thus necessitating the destruction of an unusually large mass in order to obtain an analyzable quantity of iron.

An alternative submicrogram procedure for iron determination in serum is that of Burch, Lowry, Bessey, and Berson (12), in which the color of ferric thiocyanate is developed. In general analysis, this method is considered less suitable than the *o*-phenanthroline or α,α′-bipyridyl methods because of the well-known reducing action of thiocyanate on the ferric ion (13), as well as the

interference of phosphate and other materials on the color formation and other effects which have been well-summarized by Woods and Mellon (14). It must be pointed out that Burch et al. took suitable precautions against many of these effects by extracting the ferric thiocyanate with isoamyl alcohol containing hydrogen peroxide, which prevented the reduction of ferric ion by thiocyanate. They also reduced the iron with ascorbic acid, after reading in the Lowry and Bessey cell, and remeasured the absorption of the reduced solution. The iron in 20 λ of serum could be determined with an average recovery of about 90%. By combination of the acid and thiocyanate reagent, a considerable simplicity of operation was achieved.

Determination of Manganese

Of the several procedures available for determination of this element, that which depends on its oxidation to permanganate and spectrophotometric evalution of the latter is perhaps the most dependable, (15), though it suffers from a low sensitivity. It is highly specific and relatively simple to perform.

The sample, which for measurement in a 5-cm. cell may contain from 70 mγ to 3 γ and which is free of organic material, is strongly acidified with concentrated sulfuric acid added in a volume at least twice that of the sample. To this is added a fresh solution of potassium periodate (or metaperiodate) containing from 500 to 1000 times the weight of the manganese in the sample. The solution is heated in a boiling-water bath for 10 minutes, cooled to room temperature, and diluted to 200 λ or to 1 ml., depending on the cell to be used. After thorough mixing, the absorption at 522 mμ is measured in the capillary absorption cell with the spectrophotometer. In the presence of excess periodate the color is almost indefinitely stable and is subject to little interference except possibly from other colored materials present in the sample originally which absorb in the same spectral region. Any such material would usually be destroyed in preliminary ashing or digestion. The color conforms closely to the Beer-Lambert law.

Biological materials require destruction of organic material, which may be performed as described for the determination of iron.

Determination of Copper

Copper is conveniently determined in biological materials by means of its compound with diethyldithiocarbamate (1). The reaction is slightly less sensitive than that with dithizone, but the technical details of its application are somewhat simpler, and it probably is more readily adapted to the submicrogram scale. The compound of copper with diethyldithiocarbamate is insoluble in water and readily extracted by a variety of organic solvents in which solution the color absorption may be measured. The range of application of 5-cm. absorption cells is about 10 mγ to 2 γ. Because the sample is extracted, the volumes of materials placed in the aqueous phase are not critical but should be kept as small as convenient in order to provide a favorable extraction distribution.

The sample containing copper and not more than a small amount of iron or manganese is treated with about half its volume of 20% ammonium citrate solution. The *p*H is adjusted to 9 to 9.2 with ammonium hydroxide, by means of a little thymol blue and by comparison against a reference buffer of *p*H 9 to 9.2. A volume of 0.1% sodium diethyldithiocarbamate reagent not in excess of the sample volume is added, and the precipitated copper compound is extracted in a microextractor as described earlier, with carbon tetrachloride or isoamyl acetate. With the former solvent, the extractor shown in Fig. 61 or 62 should be employed. Isoamyl acetate is lighter than water and may be separated either by the extractor shown in Fig. 63 or that in Fig. 64, depending on the size of capillary cell. The volume of solvent must be no greater than that of the volumetric flask corresponding to the cell capacity, i.e., 1 ml. for the 4-mm. cell, 200 λ for the 2-mm. cell, or 50 λ for the smallest cells. The latter would yield a lower limit of range smaller than that indicated above.

The colored compound dissolved in the organic solvent is placed in the capillary cell, and the absorption at 440 mμ is measured. The choice of solvents is not critical, though Drabkin (16) recommends isoamyl acetate because it is less volatile than carbon tetrachloride and is lighter than water. This factor carries no advantage when the analyst is equipped with proper extraction apparatus.

Determination of Phosphorus

Of the various modifications of the molybdenum blue method commonly employed for determination of phosphorus, that of Berenblum and Chain (17) has been adapted most successfully to the determination of microgram and submicrogram quantities. It consists essentially of the formation of phosphomolybdic acid, extraction of the heteropoly acid with butyl or isobutyl alcohol, followed by reduction with stannous chloride in the alcohol solution. Virtually all interference with the method is eliminated by this procedure; the conditions of formation of molybdenum blue are completely standardized, which is very important because of the colloidal nature of this material in aqueous solution; and, in the alcohol phase, the conformity of the solution to the Beer-Lambert law is very close over a wide range. Solutions containing phosphates usually require digestion, because the interest in this material is largely from the standpoint of biological materials.

