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PHOTOMETRIC CHEMICAL ANALYSIS

VOL. II

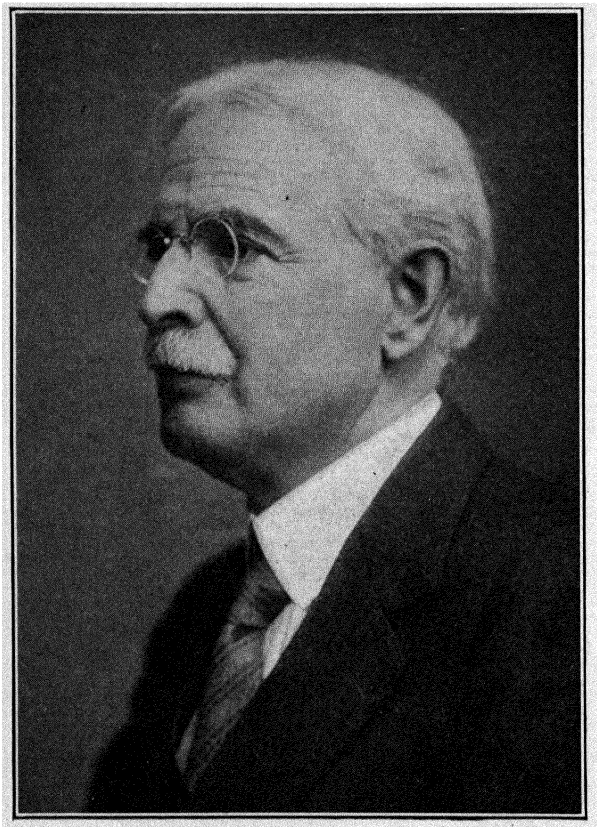
NEPHELOMETRY

PHOTOMETRIC CHEMICAL ANALYSIS

VOLUME I

COLORIMETRY

771 pages, 6 by 9. Cloth.



Frontispiece.

THEODORE WILLIAM RICHARDS

1868–1928

Professor of Chemistry 1894–1928 and Director of the Gibbs Memorial Laboratory 1912–1928, Harvard University. Known for his work on atomic weights, winner of the Nobel Prize in Chemistry for 1914, past President of the American Chemical Society, and founder of Nephelometry.

1—12—12—HMD

ARTHUR MICHAEL
THEODORE WILLIAM RICHARDS
ELMER PETER KOHLER
GREGORY PAUL BAXTER, Chairman
LAWRENCE JOSEPH HENDERSON
ARTHUR BECKET LAMB, Director
GEORGE SHANNON FORBES
GRINNELL JONES

HARVARD UNIVERSITY

DIVISION OF CHEMISTRY

GEORGE SHARP RAYMER
CHARLES HENRY WHITE

Cambridge, Massachusetts, U S.A.

Oct. 11, 1912.

Mr. P. A. Kober,
Roosevelt Hospital,
New York, N. Y.

Dear Mr. Kober:

Please pardon my long delay in answering your letter. It came during the hectic time of moving the family home from the country and was unfortunately mislaid. I shall be very glad to have you use the nephelometer in any way. Unfortunately I have no reprint left of the article in the American Chemical Journal, volume 31, page 235, March, 1904, because of the considerable demand which there has been for separate copies. The maximum accuracy to be obtained under the most favorable conditions is perhaps one-half of one percent as the average of many readings. With a single reading I can hardly be sure of the result to within 3 percent even after practice. The readings are very easy to make, however, and there is no difficulty about making twenty which give a very satisfactory and trustworthy average.

Sincerely yours,

T. W. Richards

PLATE I.

3—1912—3000

ARTHUR MICHAEL
THEODORE WILLIAM RICHARDS
ELMER PETER KOHLER
GREGORY PAUL BAXTER, Chairman
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DIVISION OF CHEMISTRY

GEORGE SHARP RAYMER
CHARLES HENRY WHITE

Cambridge, Massachusetts, U.S.A.

Dec.17, 1912.

Dr. P. A. Kober,
Harriman Research Laboratory,
New York, N. Y.

My dear Dr. Kober:

Many thanks for your article which I have found very interesting. I think your idea of turning the calorimeter into a nephelometer is a good one and a distinct improvement over the somewhat crude apparatus which we have used. It is possible that in your enthusiasm you have overrated the difference in the two, however. I am inclined to think that the phenomena to be observed by a nephelometer are not trustworthy to within a smaller range of accuracy than that given by our instrument; therefore, that the improvement is chiefly one of convenience than one of real accuracy. I have taken the liberty of putting on the margin in pencil one or two slight suggestions in the second and third pages.

I am delighted that you are finding Nephelometry of use to you in your work.

Very cordially yours,

T. W. Richards

The range of accuracy of the old Nephelometer is apparent from the figures given in the paper Am. Chem. Journ 31, 235 (1904).

PLATE II.

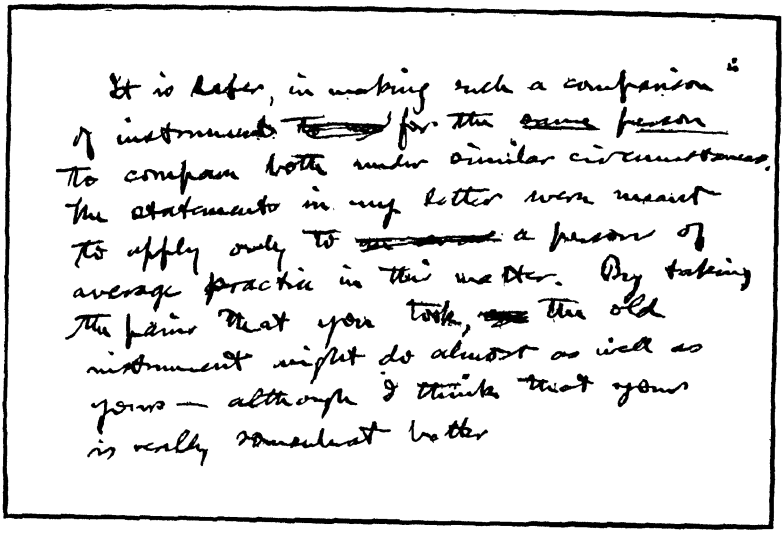


PLATE II.—*Continued.*

PHOTOMETRIC CHEMICAL ANALYSIS

(COLORIMETRY AND NEPHELOMETRY)

BY

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Professor of Chemistry, University of Virginia

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VOLUME II

NEPHELOMETRY

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PREFACE

NEPHELOMETRY offers a means of determining directly the mass or weight of precipitate in suspension, i.e., without filtering, washing, drying, and weighing. The method is based upon the measurement of the brightness of the light reflected by finely divided particles in suspension, very much as in an ultramicroscope. The intensity of the reflected light is a function of the amount of suspended particles, other conditions being kept constant.

T. W. Richards in the early nineties used nephelometry as a means of making corrections in certain atomic-weight determinations but it was not until 1912 that Philip A. Kober used it for the first time as an analytical method and not simply as a means of correcting other analytical procedures. About the same time as Kober's first work, but entirely independently, W. R. Bloor (1913) used nephelometry as a quantitative method. During the past fifteen years rapid strides have been made both in the development of new nephelometric methods and in the improvement of instruments. Kleinmann in Germany has made important contributions. The rapid growth of nephelometry is indicated by the large number of papers on this subject that have appeared within the past few years.

The purpose in writing the second volume of this treatise has been six-fold: (1) To give an accurate account of the development of nephelometry, (2) to present an impartial discussion of the present status of the theory of nephelometry, (3) to give detailed working-directions for using a precision nephelometer, (4) to discuss nephelometric research, (5) to give procedures for the determination of a number of inorganic and organic constituents, and (6) to give an accurate and fairly complete survey of the literature on nephelometry.

The book has been written for advanced students in chemistry and for research workers in biological, medical, pharmaceutical, and industrial chemistry. It is hoped that it will prove useful not only as an aid in the various fields of applied chemistry but also in stimulating research in developing new nephelometric methods of analysis.

The author has received many helpful suggestions from Dr. Philip A. Kober, who read the manuscript and portions of the galley proof. He also contributed a chapter on nephelometric research and assisted with the translation and editing of Dr. Kleinmann's contributions to this volume. Professor Robert F. McCrackan has rendered valued assistance in reading the galley proof. Professor Jesse W. Beams, the author's colleague, checked a number of the equations given in the chapter on nephelometric theory.

Thanks are due the Precision Scientific Co. who furnished several cuts.

The author is very grateful to his wife, Françoise Cheely Yoe, who typed much of the manuscript and assisted in the proof-reading.

J. H. Y.

UNIVERSITY, VIRGINIA,
January, 1929.

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ERRATA

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5, footnote, "J. Lab. Clin. Med." should appear only once.

6, line 20, omit "reasonably."

28, line 14, "scales" should read "scale."

37, line 20, "plunger" should read "plungers."

37, line 22, "on both sides" should read "between the two halves."

80, the first equation should read $S = \frac{Y}{X} - \frac{(1 - X)YK}{X^2}$.

81, the first equation should have " = " after S instead of " − ."

87, in footnote 12, last line, "showed" should read "shown."

95, line 37, "$x Sb_2S_3 \cdot yS^{--}$" should read "$x Sb_2S_3 yS^{--}$."

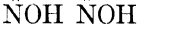
97, line 3 from bottom, "trisulfide" should read "sulfide."

134, line 18, and page 136, line 3, formula for borax should be

"$Na_2B_4O_7.10H_2O$."

167, line 9, "9.2" should be "0.2."

248, the formula for dimethylglyoxime should be



378, line 10, "other" should read "ether."

380, Note 1, line 5, "residue" should follow "insoluble."

394, line 6, "acid" should follow "nitric."

445, line 2, "poassium" should read "potassium."

NEPHELOMETRY

PART I

Introduction, History and Description of Early and Later Types of Nephelometers, Kober and Kleinmann Precision Nephelometers, General Directions for Using a Precision Nephelometer, Theory of Nephelometry, and Nephelometric Research

CHAPTER I

INTRODUCTION

In general, it may be said that the progress of chemistry has been dependent upon the development and refinement of quantitative methods of analysis. Indeed, the introduction of the analytical balance marked a new era in the science of chemistry. No other single instrument has contributed more to the development of chemistry than the analytical balance. From time to time, other instruments have been devised for the quantitative study of the chemical elements, their compounds, reactions, physical constants, and even their internal structure. Among the older instruments we may mention: the graduated tube, burette, pipette, and the graduated flask. More recent instruments include: electrolytic apparatus, electrometric apparatus, interferometer, colorimeter, nephelometer, apparatus for X-ray analysis, and mass-spectra apparatus. The two last-named instruments are, indeed, quite recent, but they are not employed in the ordinary quantitative analysis. Although the first nephelometer was introduced over thirty years ago (1894),

its general use in quantitative analysis did not begin until eighteen years later (1912), when Kober showed that nephelometry could be made sufficiently accurate for most analytical purposes. Within a few years several types of nephelometers were on the market at a reasonable price and these now take their place among our instruments of highest precision and accuracy. An idea of their extreme sensitiveness is gained from Kober's and Egerer's [1] nephelometric reagent for phosphorus, which will detect 1.0 part in 333 million parts of water; Graves' [2] nephelometric reagent for ammonia, which will detect 1.0 part in 160 million parts of water; and Marriott's [3] nephelometric reagent for acetone, which will detect 1.0 part in 100 million parts of water. Many substances can be quantitatively determined nephelometrically when their concentration is of the order of a tenth to one part per million parts of water. Indeed, the nephelometer is limited to the measurement of substances in *low concentration*, usually not stronger than 100 milligrams per liter. To apply the method to larger amounts of substances it is only necessary to dilute suitably. There are certain other limitations but these will be discussed subsequently.

Quantitative analysis until recently has depended chiefly upon the formation of a slightly soluble precipitate which can be separated from its surrounding medium either by sedimentation or by filtration. The precipitate is then washed, dried, and weighed. This method, as all analysts know, is long and often quite tedious, and requires considerable practice, patience and skill. The requirements of the precipitate are many. It must contain all of the constituent, i.e., it must be insoluble or practically insoluble; it must be pure; and must have a definite and known composition. Moreover, the precipitate must be in a form permitting reasonably rapid filtration and highly accurate weighing. It must not change in composition upon drying or being ignited, or if it does, it must change to a substance of definite and known composition and be in a condition suitable for weighing. For-

[1] J. Am. Chem. Soc., **37**, 2375 (1915).
[2] *Ibid.*, **37**, 1171 (1915).
[3] J. Biol. Chem., **16**, 289 (1913).

tunately, the chemist is not dependent upon gravimetric methods entirely. Frequently a volumetric or a colorimetric method may be used. Often these are just as accurate and much shorter. However, most determinations depend upon gravimetric methods. Obviously, a direct method of estimating the mass or weight of precipitate in solution (or rather, in suspension), i.e., without filtering, washing, drying, and weighing, would be highly desirable. Such a method we now have in *nephelometry*. Both the name and the original apparatus are due to Richards.[4, 5] The word *nephelometry* is derived from the Greek, νεφέλη, meaning a *cloud* and μέτρον, meaning *measure*. The method is based upon the measurement of the brightness of the light reflected by a cloud—in other words, by finely divided particles in suspension—very much as in an ultramicroscope. The intensity of the light reflected is a function of the amount of suspended particles, other conditions being kept constant.

[4] Proc. Am. Acad. Arts Sci., **30**, 385 (1894).

[5] It is interesting to note that T. P. Blunt, Chem. News, **33**, 7 (1876), suggested that judgment by turbidity might be provisionally termed "nephelometry" and said this method of analysis might be widely extended.

CHAPTER II

HISTORY AND DESCRIPTION OF EARLY AND LATER TYPES OF NEPHELOMETERS

HISTORY

BEFORE taking up a description of the various types of nephelometers we shall consider the apparatus used by the older investigators. Although Gay-Lussac invented the volumetric method of estimating silver, apparently he did not employ any special apparatus for judging the amount of opalescence. Mulder,[1] however, roughly compared opalescent silver chloride suspensions obtained in the supernatant liquors, or the filtrates, in his atomic weight determinations. This he accomplished by keeping the flasks, containing the silver chloride, in metal boxes blackened inside, and from time to time raising them before the diffused light of a window. Thus he roughly judged the amount of opalescence. Sometimes he decanted the supernatant liquors into small tubes and the amounts of opalescent precipitate were gauged roughly with the eye (Fig. 1). His comparisons were wholly a matter of judgment; he used no measuring device. His experiments, however, mark the first step in the development of nephelometry. The second step was made by Stas,[2] in 1894, who used a series of tubes with perfectly plain bottoms (Fig. 2). Four tubes, about 4 cm. in

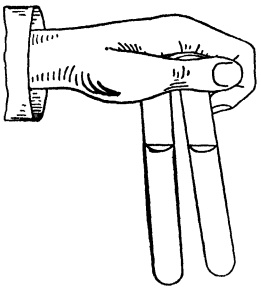


FIG. 1.—First Step in the Development of Nephelometry. The amount of precipitate was gauged roughly with the eye. (1859, Mulder.)

[1] Die Silber Probiermethode, p. 23, Trans., Grimm, Leipzig, 1859.

[2] Œuvres, **1**, 155 (1894).

diameter, were supported adjacent to each other upon a shelf over holes of the same diameter as the tubes, with an illuminated scale beneath the holes. Everything above the shelf was kept in darkness. When the marks on the scale viewed through two heights of opalescent solution appeared the same, Stas assumed there was an equal weight of suspension in the two tubes. The great disadvantage of this instrument is that the light is transmitted through the precipitate. If, instead, the reflected light is observed, when a powerful beam is allowed to fall upon the precipitate obliquely, the delicacy of the estimation is greatly increased. It was upon the latter fact that Richards [3] in 1894 devised the instrument which he named the "nephelometer."

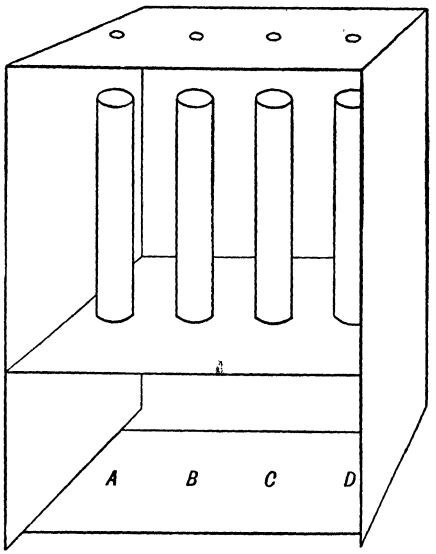


FIG. 2.—Second Step in the Development of Nephelometry. The amount of absorbed light was compared to that of a standard. Used by Stas in 1894. Light was reflected from illuminated scale through tubes, containing precipitate, to the eye.

EARLY TYPES OF NEPHELOMETERS

First Nephelometer (Richards, 1894).—The first real nephelometer, as above mentioned, was made by Richards [4] to compare opalescent precipitates in connection with his atomic weight work on strontium and was described in a paper on the latter subject. A diagram of the instrument is shown in Fig. 3. Two test tubes were arranged almost vertically, but slightly inclined toward one another so that the eye could look into both. Around the

[3] Proc. Am. Acad. Arts Sci., **30**, 385 (1894).
[4] *Loc cit.*

test tubes were two opaque sliding jackets. When the slides were adjusted so that the incident light was cut off in such measure as to give equal opalescence in the two tubes, the precipitate was taken as inversely proportional to the lengths exposed to the light. This relation does not hold accurately for dense precipitates, since the nearer portion partly hides the more distant ones; but for slight opalescence the error is not great, especially

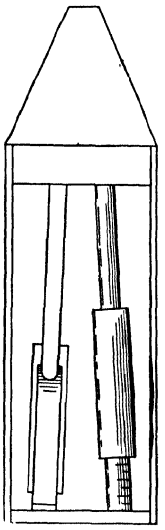


FIG. 3.—First Nephelometer. Made by Richards in 1894. Substance contained in two test tubes; observed from above. Sliding jackets (graduated) were used to match the light reflected.

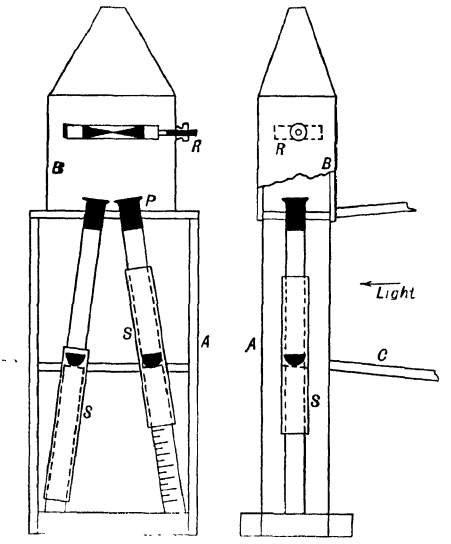


FIG. 4.—Second Nephelometer. Made by Richards and Wells in 1904. The sensitiveness of the instrument was increased by painting the ends of the tubes and inserting two prisms.

when the lengths are not very different. With care, considerable accuracy could be attained, but to secure good results, long practice was necessary.

Second Nephelometer (Richards and Wells, 1904).—In order to reduce the uncertainty of comparison due to the inevitable distance between the centers of the two test tubes, Richards and Wells [5] modified the first nephelometer and made a number of improvements. The instrument is shown in Fig. 4. It consists

[5] T. W. Richards and R. C. Wells, Am. Chem. J., **31**, 235 (1904).

essentially of three separate parts: the main frame *A*, which holds the tubes in position, a movable top *B*, containing adjustable prisms, and a large box *C*, in which is a source of light.

The tubes destined to hold the solutions under examination were test tubes of clear glass free from striations, containing each 0.032 liter, and painted outside around the top and bottom with black asphalt paint. These opaque bands form the most convenient method of obliterating reflections from the meniscus and the curved bottom of the test tube. The space between the lower edge of the upper band and the bottom of the tube was exactly the same in each case. The lower edge of the upper band projected below the wooden support, *P*, in order to provide a sharp line of demarcation between light and darkness, as well as to allow the tubes to be shut into complete darkness by the sliding jackets, *S*,*S*. The tubes rested upon equal wooden pillars (which guided the sliding jackets) and projected well above the support, *P*, in order to preserve their cleanliness. The sliding jackets were also of glass, thickly painted. They moved up and down over the test tubes, shutting off as much light as was wished and being held in any desired place by a brass spring. When raised, they disclosed below two scales which indicated exactly the lengths of the tubes above exposed to the light.