The sample, which may contain as little as 8 mγ (if measured in a 2-mm. absorption cell), is digested in a microgram digestion vessel similar to a Kjeldahl flask with a total bulb capacity of not more than 1 ml. A few drops of nitric acid are used, with occasional small measured amounts of 60% perchloric acid added to the cooled digest. The total volume of perchloric acid should be known; it is usually not over 25 λ. A little more nitric acid may have to be added during the boiling period. After about 20 minutes, the mixture is evaporated almost to dryness and cooled. The digest is diluted to a final acidity of 0.3 to 0.5 *M* with dilute sulfuric acid or water. The strength of acid added must be determined in relation to the digestion conditions and the total volume desired. The acid remaining in the digest is almost equivalent to the amount of perchloric acid added. The total volume is not critical because of the extraction step but must be kept small when the amount of phosphorus is low. Usually the volume may be made to 0.5 to 1 ml., but less may be desirable in the lowest range. Trial runs in which the residual acid is determined by microtitration are necessary to obtain the exact conditions for the dilution. If the acidity of the diluted digest is too high, extraction of uncombined molybdic acid results; if too low, silica interferes. Regardless of the final acidity, a reasonable degree of standardization is necessary in adjusting the acidity in order to give low and reproducible blanks.

To the diluted digest is added half its volume of 2 *N* sulfuric acid and an equal volume of 5% ammonium molybdate solution which has been stored in a paraffin-coated bottle to prevent formation of silicomolybdate. The solution is thoroughly mixed, after which it is ready to be extracted.

The extraction is carried out with a volume of butyl or isobutyl alcohol which is not in excess of half that of the aqueous phase and which may be considerably smaller in quantity. Because of the small volumes often required, it may be impossible to employ any of the extractors described. It is possible to perform a satisfactory extraction in a micro test tube, a pipet being used to mix the solutions and to separate the alcohol layer. The capillary extractor shown in Fig. 64 requires a larger ratio of solvent to aqueous volume than is desirable. A single extraction suffices to remove virtually all the phosphomolybdic acid because of its favorable distribution coefficient. Multiple extractions increase the amount of uncombined molybdic acid which is extracted without appreciably raising the recovery of phosphate.

The phosphomolybdic acid is reduced by mixing the alcohol solution with three times its volume of a freshly diluted stannous chloride solution made as follows: A stock solution is made by dissolving 10 g. of stannous chloride in 25 ml. of concentrated hydrochloric acid, and a sample of it is diluted daily to 200 times its volume with 1 *N* sulfuric acid.

The extract containing molybdenum blue is diluted to the appropriate volume (1 ml. or 200 λ) with ethyl alcohol and mixed, and the absorption is measured with the spectrophotometer at 720 mμ.

Determination of Ammonia Nitrogen

The sodium phenate-hypochlorite method of Van Slyke and Hiller (18) may be satisfactorily modified in line with the investigation of Russel (19) to the submicrogram determination of ammonium ion. It may then be combined with submicrogram diffusion methods similar to but smaller in scale than those described in the literature or in this volume. The lower limit of sensitivity with the 200-λ capillary cell is apparently about 0.5 mγ of ammonia nitrogen; thus analysis is permitted with quantities of about 10 mγ of ammonia as a lower practical limit.

Manganous sulfate in the ratio of about 50 γ/100 λ is added

to the sample in the form of a concentrated solution. To the mixture is then added 20 λ of sodium phenate solution made as follows: 2.5 g. of reagent-grade phenol is added to a 10-ml. volumetric flask, followed by 5.4 ml. of 5 *N* sodium hydroxide solution, with cooling under the water tap, the flask then being filled to the mark with ammonia-free water. Phenate solution is made fresh at least every week and is stored in the refrigerator in colored glass containers.

A fresh bottle of commercial sodium hypochlorite (Clorox or equivalent) is treated with a few drops of strong sodium hydroxide. A 10-λ volume of this reagent is added to the sample after the sodium phenate. The volume is increased to about 100 λ with ammonia-free water, and the solution is heated in boiling water for 10 minutes. After cooling, the volume is made to 200 λ, mixed, and measured in the capillary cell at 625 mμ.

Extreme care is necessary to avoid ammonia contamination in work involving such small quantities. The air in many laboratories contains appreciable amounts of ammonia, and it has been found that even the smoke of a cigarette in the room is sufficient to introduce relatively large errors. All water must be redistilled in a glass apparatus from dilute sulfuric acid, and the water and all reagents must be protected by acid traps from atmospheric contamination. It is desirable to wash all glassware with ammonia-free water immediately before use and to store it in a closed vessel between times.

Determination of Iodine

The determination of submicrogram quantities of iodine is most sensitively performed by employing a ceric-arsenite system in which the iodine acts as a catalyst for the oxidation-reduction reaction (20–22). The method is not especially precise, but it is quite delicate. Because the analysis is based on catalysis rather than on direct measurement of monochromatic light absorption, the liquid system is not small and does not require capillary absorption cells. Whether such cells would allow the determination of still smaller quantities of iodine is not clear. The usual range of the determination as commonly performed is from 0.005 to 0.1 γ of iodine.