Upon the frame rested the small box *B*, which shut out all light, and yet was easily removable. In this box was a small frame which could be adjusted to any position by means of the set screw *R*. This frame contained two 15° prisms with their thin edges together, the edges having been ground slightly by an optician until they fitted closely. The effect of the prisms when looking downwards through them is to bring into view, side by side, semicircular images of about half of each tube, so that the two halves appear scarcely larger than a single tube, the dividing line being barely visible. The appearance of the image resembles the field of a half-shadow polarimeter. The success of the instrument lies in this arrangement of the prisms. Different observers can judge the equality of two tubes to within a few per cent, an accuracy impossible with the older nephelometer where one had to estimate the small differences at a distance; under favorable

conditions successive readings of the scale by one observer will not vary so much as a millimeter.

Third Nephelometer (Kober, 1912).—Up to this time the Richards instrument and previous apparatus were designed especially to estimate small quantities of substances in filtrates in order to obtain a correction for certain atomic weight determinations. For this purpose it served excellently, but it had one disadvantage as an instrument for general analytical work, namely, best results were obtained only by taking the average of a large number of readings.

As a matter of fact Richards [6] believed nephelometry was not suitable for general analytical work, owing to its inaccuracy. Richards' erroneous opinion was based upon his experience with silver chloride suspensions, which Kober [7] and Kleinmann [8] later showed are, unlike most suspensions, very difficult to obtain in a condition suitable for nephelometric measurements. More recently, however, Lamb, Carleton and Meldrum [9] developed a special technique which produced silver chloride suspensions suitable for nephelometric work.

Kober considered that an instrument similar to Richards' but of greater accuracy and one which would yield reliable results with a few readings, comparable to those obtained with a Duboscq colorimeter, would greatly enhance the value of the nephelometer for the chemical analyst, and so in 1912 he built a nephelometer from a Duboscq colorimeter with wooden attachments made from small packing-box boards. (Fig. 5.) The only changes required were a coat of black asphalt paint on the plungers, a special nephelometric tube and a receptacle for it. This instrument

[6] "This instrument is not intended for determining large amounts of substance which deposit easily from solution; ordinary quantitative methods serve much better in such cases. Its great usefulness appears when one is required to determine minute traces of precipitates which obstinately refuse to settle, or to be caught by an ordinary filter or Gooch crucible. It may be used not only with silver chloride, but also in many other cases in which a finely divided precipitate reflects light; and it provides an unusually sensitive means of detecting very faint cloudiness in a liquid." Richards, Orig. Com. 8th Intern. Congr. Appl. Chem., **1**, 426 (1912).

[7] J. Ind. Eng. Chem., **10**, 564 (1918).

[8] Inaugural-Dissertation (Berlin), **1919**, p. 119; Biochem. Z., **99**, 115 (1919).

[9] J. Am. Chem. Soc., **42**, 251 (1920).

eliminated errors due to the meniscus and had a much better optical equipment.

Fourth Nephelometer (Kober, 1913).[10]—The third nephelometer gave good results but the wooden parts soon began to warp due to moisture; so in the next instrument the wooden parts were replaced by metal ones. In other respects this nephelometer is

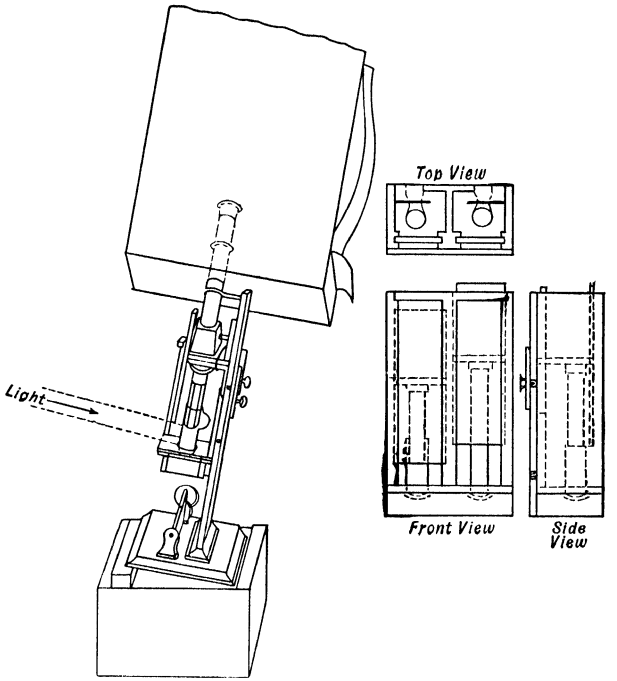


FIG. 5.—Third Nephelometer. Made by Kober in 1912, from a Duboscq colorimeter with wooden attachments. This instrument eliminated errors due to the meniscus and produced a much better optical equipment.

similar to the third. In addition to increased accuracy over the Richards instrument, it has the great advantage of being still

[10] During the summer of 1913 W. R. Bloor working in the chemical laboratory of Queen's University, Kingston, Canada, converted a Schreiner colorimeter into a nephelometer and used it in working out a method for the determination of fat in milk. This method was reported "by title" at the Rochester meeting of the American Chemical Society in the fall of 1913. Later in St. Louis, Mo., he continued his work on the nephelometric method for fat in milk and finally reported his results in the J. Am. Chem. Soc., **36**, 1300 (1914).

usable as a colorimeter. The change from colorimeter to nephelometer and *vice versa* can be made in a few minutes. Although the instrument gives very good results, it has several disadvantages: (1) it is limited to about 40 mm. height of liquid; (2) the plungers require repeated painting on account of the solvent action of some liquids and the peeling off of paint through use; (3) dissolved or minute flakes of paint might introduce an unexpected error; and (4) its cost is relatively high.

LATER TYPES OF NEPHELOMETERS

Bloor Nephelometer (1915).—The Bloor[11] nephelometer is an improved Richards type of instrument made from a Duboscq colorimeter by removing the plungers and adding several simple and easily constructed parts. Although his apparatus was a decided improvement, the original criticisms against the Richards instrument are still valid; that is, there are sources of error due to variations in the meniscus and in the indirect reflection of light from suspended matter in the lower part of the tubes.

An apparent advantage of the Richards type is that the solutions are not stirred or agitated by the plungers while obtaining a photometric balance, if we assume that agitation tends to agglutinate the suspensions, and ignore the fact that agitation also tends to keep any agglutinated particles in the suspension from settling. That this advantage is more apparent than real is due not only to the fact that all liquids are considerably more agitated, and practically to the same extent no matter what instrument is used, when adding the suspensions to the nephelometric containers, but also to the fact that the agitation produced by moving a plunger slowly up and down while making a reading is so slight that it must be negligible in most cases. However, any advantage derived by freedom from agitation is wholly lost by sources of errors, introduced in this instrument elsewhere as pointed out above.

Marshall-Banks Nephelometer (1915).—These authors[12]

[11] J. Biol. Chem., **22**, 145 (1915); see also Bloor, ibid., **17**, 377 (1914).

[12] J. T. W. Marshall and H. W. Banks, 3d, Proc. Am. Phil. Soc., **54**, 180 (1915); *cf.* P. A. Kober, J. Biol. Chem., **29**, 156 (1917).

describe their apparatus as having equal columns of suspensions, and actually measuring the reflected light with a suitable photometer. The photometric part of the apparatus consists of a wedge of neutral tinted glass, while the optical arrangement for observing the two beams of light consists of a simple arrangement of mirrors by which a field far more sensitive than that of a Duboscq colorimeter may be obtained. It may be noted that a smoked glass wedge was used for photometric work in astronomy[13] in 1882 but was not adopted by photometrists. Also, in the Donnan–Kohler colorimeter[14] an arrangement of mirrors similar to that in the Marshall-Banks instrument is used, but, as stated in the book, an empirical correction is required on account of the loss of light by reflection of the glass of the front mirror, and also on account of the unequal paths the light from the cups must travel.

Lenzman-Kober Nephelometer-Colorimeter (1915).—This instrument[15] marked a decided advance in nephelometer and colorimeter construction and practically eliminated the number of defects of the then current model of the Duboscq colorimeter, with the exception of the inequality of illumination in the fields.

Kober Nephelometer-Colorimeter (1917).[16]—The Lenzman-Kober instrument when used as a nephelometer has a disadvantage in that it uses automatic black cloth curtains necessary to give a black background for opalescent solutions. Furthermore, it has a rack and a pinion for raising the plungers, which are difficult to make and usually give an appreciable lost motion. By employing a screw arrangement, as shown in Fig. 6, Kober eliminated both the black curtains and the lost motion of the rack and pinion, thus considerably simplifying the construction. With the double-milled head, a double adjustment is practically secured: the smaller diameter gives a quick action for rough adjustment, and the larger diameter gives a slow action for fine adjustment. Moreover, the screw arrangement is in the back of the instrument, away from possible contact with corroding liquids

[13] E. C. Pickering, Nature, **26**, 259 (1882).
[14] Described in Abderhalden's Handb. biochem. Arbeitsmethoden, **1910**, i, 648.
[15] Cf. P. A. Kober and Sara S. Graves, J. Ind. Eng. Chem., **7**, 843 (1915).
[16] Kober, J. Biol. Chem., **29**, 155 (1917).

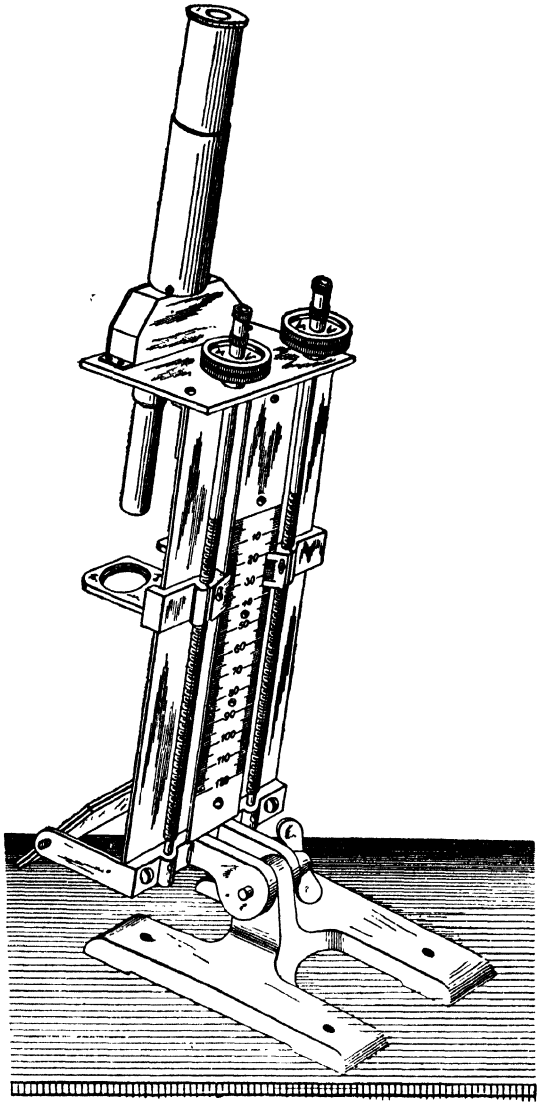


FIG. 6.—The Kober Nephelometer-Colorimeter (1917). Showing screw arrangement with adjustable verniers, also the double-milled head. There are no open spaces through which dust may enter and light escape when fitted to the lamp house (see Fig. 11).

overflowing from the cups, and the scale is removable, thus making it possible to repair it, or to replace it by a new one. The scales are made with a dividing machine and are therefore usually very exact.