Iodine-free solutions of arsenious acid and ceric ammonium sulfate are required. The first is made by dissolving 3.71 g. of

arsenic trioxide in 50 ml. of iodine-free 1 *N* sodium hydroxide, adding 200 ml. of redistilled water, and neutralizing with 70% arsenic- and nitrogen-free sulfuric acid solution. Then, 54 ml. of the 70% acid is added in excess, and the volume is made to 500 ml. To this solution is added 3.125 g. of iodine-free sodium chloride. The ceric sulfate solution is made by dissolving with stirring 12 g. of ceric ammonium sulfate in 500 ml. of 3.5 *N* iodine-free sulfuric acid solution. The ceric and arsenite solutions when mixed should not react appreciably in a period of several hours. Any iodine contamination allows the reaction to proceed at a measurable rate.

The iodide-containing solution in an amount of 5 ml. is measured into a spectrophotometer absorption cell of appropriate volume, and 0.4 ml. of the arsenious acid reagent is added. The cell is warmed in a water thermostat to exactly 37° C. When temperature equilibrium is reached, 0.5 ml. of the ceric ammonium sulfate solution is added and the time noted. The cell is returned to the thermostat and allowed to incubate for exactly 15 minutes, after which the absorption at 480 mμ is read immediately. The readings are calibrated against variable quantities of iodide solution in the correct concentration range. If all reagents are added accurately, the temperature is maintained constant, and all additions and readings are accurately timed, it is possible to measure submicrogram quantities of iodine with a precision of better than ±10%, which is sufficient for many biological purposes.

More suitable submicrogram procedures for iodine determinations are undoubtedly possible and should be developed. The spectrophotometer has rarely been used for the catalytic determination of iodine by this procedure, the filter photometer with a blue or violet filter being ordinarily employed. It is probable that the instrument is not the limiting factor in the accuracy of the method, but it is true that absorption in this region is not sensitively or precisely measured with filter photometers which are photocell instruments with low sensitivity in the violet region of the spectrum.

Determination of Lactic Acid

The method for step-photometric determination of lactic acid described by Miller and Muntz (23) was applied by them to as little as 0.1 γ of this constituent and showed a maximum accuracy

in the range of 2 to 10 γ. The volumes were slightly in excess of 1.5 ml., and the optical path was 5 mm. in length. An increase in sensitivity of somewhat better than tenfold should result from use of a 1-ml.-capacity, 5-cm. capillary absorption cell, and it appears possible to reduce the scale of operation, possibly to the extent of employing the method with volumes of 200 λ, in which case the sensitivity would be such as to permit analysis of quantities of the order of 5 to 50 mγ.

To the sample containing lactic acid is added 7.5 times the volume of concentrated sulfuric acid while the sample is being chilled in ice water. The sample should be contained in a small glass-stoppered test tube. The tube is sealed, a little of the sulfuric acid acting as a lubricant, and the contents are mixed. It is then heated in a boiling-water bath for 5 minutes, after which it is cooled for 10 to 15 minutes in a deep ice bath. On removal, the tube is rotated in a nearly horizontal position to collect the liquid and drained in a vertical position before it is opened. Immediately, a small quantity adjusted to the scale of operation (8 mg. in the original method) of dry, specially purified, powdered *p*-hydroxydiphenyl is added with a small funnel to prevent its collecting on the sides of the tube. The tube is stoppered, and the contents are thoroughly mixed. After an hour at room temperature, the tube is heated in a water bath for exactly 90 seconds, removed, and cooled to room temperature. The color, which should be clear blue violet, is stable for several hours.

The *p*-hydroxydiphenyl is purified by several recrystallizations from acetone with heating and cooling. The final crystallization is made from absolute acetone, and the crystals are carefully dried in the absence of dust or fumes.

In order to adapt the method for the spectrophotometer, the colored solution probably requires dilution to volume with concentrated sulfuric acid and transfer to the capillary cells of the instrument. The absorption maximum is in the neighborhood of 580 mμ. The method is claimed to be highly specific, not being disturbed by the presence of carbohydrates, as are most procedures, and to be rapid in operation.

Determination of Ascorbic Acid

Lowry, Lopez, and Bessey (24) developed a submicrogram procedure for ascorbic acid in blood serum, adapted to 50-λ final

volumes, which they measured in the Lowry and Bessey type of absorption cell. The ascorbic acid in 10 λ of blood serum could be determined with close agreement to the values obtained from various other procedures. Presumably, the method is equally applicable with capillary absorption cells if the volumes are increased correspondingly. By so doing, the sensitivity is at least as great, and possibly there are technical advantages to handling the larger volumes.

To the sample consisting of 10 λ of serum is added 40 λ of an acid charcoal suspension made by placing 1% norit in 5% trichloroacetic acid. The mixture is made in the bottom of a small test tube, 6 by 50 mm. in dimensions. After the contents are mixed, the tube is capped with a piece of Parafilm or a rubber stopper and centrifuged for 10 minutes at 3000 r.p.m. A 30-λ aliquot of the clear supernatant fluid is removed to another similar test tube, and 10 λ of a thiourea-dinitrophenylhydrazine reagent is added and mixed. The reagent is made by dissolving dinitrophenylhydrazine to the extent of 2% and thiourea to the extent of 0.25% in 9 *N* sulfuric acid. If a precipitate develops, the solution is cleared by centrifugation. The reagent is stored in a refrigerator and is discarded after a month.