Additional improvements in the Kober instrument may be summed up as follows: (1) One or two adjustments of the adjustable verniers eliminate any inaccuracy of the zero-point; (2) no open spaces are to be found between the top of the eye-piece and the liquids in the cups so that dust is excluded; (3) the scale and instrument are so constructed that any height of

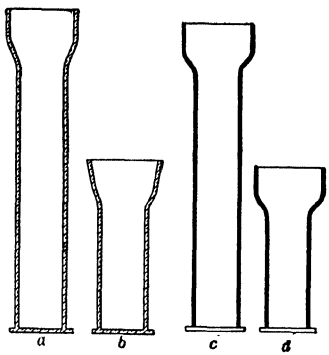


FIG. 7.—Colorimetric Cups. (*a*, *b*) Transparent and optically clear bottoms fused to the sides; (*c*, *d*) black glass sides which eliminate the colorimetric light shield.

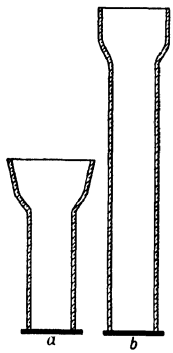


FIG. 8.—Nephelometric Cups. (*a*, *b*) Black glass bottoms fused to transparent sides. Diameter of cups just ½ mm. larger than that of the plungers.

liquid up to 110 mm. can be measured; (4) the colorimetric cups (Fig. 7) in this instrument, like the nephelometer cups (Fig. 8) with which they are interchangeable, are fused instead of cemented and are therefore of one piece of glass, usable for all solvents. (5) The black glass plungers (Fig. 9) with fused-in, optically clear bottoms, not cemented, as they might seem, and a few have believed them to be, make its use as a colorimeter and nephelometer a matter of equal ease and accuracy, entirely eliminating the use of asphaltum paint for this purpose. (6) The new design of a field (Fig. 9) similar to that of Lummer-Brodhun, a square within a circle, makes its use, when correctly made and

adjusted, much more comfortable for the eye and more sensitive.

The Lamp House.—In nephelometry accurate determinations are dependent upon a uniform and constant light thrown upon the tubes. By a careful standardization of the light, errors due to uneven distribution of the light rays may be eliminated, but errors due to fluctuation in the distribution are beyond control. An ideal light for nephelometry would be one having its rays both parallel and uniform. The theoretical requirements for getting such a light are: (1) a point source of light, that can be attached to the ordinary 110-volt lighting circuit, and (2) an optical system for making the rays parallel, the simplest system being the Ramsden ocular shown in Fig. 10.

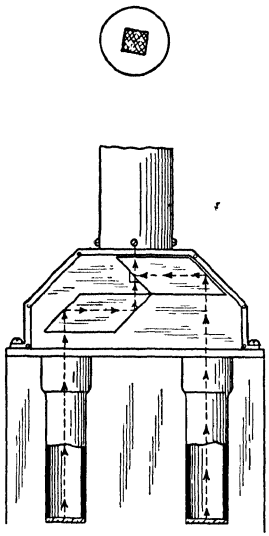


FIG. 9.—Prism Arrangement and Plungers. Black glass plungers with optically clear glass ends, fused together. Prism arrangement, which gives a field similar to that of Lummer-Brodhun.

If we exclude arc lights, the simplest form would be a very compact filament lamp. But since a convenient source of low voltage (6–20 volts) is not available the task of obtaining parallel rays of light is very difficult, if not impossible. The arrangement, as shown in Fig. 11, of a concentrated

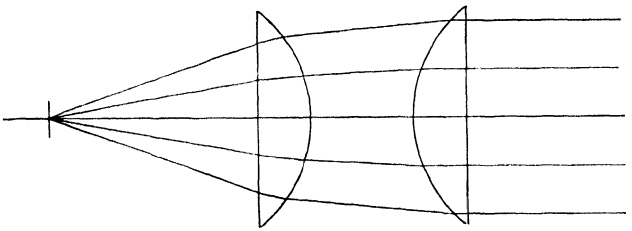


FIG. 10.—Ramsden Ocular. Two plano-convex lenses, the convex sides facing each other.

tungsten filament 110–120-volt lamp (stereopticon) with a condenser gives a very strong light, suitable for most work. A

plane of glass is interposed between it and the condenser, so as to provide an air space, to prevent unnecessary heating of condenser and nephelometer. The lamp house can then be directly connected to most lighting circuits. The reflectors (Fig. 12) of the lamp house are made in pairs, so that the light which falls on each side of the colorimeter can be adjusted to produce equality in the field.

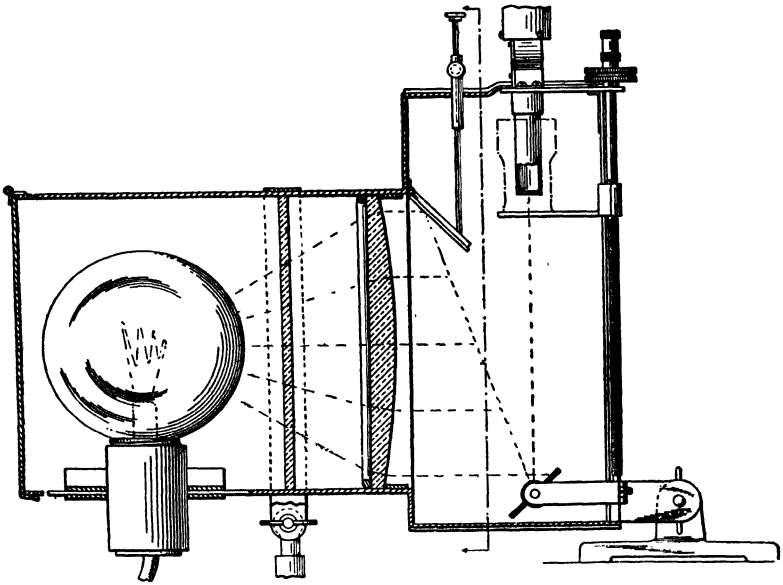


FIG. 11.—Lamp House and Instrument. Showing the concentrated filament lamp, air space, condenser, and lamp house. When the doors (not shown) are closed, no light is visible except in the eyepiece. The inclined angle of the instrument, which allows air bubbles to escape from underneath the plungers, and the exact position and angle of reflectors are not shown in the sketch.

Removable light filters made of the so-called " daylight " glass, or of a light blue glass, may be attached underneath the cup holders to remove any yellow in the artificial light, thus making it possible to measure yellowish liquids. These filters may remain on the instrument without interfering with nephelometric work and, hence, permit a permanent set-up equipped to measure quantitatively either colored or opalescent liquids with equal facility and almost equal accuracy.

Kober Improved Nephelometer-Colorimeter (1921).[17]—After several years of use, the 1917 model of the Kober instrument was improved in several respects. The advantages of the improvements are: (1) The elimination of the fatigue and annoyance due to stooping to read the scale of Duboscq instruments; (2) an enlarged and well-illuminated scale read through an eyepiece of the same focal length as the telescope; (3) a more convenient position for the milled heads operating the stages; and (4) a micrometer arrangement for setting the zero-point which can be locked in any position.

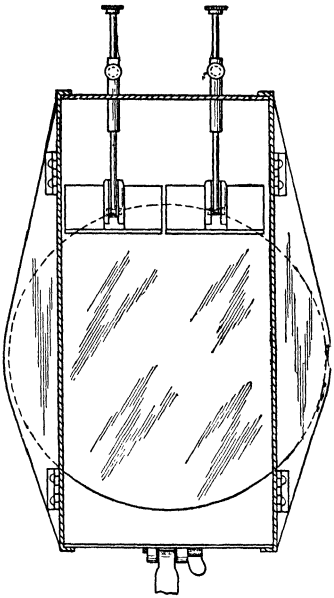


FIG. 12.—Colorimetric Reflectors. Showing two separate reflectors by means of which the light reaching the eyepiece may be adjusted to equality.

The improvements in the 1917 instrument are: The milled heads, formerly at the top of the instrument, are placed at the bottom, which allows the hands to rest on the table or other support and the adjustments to be made with the fingers. An auxiliary scale is provided at the top of the instrument consisting of: (1) two scales engraved upon the side away from the operator, fastened to the movable stages, so that when the stage is being moved up or down, the scales move with it; and (2) a stationary vernier, protruding beyond the top plate, also engraved upon the side away from the operator, fastened to the top of the instrument. A mirror facing the operator at an angle of 45° is placed in front of the protruding scale and vernier, so that an image of the two is reflected vertically. A magnifying glass of the same focal distance as the telescope, serving as a second eyepiece, has been placed close

[17] Made with the assistance of R. E. Klett and manufactured by the Klett Mfg. Co., New York City. The instrument is now sold as the Klett Colorimeter-Nephelometer.

beside the regular eyepiece, directly above the mirror, showing the image of the scale enlarged in good light.

The setting of the zero-point is easily and accurately accomplished with a micrometer arrangement, involving a milled head working against a spring. This convenient method of zero-point adjustment, together with the very simple method of using the instrument, the method of Lamb, Carleton, and Meldrum, where the height of the standard solution is kept constant makes the operation of the instrument and the calculation of results extremely simple and easy without, however, sacrificing accuracy or deviating from the fundamental basis of either colorimetry or nephelometry. See p. 46.

In Fig. 13 is shown the instrument attached to a lamp house. For a detailed description of the nephelometer-colorimeter see Photometric Chemical Analysis, Volume I (Colorimetry), p. 28.

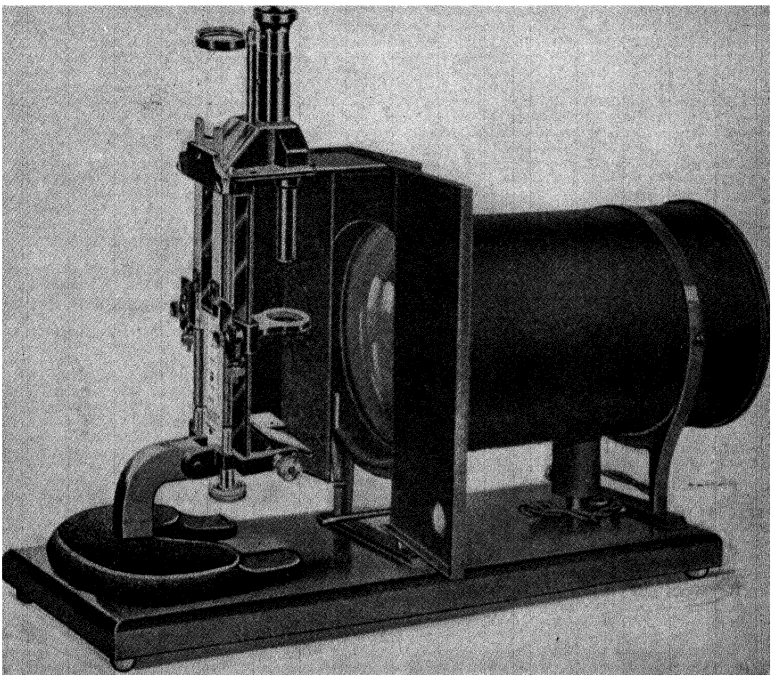


FIG. 13.—The Kober Nephelometer (1921) and Lamp House. Showing the split reflectors as well as the front of the instrument illuminated by the light from the lamp house.

CHAPTER III

KOBER AND KLEINMANN PRECISION NEPHELOMETERS

THE KOBER PRECISION COLORIMETER-NEPHELOMETER [1]

WHEN colorimetery or nephelometry is used at all, in routine analytical work or in research problems, it is apt to be used almost continuously. As a matter of fact the large number of analyses that can be made in a day with either of these methods constitutes one of the advantages that photometric methods have over the older and longer methods. In such continuous use of a photometric instrument, the greatest burden falls upon the eyes. Therefore any instrument which will help to save the use of the eyes and make the work more comfortable for the analyst not only is greatly appreciated, but also actually saves time and promotes greater accuracy.

Here and there in the literature a few authors ask, in effect, why design and build instruments of greater accuracy when the reproducibility of the color reaction or of the nephelometric suspension is not so accurate? The answer to this criticism is that reproducibility is dependent upon the particular method developed which it is always possible to improve and upon the skill of the analyst which also can be improved. Chemists never use a crude balance in preference to a sensitive balance even though their analytical work often yields an accuracy much below that of a sensitive balance. Modern analytical workers demand accuracy and comfort when using a scientific instrument just as most people demand something more than the plainest when purchasing transportation. For those who use an instrument for a short period of time only or a few times a year this may be a luxury, but for those who use it continuously it is a necessity.

[1] Made by the Precision Scientific Company, Chicago, Ill.

In this instrument the objective is to avoid the reading of the vernier for routine work. Kober believes the reading of the vernier is just as fatiguing to the eyes as, if not more so than, the use of the eyes while making a photometric balance in the eyepiece. In recent instruments of this kind a " top-reader " has been provided which eliminates the bending over or stooping of the observer while reading the scales. While these " top-readers " are an advantage over the vertical scale reading, yet the eye-fatigue due to reading the vernier has not been diminished to any extent.

To eliminate the vernier or scale reading requires a device which automatically gives the heights of solutions compared in millimeters and fractions directly as in a counter. To accomplish this purpose, a number of new departures from the usual construction of such photometric instruments are made:

(1) Instead of the usual raising and lowering mechanism of a screw or rack and pinion, a hydraulic system is introduced. The weight of the cup-holder, of the vertical piston, and of the water in the vertical cylinder always produces a back pressure sufficient to eliminate any lost motion and makes delicate adjustments possible. This permits the adjustment knobs as well as scale readers or counters to be placed in the base of the instrument directly in front of the observer. The adjustment knobs can be operated by hands resting on the table. This permits more gradual and better controlled adjustment of the heights of the solution. A further advantage is possible from this arrangement in that the cup-holders can be rotated away from the instrument and the suspension or liquid carefully examined in the strong light before it is put into position underneath the optical plunger.

(2) The counter enables the operator to read the height directly without the use of a vernier. This not only saves eye-fatigue but also eliminates errors of reading the scale.

(3) The knobs operating the colorimetric reflectors are placed in the back of the instrument so that accidental displacement of the mirrors while making the photometric balance is practically impossible.

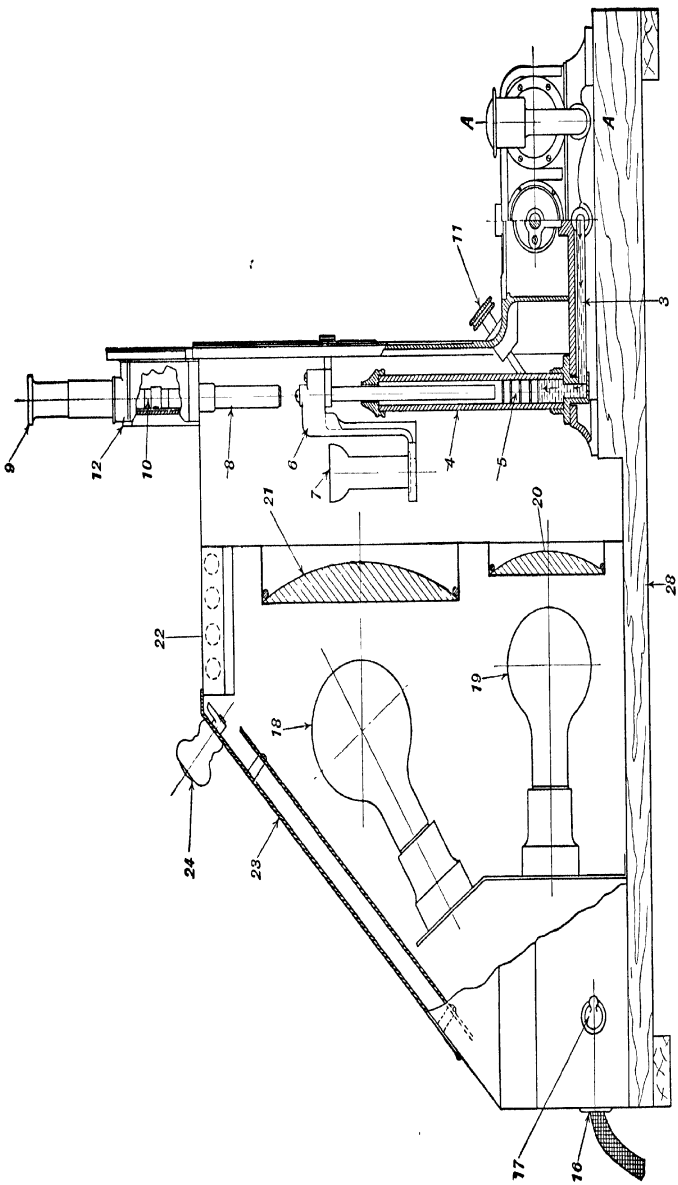


FIG. 14.—Kober Precision Colorimeter-Nephelometer. Cross-section view of instrument and lamp house. (4) Vertical cylinder; (5) vertical piston; (6) cup-holder; (7) cup; (8) optical plunger; (9) eyepiece; (10) prism; (11) knobs for adjusting split reflectors; (12) prism house; (16) lamp cord; (17) three-way switch; (18) nephelometric lamp; (19) colorimetric lamp; (20) colorimetric condenser; (21) nephelometric condenser; (22) air vents of lamp-house; (23) lamp-house door and baffle plate; (24) knob for lamp-house door.

(4) In the lamp house, two separate lights are provided; a small special lamp for colorimetric work and a more powerful light for nephelometry. A three-way switch operating either lamp or extinguishing both is shown on the side of the lamp house. Each lamp is also provided with a separate condenser. A door in the rear of the lamp house permits easy access to the lamps and condensers for the purposes of cleaning and replacement.

The instrument is very solidly built to avoid the vibration of parts. The prism house can be easily opened and the prism, of Albrecht-Hüfner design which eliminates the use of balsam and any unstable parts, can be easily cleaned and correctly replaced.

This arrangement of the instrument permits a minimum of horizontal distance between the cups, requiring therefore for either colorimetry or nephelometry an exceptionally small cone of light.

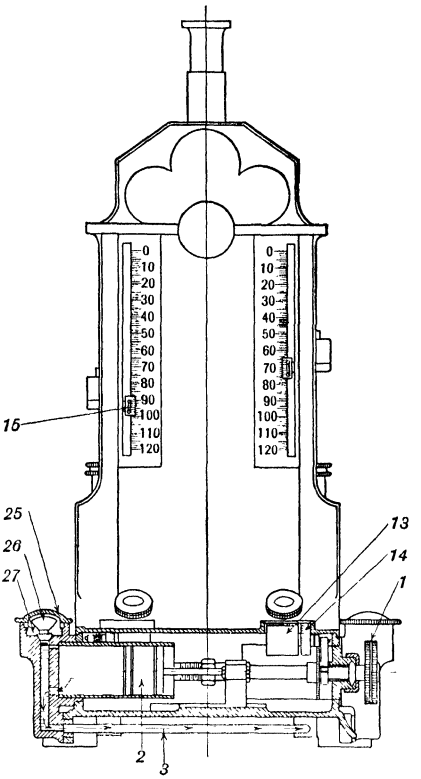


FIG. 15.—Kober Precision Colorimeter-Nephelometer. Cross-section view of instrument base. (1) Adjustment knobs for raising and lowering the cup-holders; (2) large piston operated by (1); (3) metal tube connecting large cylinder with vertical cylinder (4) in Fig. 14; (13) counter; (14) fraction counter; (15) vernier and scale; (25) screw cap of filler; (26) valve of filler; (27) filler.

The counter is standardized with the vertical scale by removing the screw cap of the filler (25), opening the filling valve (26), and operating the adjustment knob (1) until the counter coincides with the vertical scale reading, held at

zero or some other convenient point, and then closing the valve tightly.

Figure 14 shows a cross-section view of the side of the instrument and lamp house, while Fig. 15 shows a cross-section of the base. Fig. 16 shows the exterior of both instrument and lamp house.