The capped tube containing the sample and the reagent is incubated at 38° C. for 3 hours. It is then chilled in ice water, and 50 λ of ice-cold 65% sulfuric acid solution is added, mixed thoroughly, and transferred to the spectrophotometer cell. The reading is taken at 520 mμ and compared with a calibration curve prepared by the same procedure.

The presence of any floating charcoal is to be carefully avoided because of its light scattering and absorption.

Determination of Protein

Of the various spectrophotometric methods that may be applied to the determination of the protein content of biological materials, the biuret method appears to be fundamentally most sound (25). It is not a very sensitive method, and experience shows several limitations in its application. There is a strong tendency for protein to be coprecipitated with copper hydroxide in the classical method (26) in which the protein solution is mixed with copper sulfate and made alkaline with sodium hydroxide. Stabilization of the solution by addition of ethylene glycol, recommended by

Mehl (27), appears to lead to color instability as discussed by Weichselbaum (28). Tests of the method detailed by this author give reproducible and accurate results, and the method is adaptable with some modification to use with capillary cells.

The reagent of Weichselbaum is modified slightly to reduce the copper content as far as possible, thereby lowering the blank. It is made from 0.75 g. of $CuSO_4 \cdot 5H_2O$, 2.25 g. of sodium potassium tartrate and 1.25 g. of potassium iodide dissolved in 0.2 *N* sodium hydroxide to make 100 ml. of solution.

The protein sample containing from 0.5 to 25 γ is placed in the appropriate volumetric flask (200 λ for smaller amounts, and 1 ml. for amounts greater than 4 γ). To it is added 0.2 *N* sodium hydroxide to fill not more than $\frac{4}{5}$ of the volume of the flask. To the 200-λ flask is added 40 λ of the copper reagent; or to the 1-ml. flask is added 200 λ of the copper reagent. After the sample is mixed, the flask is filled exactly to the mark with 0.2 *N* sodium hydroxide and allowed to stand in the water bath at 37 ° C. for 30 minutes. After it cools, it is transferred to the absorption cell, and the absorption is measured at 540 mμ. The reading is compared with a calibration graph prepared with pure dry protein.

MICROPHOTOFLUOROMETRY

An outstanding advance in the analysis of submicrogram quantities is made possible by the development of the microphotofluorometer by Lowry (29). This instrument is based on the application of the multiplier phototube (30) to the measurement of extremely small light intensities, a principle of electronics which is destined to assume greater importance in analytical procedures, both for light absorption and X-ray absorption measurements and for radioassay. The multiplier tube allows amplification in a single tube of as much as a millionfold, which is achieved without corresponding increase in the "noise" factor as occurs in conventional amplifiers. When this tube was applied to the Coleman photofluorometer, the sensitivity was increased a thousandfold with an increase in stability. Since the fluorescence reactions are characterized by a high inherent sensitivity, this development made possible the measurement of as little as 0.2 mγ of riboflavin and differentiation of it from its natural derivatives (31). This is probably the most sensitive analysis so far per-

formed with any material and was achieved with a reproducibility of 3 to 5% which compares favorably with the reproducibility of many large-scale methods and most small-scale analytical procedures.

It is to be expected that further important applications of this and similar instruments of fluorescence analyses will be forthcoming and that the technique will assume a position of real importance in the submicrogram analytical field. Not only are a number of important biological constituents fluorescent in their own right, but several, e.g., aluminum, can be combined with reagents to yield fluorescent compounds. There is often a low degree of specificity in such reactions, because if more than one fluorescent material is present at the same time the photosensitive element will respond to both. This may be partially corrected by means of filters and by application of chemical and physical separations to eliminate interference.

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CHAPTER 10

Physical Methods

In the field of microgram chemistry it is necessary at times to determine the physical constants of very small quantities, though not necessarily as a portion of a direct quantitative analysis. Many compounds have been prepared in microgram quantities in connection with the study of the chemistry of the transuranic elements, the details of which may be found in specialized publications of the Manhattan project. In order to study these small preparations, many physical constants were determined, thereby demonstrating the utility of well-established techniques when applied to new problems. In the field of biochemical microgram analysis it is often equally desirable to determine physical properties. Such procedures as the determination of melting points, boiling points, refractive indexes, and density have been used routinely with milligram quantities for many years. Their application to microgram quantities requires merely the adaptation to the smaller amounts, and some of these adaptations also are standard procedures that have been employed in some laboratories over considerable periods of time. Here are discussed some of the alterations in standard technique which are necessary when the quantity of material is so small as to preclude the use of conventional procedures.

MELTING-POINT DETERMINATION

Capillary-tube methods normally require more material than is available to the microgram chemist. Hot-stage methods for use with the microscope, however, are entirely suitable even for the most minute quantities. Various designs of melting-point blocks for the microscope stage have been described, and some of them are available commercially. They depend on heating electrically a block of metal carrying the sample and a thermometer bulb (or thermocouple). The compound is observed microscopically to determine when it melts, and any sample that can be viewed with the microscope can be measured.

Most commercial melting-point hot stages have a vertical well through the entire block to allow the use of transmitted light. This design separates the sample a significant distance from the hot metal and gives rise to a lag in the transmission of heat and consequently in the actual melting or in the thermometer reading or in both. It may be necessary to calibrate the instrument against pure compounds of accurately known melting points in order to correct for this effect. Such a method also requires a constant rate of heating, which is not simple to attain in practice.

An alternative is to employ a block designed similarly to that shown in Fig. 110. In this design the ability to use transmitted light has been sacrificed in order to minimize the differences between the actual melting temperature and the simultaneous thermometer reading. A small spotlight provides sufficient il-

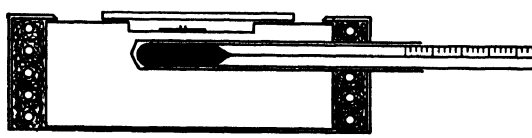


FIG. 110. Microscope melting-point block.

lumination for the lower powers of the microscope which are adequate for all purposes. The block of this instrument may be machined from aluminum, copper, or Duralumin, with a shallow well in the top, and a horizontal thermometer well so situated that the bulb of the thermometer is directly under the central well and separated from it by a thin layer of metal only. Heat is applied by a continuous helical resistance wire wound around the entire block. The movement of heat in the block is radial, from the entire periphery, with the sample and thermometer bulb at the center. The input of heat is then symmetrical and as nearly uniform as possible. The lag in this instrument is negligible if the temperature is not advanced too rapidly.

The sample is usually placed on a small fragment of thin cover slip to separate it from the metal of the block, and the well is covered with a piece of microscope slide to immobilize the enclosed air. As the melting point is approached, the rate of heating should not exceed 1 or 2° per minute, at which rate there is no appreciable error caused by lag. For accurate determination, the

stem correction should be made for the thermometer, as is true of all melting-point determinations if a temperature gradient exists in the thermometer stem.

BOILING-POINT DETERMINATION

The boiling point of fractional milligram quantities of liquids may be determined with reasonable accuracy by the method described by Emich (1). In order to use as little material as possible, the boiling-point tubing must be drawn somewhat finer than he described it. The first operation consists of drawing a thin-walled capillary tube which may have a diameter as small as a few tenths of a millimeter and pulling it to a still finer tip. The drawing of the tip must be done with a very small luminous flame, and the thread should be drawn to a length of 5 mm. or more and broken off. This tip, when dipped in the material whose boiling point is to be determined, allows the material to flow into the tube by capillary action after which the fine capillary is sealed.

It is sometimes troublesome to seal off the fine tip. The operation is best carried out by passing the thread through a very hot needle flame of an injection-type micro blast lamp (Fig. 55) which is most readily constructed from glass. The end of the fine tip may be grasped with forceps, and the high temperature in a narrow zone gives an immediate seal without excessive heating of the remainder of the tube. Incorrect heating may displace the liquid completely from the tube. On cooling, the liquid should return so as almost to fill the tip, leaving only a tiny bubble. The charged tube is placed on a thermometer as in melting-point determination and immersed in a bath of glycerin or other high-boiling liquid and heated slowly with constant stirring until the upper level of the liquid in the capillary reaches the level of the bath itself, at which point the thermometer is read. The recommendation of Emich that the bath be made from a glass beaker which is insulated by asbestos cloth around the outside and contains two windows on opposite sides for observation of the sample is advantageous. This procedure is applicable to fractional milligram amounts. The accuracy obtainable is normally less than that of the macro methods but is sufficient for many purposes.

DENSITY DETERMINATION

For the determination of density (or specific gravity), two types of procedure are suitable for minute amounts: (*a*) flotation in liquid of known density, and (*b*) displacement methods. The choice of method depends on the density region of the sample and on its solubility.

The heaviest liquids available for flotation methods have densities somewhat in excess of 3.25, which sets the upper limit on the measurable density of the sample. Since most heavy liquids are organic, materials soluble in them cannot be measured by flotation. Samples with a density greater than about 3.25 and materials soluble in organic solvents but insoluble in water may require the displacement method which is more difficult to perform.

Flotation Methods

Depending on the circumstances, there is a choice between two or more techniques for performing density determinations by flotation. The first method depends on adjusting a liquid mixture to a density which is identical with that of the solid suspended in it, after which the absolute density or specific gravity of the liquid is determined by weighing a measured volume of the liquid in a calibrated pycnometer at a controlled temperature. This method is most convenient when the number of samples to be measured is small and where comparison between them is less important than obtaining an absolute value.

The second procedure depends on the use of a tube containing a density gradient such as that described by Linderstrøm-Lang (2). This method is more convenient for comparison of a number of samples with each other and particularly where large numbers of samples must be measured (3–5). Both methods require properly chosen mixtures of heavy liquids. For any given sample, at least two liquids are needed, one heavier and one lighter than the sample in question. For the greatest sensitivity the difference between the two liquids should be as small as possible. If there is no indication of the density of the sample, it may be necessary to choose liquids which differ considerably in their values. Table 12 shows the densities of a variety of organic liquids which are suitable for use in flotation methods.