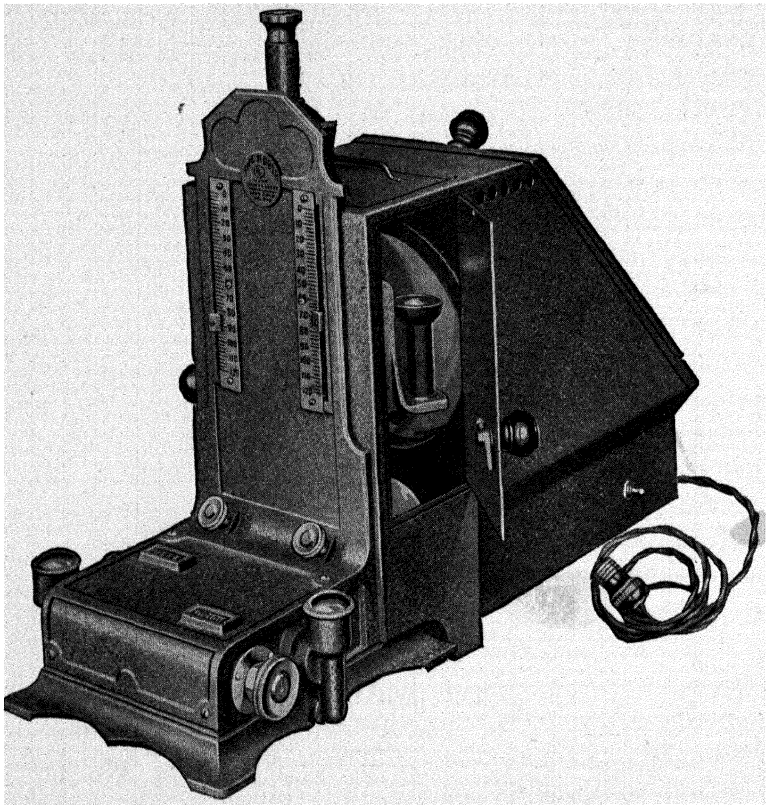


FIG. 16.—Kober Precision Colorimeter-Nephelometer. Exterior view of instrument and lamp house. Showing vertical scales with verniers, colorimetric reflector knobs, counters, adjustment knobs, and fillers on the side of the instrument base. The lamp-house shows the side doors, the revolving cup-holder with cup, and three-way switch on the side of the lamp-house base.

THE KLEINMANN MACRONEPHELOMETER[2]

The working principle of this instrument consists in producing Tyndall-cones (the height of which can be varied) in two turbid media arranged side by side and measuring and comparing their luminous intensity in a line perpendicular to their axis by means of suitable optical fittings. The difference between this instrument and the instruments usually employed for colorimetric measurement, therefore, is that the light, the intensity of which is measured, is diffracted, not transmitted, light. Characteristic of this instrument is the thorough optical construction in reference to the path of light, which permits exact measurement. Thus it is possible to apply the principle, familiar to us from colorimetric methods, of varying the luminous intensity in fixed proportions by varying the " height or thickness of the layer," in our case the diameter of the Tyndall-cones. The concentration of two turbid solutions should therefore, if the law of proportionality holds good, as in colorimetric measurements, be inversely proportional to the diameter of the Tyndall-cones produced in them, when reduced to equal luminous intensity. We must, however, keep in mind that in turbid media the intensity of the Tyndall-light increases with the concentration, owing to the increase in the number of the diffracting particles, whereas in colorimetric analysis the luminous intensity on the contrary decreases with concentration. From the nephelometers hitherto constructed on the same principle—that of the applied Duboscq colorimeter—the present instrument differs mainly in certain modifications by which the optical and technical defects peculiar to these instruments are avoided.

The new nephelometer is shown in Fig. 17, an objective front view and in Fig. 18, a diagrammatic sketch of the design in side elevation.

Figure 17 and Fig. 18 show the two test tubes a and a_1 into which the standard solution and the solution to be tested are

[2] H. Kleinmann, J. Lab. Clin. Med., **12**, 629 (1927). The Kleinmann instruments, as well as all accessories, may be obtained from Schmidt & Haensch, Berlin; or from Akatos, Inc., 114-118 Liberty St., New York City.

poured. They hold about 17 cc. each. These test tubes are carried in metal casings b and b_1, in which they fit easily, so that they can be moved up and down without difficulty. The casings are fitted on spring bases adapted to slide in a suitable frame.

A beam of light is thrown on the test tubes by a lamp placed in front of the instrument and the Tyndall cones thus produced are observed and gauged in a line perpendicular to the axis of the beam. For this purpose the diffracted light is made to pass first through two solid glass cones c and c_1, identical in shape and size and cut out of adjacent parts of the same block of glass, in order to render their action on the light absolutely equivalent. To eliminate the error which may be caused by observing the surface of the liquid, the lower parts of the cylinders are immersed in it. By a suitable arrangement of diaphragms the cylinders receive light from the central part of the Tyndall cones only.

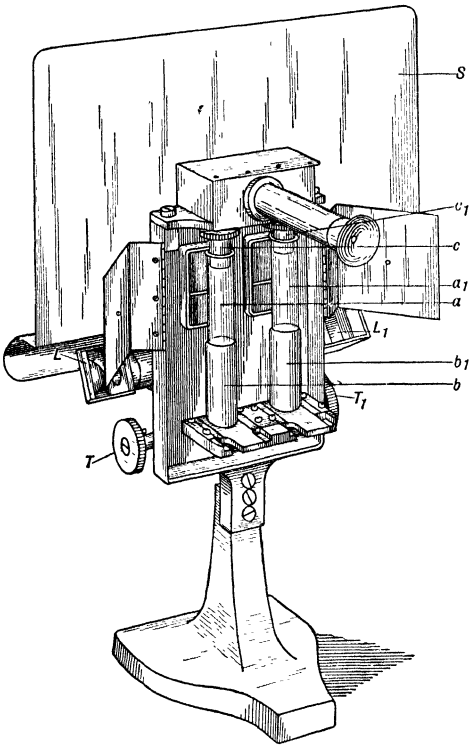


FIG. 17.—Kleinmann Nephelometer.

The sections of the tubes exposed to light and therefore also the diameters of the Tyndall cones can be varied at will by varying the height of the windows f and f_1 through which the light reaches the turbid solutions. These windows are about 4.5 cm. high and about 2 cm. wide. The bottom part of each window opening is closed by a movable metal plate with a sharp edge, fitted on the interior surface of the wall of the instrument, so that the shadow

limiting the illuminated section is very sharply defined. These metal shutters can be displaced by means of rack and pinion and the displacement read by means of verniers. The height of the windows can be varied independently for each Tyndall cone, by means of the corresponding milled screw heads T and T_1, from that of complete closure to the full height of 4.5 cm.

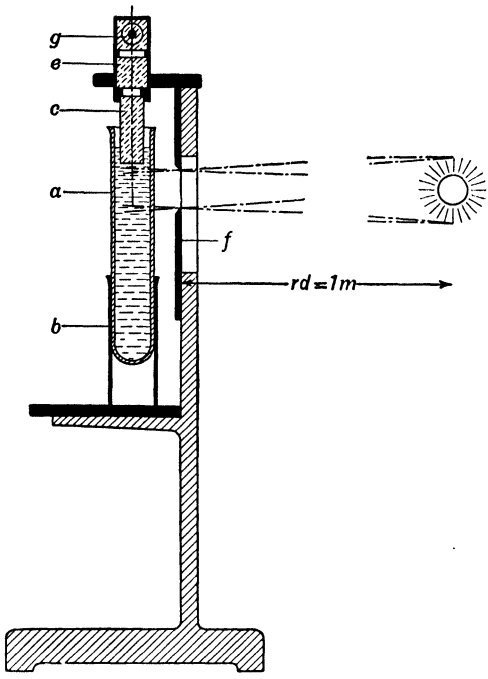


FIG. 18.*

* The cylinder c has been replaced by a cone in the latest model of the instrument.

The verniers may be conveniently read from the back, the observer's side, of the instrument in the prisms L and L_1, that receive light from the observation lamp and are adapted to be moved laterally to bring the scale into focus.

The scale is divided into millimeters and reads from zero (closed window) to 45 (window opened full). By means of the verniers the scale allows reading of 0.1 mm. The observer is screened from the light of the lamp by a removable screen s. In order to exclude the light reflected from surrounding objects the turbid solutions are enclosed in a box of blackened sheet-metal, not shown in the figures, which is permanently fitted on the instrument and can be easily opened and closed.

A frosted Osram lamp of 200–300 cp. preferably is employed as source of light. It should be installed at a distance of 75 cm. in front of the instrument in a line with its optical axis and on a level with the windows.

THE KLEINMANN MICRONEPHELOMETER

In working with material of which only small quantities are available, a macronephelometer showed a disadvantage in that about 17 cc. were required to fill the vessels used for measuring. Kleinmann, therefore, set about to design a modified form of his instrument which would allow the examination of smaller quan-

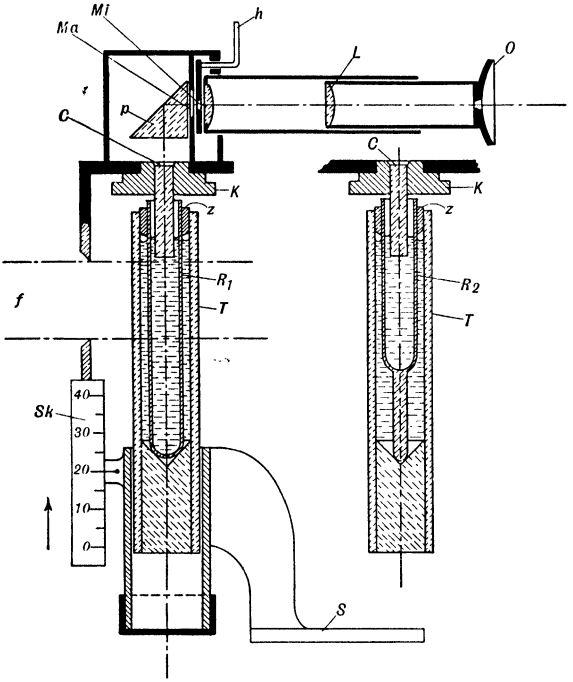


FIG. 19.

tities of solution. In coöperation with the firm of Schmidt & Haensch, he constructed a supplementary fitting to be used in the above-described nephelometer in place of the test tubes containing the solutions. By means of this modification the instrument may be used both as a macronephelometer (taking 17 cc. of solution) and as a micronephelometer (taking smaller quantities, down to about 5 and 3 cc.).