TABLE 12. DENSITY OF VARIOUS FLOTATION LIQUIDS

Methylene iodide	3.325
Bromoform	2.89
Iodobenzene	1.832
Bromobenzene	1.499
2-Chloronaphthalene	1.194
Nitrobenzene	1.158
Dibutyl phthalate	1.048
Kerosene	0.82

1. **Pycnometric Flotation Method.** A small tube, about 8 mm. in outside diameter and 5 cm. in length, is partially (about one-third) filled with a liquid which may be either heavier or lighter than the sample measured but not greatly different from it. The sample, which may be a fragment of solid or a drop of immiscible liquid, is added. After thorough wetting, which in somewhat porous materials may require evacuation of the tube, the sample sinks if it is heavier or floats if it is lighter than the liquid in the tube. Depending on which event occurs, a heavier or lighter liquid, respectively, is added in small quantities and thoroughly mixed by twirling in it a glass zigzag stirring thread containing a number of successive sharp bends. This is inserted to the bottom of the tube and twirled between the thumb and forefinger. When sufficient of the liquid of opposite sign has been added to produce a density exactly equal to that of the sample, the sample remains in suspension at whatever point it comes to rest. It should be observed for a short time to detect slow rise or fall in the tube. All heat effects must be avoided and evaporation from the surface prevented by stoppering the tube.

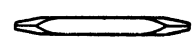


FIG. 111. Capillary-tipped pycnometer.

When the sample is exactly balanced, a pipet-type pycnometer is used to withdraw a measured volume of the liquid which is weighed. A pycnometer of the type shown in Fig. 111 has been employed very successfully for this purpose. With the aid of a suction tube it can be filled and the meniscuses adjusted by capillary action at the minute orifices on the ends, giving a high degree of precision in filling. Variations of this general type of instrument have been described recently by Anderson (6), who studied carefully the behavior of self-filling and self-adjusted pipets and pycnometers similar to those described earlier in this

volume which have been used and have been available commercially for a number of years. The self-adjusting principle allows construction of a more satisfactory instrument than that described by Kirk and Russel (7).

The weight of the liquid contained in the pycnometer is compared with the weight of the same volume of water (or mercury) at the same temperature to obtain the specific gravity at the particular temperature. The results should be calculated to density values because of the large effect of temperature in changing the density of water and therefore of any material compared with it when expressed as specific gravity. The corresponding effect of temperature on the density of the flotation liquid is usually not known and may be large. By measuring the temperatures at all times and converting to density, most of this difficulty is overcome. The more accuracy is desired in the results, the closer must be the control of the temperature, and the larger the capacity of the pycnometer. Anderson (6) showed that a self-adjusting pycnometer of 1-ml. capacity yields a reproducibility of 1 in 40,000. Smaller instruments, necessary only for special purposes, can be made to yield essentially the same reproducibility by weighing them with the microchemical rather than the analytical balance.

2. **Density Gradient Flotation Method.** If a number of samples are to be compared with regard to their specific gravities, the density gradient provides an almost ideal arrangement for such rapid and sensitive comparison. It is less suitable than method 1 when absolute values are to be determined, though this also is possible by means of special arrangements and is advantageous for large numbers of samples.

A tube of suitable size, usually 8 mm. in diameter and from 10 to 30 or 40 cm. in length is chosen, depending on the sensitivity of the gradient desired. Tubes larger in diameter are to be avoided when possible because of the difficulty of preventing convection currents as the tube diameter increases.

Two liquids are chosen, one heavier and one lighter than the objects to be compared. To the bottom of the gradient tube is added a portion, e.g., 2 ml. of the heavier liquid. On top of it is carefully placed without mixing a portion, e.g., 1 ml., of a mixture composed of three parts of the heavy liquid with one part of the light. On top of this is placed without mixing another

similar portion composed of equal parts of the heavy and light liquids. Another similar portion composed of one part of the heavy liquid with three parts of the light is placed on top of this, and finally a double portion, e.g., 2 ml. of the light liquid is placed on top without mixing. After this stands for about 24 hours or even longer, diffusion establishes a uniform gradient of considerable length and, therefore, sensitivity. A longer and more sensitive gradient may be prepared by adding more intermediate layers with corresponding compositions. Frequently as many as seven or eight such intermediate layers are added for very sensitive distinctions of density of floated objects.

Into the tube are dropped the objects to be compared, and the levels at which they float after reaching equilibrium are noted. If they float at the same level, they are equal in density to an extent that would not be detectable by absolute measurement. During the measurement, it is very essential that all hot objects be kept away from the tubes and that the tubes should not be handled, owing to the effect of heating which causes convection currents. A fluorescent light source may be employed for observation. If difficulties from heating are encountered, it is necessary to surround the tube with a water jacket.

If absolute values are required, a set of standards must be placed in the tube with the unknowns. These are often (2) made from salt solution such as aqueous potassium chloride, particularly when the densities of the sample are not greatly different from that of water.

With samples which are heavier than can be matched by dissolving salts in water, it is possible to use glass beads or glass spheres for a density gradient over any desired range up to the maximum specific gravity of glass itself. The preparation of such standards is somewhat time-consuming, but, once made, they have the advantage of being relatively permanent. Glass beads are suitable only in the range covered by the specific gravity of commonly available glass, from about 2.46 to at least 2.60. For specific gravities lower than this, a hollow bead is formed by melting the end of a capillary tube to close it and pulling it off in a very small flame with entrapped air bubbles contained in it. Depending on the size of the air bubble, the density of the standard is determined primarily by the size of the air bubble contained

in the sphere as compared with the mass of glass used. Such standards are entirely empirical and require calibration before they can be applied. Suitable standards are chosen directly by test in the gradient and calibration by method 1. When such a set of density standards is available, the gradient tube becomes applicable over a wide range and with great convenience for the measurement of the density of many types of materials. The standard beads automatically adjust their positions to any change in the gradient, thus providing a flexible frame of reference for the gradient.