For this purpose the test tubes R_1 and R_2, Fig. 19, of smaller diameter and shorter than the tubes ordinarily used, are pro-

vided, R_1 holding 4–5 cc. of solution and R_2 about 3 cc. The latter is fitted with a glass stopper. Two glass cylinders for submersion in the solution are further provided, of smaller diameter than the cylinders ordinarily used, to fit the narrower test tubes and adapted to be fitted in their places by screw heads. A diaphragm *Mi* interposed in the path of the rays adapts the latter to the reduced diameter of the cylinders. (This diaphragm is now fitted in every instrument, so that the supplementary fitting for microanalysis may be used if desired without alterations in the instrument.)

Owing to the short radius of curvature of the test tubes R_1 and R_2, Figs. 20 and 21, the light rays would on entering be so strongly deflected by refraction that the illuminated space, indi-

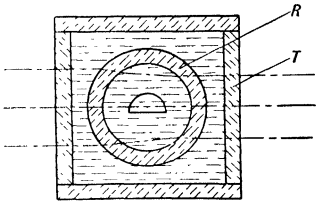


FIG. 20.

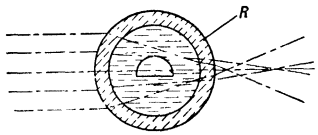


FIG. 21.

cated in Fig. 20 by a circle, would become too small to yield sufficient light for observation. The test tubes are therefore enclosed in glass casings, which are filled with the solvent used in preparing the solution contained in the test tubes. This arrangement (acting similarly to the immersion of the object glass of a microscope) diminishes the refraction, as is shown by Fig. 21, and the path of the light rays is sufficiently extended to enclose the immersed cylinder.

Test tube and casing are supplied mounted, ready for fitting into the nephelometer. The disposition of the parts will be easily understood from Fig. 19. By means of this, material which is procurable in extremely small quantities only, such as immunized sera and the like, can be subjected to investigation. The minimum quantity which can be estimated by nephelometric methods is thus reduced to one-sixth of that hitherto required.

MANIPULATION OF THE KLEINMANN NEPHELOMETER [3]

The manipulation of the instrument is simple but requires, however, painstakingly exact observation of the directions given here, particularly in regard to cleanliness and subtility of the operations.

The measurements are carried out in a dark room in which during a measurement no lights are present except those used for illuminating the nephelometer. It is also desirable that the working table and walls be painted a dull black. The apparatus is set up on a firm table, which must be long enough so that the source of light can be put about one meter from the instrument. After the instrument is properly placed before the light source, both it and the light are fastened to the table by means of metal strips screwed to the table.

Setting up the instrument begins with the screwing in of the glass plungers. These are carefully wiped with a chamois cloth. Particular attention should be paid to keeping them clean, as well as to keeping them from getting cracked or scratched. They should be cleaned with a soft cloth, best with a chamois cloth, after each use of the instrument. Now and then they should be unscrewed from the instrument and given a thorough cleaning. Damage to the bottom surface of these plungers makes a measurement practically impossible and necessitates replacement of both plungers.

After the plungers are screwed into the instrument the test tubes are placed in the jackets and firmly pushed into place. The tubes also must be very carefully cleaned. This is best done by means of a sulfuric acid-chromate cleaning solution. The tubes are cleaned with alkali when protein solutions have been used in them, and with alcohol and ether when fat solutions have been used, and so on, according to the substance used. Finally they are rinsed out with distilled water and wiped on the outside with a chamois cloth which is free from lint. It is not desirable to dry them on the inside. This is done only when a series of experiments is concluded and the instrument is to be put aside for the

[3] Private communication to the author from Dr. Hans Kleinmann.

time being. Brushes or cloths invariably leave small fibers and other particles on the surface of the glass. It is therefore preferable to leave the tubes wet and to rinse them once or twice with the solution to be used in them. The tubes must be handled only at the top or bottom and not by their sides, which would leave finger marks. After setting them in place and elevating them, the tubes are cleaned once more on the outside with chamois, as the illumination from the light source shows up impressions, deposits, etc., more easily. To accomplish this the tubes are grasped either above or below the Tyndall beam and slowly turned. Finally they are so placed that the mark which measures the top of the solution is turned toward the observer. In this way the same parts of the tubes are always exposed to the light source.

As soon as the tubes are perfectly clean, the instrument is adjusted with relation to the light source. This adjustment is made when mounting the instrument on the table and at the beginning of each series of determinations, as a control. For this purpose the apparatus is so placed that equal illumination is obtained when both tubes are equally filled.

A stable, suitable suspension (glycogen suspensions are recommended, see p. 32) is placed in each tube up to the mark. The tubes after being firmly pressed into the jackets are placed in the instrument by mounting the jackets in the slots, which are then slid in until they meet the slug. The tubes being held at their upper edges are raised until the plungers are immersed, that is, until the tubes are in contact with the conical plungers. Care must be taken that no air bubbles get underneath the plungers when they are immersed in the liquid, or that air bubbles form during the measurement, due to heat or otherwise. Sudden changes in the amount of light during a measurement indicates the presence of air bubbles. If the air bubbles stick to the plungers in such a manner that they are not removed by moving the plunger in and out of the liquid they may be removed by wiping the bottoms of the plungers with a soft leather.

After the cups have been filled with a suspension and raised into position without air bubbles, the light source is placed about one meter from the instrument in as symmetrical a position as

possible, during which it is to be noticed that the height of the light source is that of the nephelometric apertures. If no definite horizontal lines are seen on the edges of the Tyndall beam, but only slanted shadow lines, the height of the lamp must be regulated so that horizontal illumination of the instrument is obtained. Then the shutters of the instrument are opened to the same height on both sides and as far as possible—about 40 mm.

The eyepiece is sharply focused on the field by movement of the ocular and then the instrument is turned to one side or the other until both sides of the field are equally illuminated. The tubes are then lowered and exchanged and then raised again. If the illumination was symmetrical, both sides of the field are again equally illuminated. If they are not, the instrument must be moved about again in reference to the light source, the tubes exchanged and the fields compared, until an exchange of tubes does not disturb the equality of the two fields. The position of the instrument and the lamp is now suitably fixed to the table, but it is necessary to test the correct position of the instrument every time a new series of measurements is made, by putting the same solution in both tubes, since jarring or shaking may produce a displacement which might introduce errors.

Sometimes it happens that with the greatest care and cleanliness it is impossible to find a position for the instrument where the exchange of tubes produces an equal illumination of the fields. There are two possible causes. First, the suspension is not uniform or stable, so that the Tyndall beam is not of the same intensity in each case. This can be overcome by the use of a new suspension. The second possibility is that the tubes do not match. The tubes are not made of optical glass but are manufactured by being drawn out and hence may show up lines or imperfections, or their diameters may not be the same, thereby producing dissimilar light beams due to differences in refraction. Although the factory carefully matches each pair of cups, it is advisable to test them and to use only pairs that are well matched. Care must be taken to see that the same half of each tube is exposed to the light source, as mentioned before, which is done by having the filling mark turned to the observer at all times.

An inequality of illumination due to the tubes is recognized by the change in illumination on an exchange of the tubes. If an inequality of illumination exists, i.e., if there are unequal heights of the Tyndall beams with the same solution on both sides, in the same way, even by exchange of the tubes, the fault is due to a displacement of the optics. In such a case the instrument should be returned to the factory for readjustment.

If the illumination of the fields remains the same on exchange of tubes with the same suspension (deviations of 0.1 to 0.2 mm. can be ignored, and for the finest measurement can be eliminated by double measurement after exchanging the tubes), then the instrument is ready for use. The solutions to be measured are placed in the tubes, one of the shutters is put at a definite height and the other shutter varied in height until the illumination in the field is equal. The concentrations will be found to be inversely proportional to the measured heights. It is immaterial which solution is varied. It is best, however, always to work with the largest possible opening, especially if the suspensions are weak, so that the maximum illumination is obtained. It is not good to increase the illumination by moving the lamp close to the instrument, since in so doing parallel rays cannot be obtained. An exception can be made in measuring weak suspensions by putting the lamp closer to the instrument, if attention is given to the possibility of error (which can be easily determined). As it is not suitable to go below an opening of 10 mm., the comparative concentrations vary from 1 to 4. This is a ratio which is hardly ever used in colorimetric measurements. Although very good measurements are obtained at this ratio, it is in the interest of accuracy to make the concentration ratios closer. The smaller the difference, the smaller the error of observation.

The sensitivity of the instrument toward intensity variations of the light is exceptionally great. Yet here subjective, as well as objective, factors play a large rôle. The eye, which at first is very unsensitive to even large differences in illumination, soon attains a high sensitivity. Thus through practice the accuracy of the measurement is extraordinarily increased. It is absolutely necessary that the eye be allowed five to ten minutes to get accus-

tomed to the dark before the measurements are undertaken, and also during the measurements to allow the eye to rest in the dark.

To avoid subjective errors it is advisable to carry out each adjustment several times, say ten times, and to take the mean value of the observations as the final result. With a little practice this may be done rapidly and easily and makes possible the exclusion of an unavoidable single subjective error of observation.

Brightly reflecting objects in the immediate neighborhood (bright table or bright covers, etc.) are to be avoided. During the measurement the rear end of the box that contains the illuminated tubes is closed.

It is obvious that the solutions to be tested should be treated exactly according to the directions. The solutions must be homogeneously cloudy, unless otherwise specified. Flocculating solutions are unsuitable. Flocculation processes during the measurements are recognizable by the appearance of large flakes and are also directly detected by observation in the bright Tyndall beam.

The apparatus when not in use is covered with a cloth and protected from dust.

GENERAL DIRECTIONS FOR NEPHELOMETRY

For the purpose of obtaining the relationship between concentration and height of solution, as well as for testing out the instrument, and for the practice of the observer, solutions of glycogen are best suited. However, it is not immaterial which glycogen is used. Many glycogen preparations on the market yield on dissolving in water a dirty yellow color only instead of the blue-white opalescent suspensions, or change on dilution, even if the cloud is satisfactory, so that no proportionality is obtained between cloudiness and concentration. It may happen that on diluting a glycogen solution in the ratio of 1 to 2 no decrease of cloud is obtained. The suitability of the glycogen must be tested out. An unobjectionable preparation can be obtained only by preparing it oneself, according to Rona and van Eweyk.[4]

[4] P. Rona and C. van Eweyk, Biochem. Z., **149**, 176 (1924).