Displacement Methods

When the density of a solid sample is higher than that of any of the available liquids with which it might be compared, it is necessary to resort to some form of displacement method for the determination. The usual methods of weighing of the sample, dry and immersed, are not suitable for minute samples because of the interference of surface tension with the supporting structure, an effect which may be larger than that being determined and which is difficult to standardize. Direct determination of the volume by displacement and of the weight by direct weighing has served to obtain reasonably accurate density values in the microgram range.

Procedure. A series of fine capillary tubes with thin walls are drawn with diameters close to, but slightly larger than, the particle whose density is to be determined. These are placed vertically under the objective of a low-power microscope equipped with an ocular micrometer which measures their inside diameters. The measurement should be taken in four directions at angles of 45 degrees from each other so as to establish the degree of roundness of the capillary as well as its actual diameter. It is essential that the diameter also be uniform over a distance of about 6 to 8 mm. This may be checked by laying the capillary on its side and observing the bore microscopically over this distance, using again a calibrated ocular micrometer which should give identical readings over the length. The tube is melted together at this approximate distance from the end to form a short test tube such as that shown in Fig. 112.

Capillaries of the appropriate size with completely round peripheries and uniform diameters are chosen for the measurements.

On each of these is made a very fine scratch at a distance of about 2 or 3 mm. from the sealed-off end by means of the sharp point of a diamond pencil. The over-all length of the capillary and stem should be such as to fit well into an air-driven centrifuge of the type shown in Fig. 50, viz., about 2½ to 3 cm.

The particle whose density is to be determined is weighed accurately. This may be readily performed with the quartz beam balance described in Chapter 4. A suitable immersion fluid, e.g., water, butyl phthalate, or other liquid that will neither corrode nor dissolve the material in question, is placed in the capillary to give a column 2 or 3 mm. in length, and the liquid is centrifuged to the bottom of the micro test tube at which time its meniscus should be reasonably close to the mark scratched on the tube. The exact distance of the meniscus from this mark is measured with the ocular micrometer of the microscope. The weighed particle is then introduced into the capillary and centrifuged to the bottom of the immersion fluid. The position of the meniscus is again measured with respect to the mark on the capillary. With the diameter and consequently the cross-sectional area of the capillary known, the movement of the meniscus as compared to the reference mark may be employed to calculate the volume of the particle. This volume in conjunction with the weight provides the data from which the density can be computed. The probable error of this method is relatively large. The weight determination can be made with a reproducibility of about $\pm 0.005\,\gamma$ and a probable error of about $\pm 0.01\,\gamma$ which usually represents an uncertainty of less than 1%. The meniscus movement can be determined with care to about ±5%. The calibration of the capillary bore is not likely to be more accurate than ±5%, owing to slight deviations from roundness and to the inaccuracy of measuring the diameter, which in turn contributes to the volume error as a square power. For these reasons, a single determination may involve errors as high as 10%. With multiple observations and statistical analysis, the error can however be greatly reduced at the cost of additional time and effort.

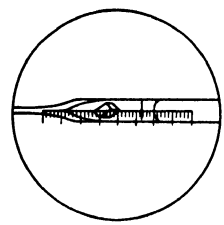


FIG 112. Density capillary displacement method.

CONDUCTIVITY DETERMINATION

Measurement of the conductivity of small volumes of solution may be of great significance in quantitative microchemistry because it is a rapid method whereby comparative concentrations, particularly of salts, can be determined. Modifications of it may be designed for the determination of absorbable materials such as carbon dioxide, the applications of which may become important in respirometry, microgram combustion techniques, and other types of method as yet undeveloped. Microconductivity has been employed to determine changes of salt content of such materials as insect blood and similar fluids which are greatly restricted in quantity. It has been applied successfully to the detection of end points in such analyses as that for sulfate by titration.

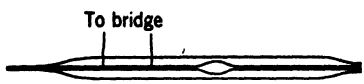


FIG. 113. Microconductivity cell, pipet type according to Craig.

It is of interest that very small conductivity cells can be and have been designed which yield as sharp maxima in the Wheatstone-bridge circuit as are shown with macro conductivity cells (8). Most notable is the cell described by Craig and Patton (9) which is shown in Fig. 113. The construction of this cell is carried out as follows: A capillary glass tube which may be as small as 0.5 mm. in internal diameter is broken. A small platinum cylinder made of thin platinum foil is constructed to slip snugly into the bore of the capillary tube. It is sealed to a fine lead wire. The electrode so formed is inserted at the break in the capillary with the wire lead against the broken surface. The two pieces of tube are placed together in the same position they had before being broken and are sealed by careful application of a small flame and internal pressure from the breath. The tube is again broken a short distance from the first break, and the operation is repeated with the second electrode. After the tube is sealed, the glass is melted around the platinum cylinder so as to fuse to it on the outside surface, giving the effect of a platinum-lined capillary tube without dead space between the glass and metal. The tube may then be made in the form of a standard capillary pipet for ease of filling and emptying. The capacity of the cell can be made readily as small as 5 to 10 λ. The electrodes are attached to a good conductivity bridge, and

the cell constant is determined in the usual manner by calibration against potassium chloride solutions of known strength. It is desirable to platinize the electrodes as is done with large conductivity cells (10). An unknown solution is measured by drawing it into the cell so that the electrodes are completely immersed in the liquid, after which the conductivity is measured by the usual method.

INSTRUMENTAL ANALYSIS

Most or all of the many general instrumental systems of analysis can at times be applied to microgram analysis, and in fact some of them are almost inherently ultramicro in their range of application. It is true, however, that none of them has equaled the spectrophotometer for sensitivity and accuracy and that few of them are as broad in their possible applications within the microgram range as the methods previously discussed.

It is not intended to embark on the tremendous task of detailing the applications of special analytical instruments to analysis of microgram quantities. It is, however, not amiss to mention briefly some of the general spheres of applicability and the limitations of a few of the more common types of instrumental analysis, and perhaps to indicate some of the future possibilities resident in the instruments discussed in connection with microgram analysis.

The Polarograph

This well-known instrument (11–13) may serve to determine most of the metals (14, 15), a considerable number of anions which are reducible at the dropping mercury cathode (16, 17), and certain organic compounds (18). The sensitivity of the instrument is such that concentrations down to about $10^{-4}\,M$ may usually be measured. If the volume of solution is sufficiently small, this allows the instrument to operate in the range of microgram analysis, though it is not usually employed for so small quantities. Microcells for use with small volumes have been described (19, 20), and there is no reason for failure to apply the instrument in this range when desired. Because polarography is a specialized type of analysis, requiring suitable experience, it is likely to remain for the most part in the hands of experts or

to be adopted by those analysts having more or less constant use for it.

Amperometry

Amperometric titrations (21) are a specialized outgrowth of polarography and are often based on direct use of the polarograph. The introduction of the rotating platinum electrode for the dropping mercury electrode (22, 23) was a major step in making the procedure more practical. Other improvements such as the application of special reference half-cells to allow operation without a superimposed potential have also simplified this application. The vibrating platinum electrode (24) as a substitute for the rotating platinum electrode makes this technique practical for very small volumes and therefore a tool for microgram analysis. This development is in an early stage, but it can be safely predicted that amperometric titrations will assume a position of considerable importance in ultramicroanalysis.

The Spectrograph

This specialized instrument has many applications that logically may be termed microgram analysis. In fact, the characteristics of spectrography are almost inherently limited to the microgram range. Its applications in the analysis for elements which emit visible or ultraviolet light when excited by an arc, spark, or flame have been well covered in the literature (25–29). As a strictly quantitative tool, the spectrograph has serious limitations of calibration and standardization which, when added to its expense and the necessity of an experienced operator, restrict its applications in general analysis quite severely. It is uniquely valuable in many research applications and is extremely useful when many similar analyses for the same constituent must be made as a control measure. It is unlikely to be of great direct importance to the ordinary ultramicroanalyst.

Radioactive Tracers

Radioactive tracers have proved of the greatest value in elucidating mechanisms of reaction, both in the biological and general chemical field (30, 31). As strictly analytical tools, they have received little attention, and the future in this field is uncertain, though possibly very significant. Isotope dilution

methods have been employed to some extent (32, 33), and tracers have been used as a supplement in determining completeness of analytical reactions (34). The inherent sensitivity of detection of radioactivity is so great that it is more than possible that ultramicroanalysis may turn more and more toward these tracers for future progress.

Micrurgy

Micromanipulative methods will become progressively more important as the size of sample and the scale of carrying out analytical operations become smaller. Numerous types of micromanipulators and special handling devices are at present available commercially, and inevitably their use will become more widespread. The limitation on manual manipulation is such that the ordinary analyst will find it difficult to carry out the necessary operations of an analysis when the volumes become smaller than a few microliters. The cytological chemist particularly will be under increasing compulsion to perform chemical studies of systems much smaller than this. Under these circumstances, manipulators and manual aids must find increasing applications and should become familiar to the ultramicroanalyst.

FUTURE TRENDS

It is apparent that the tremendous interest in nuclear chemistry and the great hazards of working with radioactive materials in any but the smallest quantities will continue as it has in the past to require more practical ultramicromethods. In this field it is to be expected that great advances in remote handling and in diminution of the scale of analysis will continue to be made. Only the foolhardy will deliberately expose himself to the dangers of large-scale operation if safe and simple small-scale methods can be applied equally well.

In the field of biology, an understanding of the chemistry of the cell is the next great frontier to be conquered. Without chemical methods applicable to the study and analysis of single cells, there will remain a constant challenge to the analytical explorer. It can be stated dogmatically that there are no insurmountable problems to prevent the ultimate chemical analysis of single cells. Much investigation remains before this goal can be achieved, but achieved it will be, and probably along the lines

already partly explored and indicated by past investigations. The field is an alluring one, and an important one. It is hoped that the techniques described in this volume may serve in some measure as signposts indicating the direction toward the understanding of the minute system in the field of chemistry.

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