In nephelometric work there are a number of precautions and principles to be observed, which are indispensable for successful work. It cannot be too often emphasized that the condition for successful nephelometric work is a high degree of cleanliness. As simple as nephelometery is, in the same degree must care of this point be observed. Dirts which do not come into consideration in other methods become sources of error here, since they give an optical effect. Therefore, in the preparation of the solutions to be used in making the suspensions, all reagents must be free from shreds, particles of paraffin and other suspended particles, and must be optically clear. Hence, the vessels used in connection with the measurements must in no case be dried with cloth or filter paper after cleaning with distilled water, but must be dried after rinsing, either in the air or in a drying oven. Moreover, pipettes and other measuring instruments must not be dried on the outside with cloth or filter paper, since they would then introduce shreds into the solution to be determined. If at all possible, avoid filtering the solution to be determined, since through filtering, filter fibers get into the solution. It is mostly on account of fine particles (large impurities can be allowed to settle) that filtering is necessary. For this purpose use Schleicher & Schüll blue ribbon filters (or similar grade), and filter back and forth until the filtrate is optically clear. If the production of the suspension takes place in a small beaker, it is necessary to cover the beaker at the end of the reaction with a cover-glass in order to exclude dust and shreds from the air. It is necessary to be very careful that the cloud of the solution is the result of the reaction and that it is a homogeneous opalescent cloud. Heterogeneous impurities are easily detected in the tubes when in the instrument, for then the Tyndall beam through the strong illumination brings them into observation. Shreds forming are easily visible in ordinary cloudy solutions. Such solutions are to be discarded and the starting material carefully tested for the source of error or trouble.

A control for the purity or cleanliness of the test tubes and the material to be tested consists in putting on one side of the instrument pure water or the solvent for the material to be pre-

cipitated, and on the other side a cloudy solution. The shutter on the water side is opened to the maximum and the shutter on the side of the cloudy solution is closed. The field on the water side in the eyepiece must then be absolutely dark, and the field on the other side when the shutter is completely closed must be equally dark. If the tubes are not quite clean or if the solvent is not optically clear or contains shreds or air-bubbles, the field is then a trace brighter, and equality of the field is attained only by opening the shutter of the side containing the cloudy solution. It is therefore to be observed that the solvent side is either absolutely dark, which is always possible, or one must estimate the fractions of a millimeter of the cloudy solution which is equivalent to that of the solvent.

If these directions are carried out, then the following can be said of the principle of determining the suspensions:

1. The degree of turbidity of the solution to be compared must remain constant within the period of observation, nor should the solution be subject to any changes of state such as the formation of flaky precipitates. This condition is a matter of course and needs no further explanation or comment.

2. Turbid media that are to be determined nephelometrically must be absolutely homogeneous at least to the naked eye. There is also an upper and a lower limit to the density of solutions susceptible of accurate measurement which may be easily determined empirically. If the turbidity of a solution is so slight that even a powerful beam of light produces only a faintly luminous cone in it, the measurements are naturally less accurate than when the Tyndall light is of ordinary intensity. On the other hand, solutions of too great density are very liable to form flocculations, not to speak of the absorption of the diffracted light by the superimposed liquid which in turbid solutions of high density begins to be perceptible. This shows the necessity of studying systematically every reaction producing turbidity which we wish to use in nephelometric investigations, as to the limits of concentration which may be employed, the stability of the suspension, etc. See Chapter VI.

3. The difference in turbidity or, what amounts to the same thing, the difference in concentration of solutions which are to be determined nephelometrically, should not exceed the ratio of 1 to 4. This is merely an empirical rule embodying the experience that with solutions more widely different in concentration the measurement becomes more difficult and less accurate. In this connection it may be noted as a most interesting fact that Lednický,[5] on the basis of diagrammatic sketches of the design of the nephelometer, discussed the theory of nephelometry mathematically and was led by his calculations to the same conclusions regarding the concentrations of the solutions to be determined.

The difference in concentration of 1 to 4 is, however, so great that it will never be exceeded or reached in practice. As a solution of unknown concentration is generally compared with a standard of turbidity, which can be made of any desired concentration, the difference in concentration of 1 to 2 between the solutions will hardly be exceeded in practice. The slighter the difference in density between the turbid media that are to be compared, the easier will the measurement be and the more accurate the result.

4. The turbid solutions subjected to analysis must possess equal dispersive power; for according to the formulas established by Rayleigh, the intensity of the Tyndall light depends upon two factors: (*a*) The number of particles present in the turbid solution, and (*b*) their size, that is, the dispersive power of the solutions.

In order to enable us to determine one of these variables, the other must be constant. As we wish to measure the concentration of a solution by comparison with another, that is, the number of particles, their dispersive power must be equal.

This condition seems very difficult to fulfill; it is, however, quite possible to realize it for a number of reactions producing turbidity. In the case of inorganic substances producing turbidity, such as chloride of silver and the like, solutions of equal dispersive power can be prepared only by special devices, such as the addition of a protective colloid. With substances possessing

[5] Kolloid Z., **32,** 12 (1923).

a very large molecule such as albuminoids, alkaloids, fatty substances high up in the series, it is comparatively easy to produce turbid solutions of equal dispersive power. That this condition is actually fulfilled is proved by the fact that a substance entering into reaction in the same concentration repeatedly gives solutions of equal turbidity. This also shows the necessity of studying a reaction methodically in all respects, before nephelometric methods can be used successfully in connection with it.

5. The optical fittings of the instrument employed in measuring must be faultless in design and construction.

A NEW TURBIDITY STANDARD

In gauging turbid solutions, especially for analytical purposes, the turbid solution to be tested is usually compared with a turbid solution of known strength, the standard solution rendered turbid by the same reaction as the solution to be tested. Suitable standard solutions for comparison are, however, in some cases difficult to procure (e.g., certain solutions of proteins). In such cases and also in chemical investigations of colloidal substances, e.g., of the variation of turbidity of the solution of a colloid during a certain period of time, it is indispensable to have a fixed and invariable standard of turbidity. Such a standard should have the following characteristics:

1. Its strength must, when once gauged, remain constant for an unlimited period of time.

2. Its strength, or the amount of light it emits, must be capable of variation to any desired extent.

3. Its color must also be capable of variation, so that it may be made to match exactly the shade or tint of the various turbid solutions.

The last adjustment is of importance, as the various turbid solutions, even those called white, although they are almost always of a bluish-white color, vary considerably in shade from blue to yellow. In order to be able to adjust turbid solutions accurately to equal brightness, it is absolutely necessary to have them of the same color or even shade of color, unless monochro-

matic light is available. There is, however, some difficulty in producing monochromatic light of sufficient brilliancy, not to speak of the inconvenience of manipulating the necessary apparatus.

A standard of turbidity consisting of a liquid or of a solution cannot fulfill the above conditions. Kleinmann therefore turned his attention to other methods of preparing a suitable and sufficiently variable standard of turbidity and finally attained his object in the following manner: The walls of a test tube, such as is used to hold the solution in the nephelometer, are frosted in a way that permits varying the thickness of the frosting layer or coating. When a tube frosted in this manner is placed in the nephelometer, the frosted white surface throws diffuse light on the bottom of the tube. On the bottom of the tube a colored preparation is spread, the color of which can be varied as required. This preparation reflects colored light upwards into the immersed cylinder of the nephelometer.

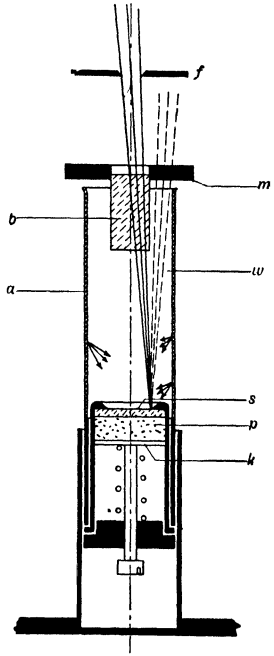


FIG. 22.

The standard of turbidity prepared on this principle took the practical form shown in Fig. 22: *a* is a small glass tube which, in order to avoid light, reflected from its walls, penetrating upwards into the submerged cylinder, is rather wider than the ordinary nephelometer tubes. The tube *a* is fitted on a short brass tube indicated by strong lines in the drawing. Into this brass extension of the glass tube a cartridge is introduced from below, which will be described in detail further on, and which contains the colored preparation. The whole is fitted into the base of the nephelometer tube in the same way as an ordinary tube and raised until the top of the tube encloses the glass cylinder, *b*.

The surface of the tube is frosted by pouring over it a solution of collodion in ether, in which an indifferent, finely divided powder, such as talcum, is suspended. This solution, on drying, leaves a film of uniform thickness covering the surface of the glass. In this manner any desired degree of frosting can be produced by increasing or diminishing the quantity of talcum, or other matter, suspended in the solution. Before the above operation is begun, the brass tube end is closed by an accurately fitting brass stopper to prevent the solution from penetrating into the interior of the tube.

When the collodion film is dry, a cartridge adapted to form the colored reflecting bottom of the standard tube is introduced into the brass tube end in place of the stopper. This reflecting bottom is formed by a powder the color of which can be varied as required and which fills the top part of the cartridge. The latter is closed off by a cover-glass, *s*, ground opaque, against which the powder is pressed by a piston, *k*. The piston *k* is kept in place by a spring and forms a uniform and evenly colored surface as bottom for the standard tube.

The colored powder used to form this bottom should be as fine-grained as possible. It will in most cases be of a bluish-green tint, as most of the turbid solutions are bluish-white. Kleinmann found a mixture of talcum, sulfate of copper and blue litmus very suitable, but, of course, any desired colored powder may be employed for the purpose. The color that is most suitable for the turbid medium in question must be found by experiment. The right shade can always be ascertained by a few experiments.

In case the light emitted by the colored bottom is not sufficiently intense to impart the right tone of color to the white light emitted by the wall of the glass tube, the collodion film itself may be colored or the tube may be lined with colored tissue paper. With paper, any desired color may be produced for the investigation of colored colloidal solutions.

The intensity of the light emitted by the standard of turbidity may be modified, not only by varying the density of the frosting film and the brilliance of the reflecting bottom, but also, and even more easily, in the same way as that of ordinary turbid

solutions, by adjusting the nephelometer window in front of the standard tube.

The standard of turbidity should not, however, stand freely in the nephelometer as shown in the drawing, but should be raised until it touches the top fitting *m*, in order that the bottom may always be at the same distance from the cylinder, *b*.

The immersed cylinder receives light exclusively from the bottom of the tube, that is, from the cover-glass, *s*. The dimensions of the tube are such that no light entering laterally through the walls of the tube can reach the cylinder directly.

A standard of turbidity prepared in the above manner can be varied in luminous intensity and color so as to match any turbid solution, and will keep indefinitely without changing. It will probably prove useful not only in ordinary nephelometric work but also in the investigation of kinetic processes in colloidal bodies.

CHAPTER IV

GENERAL DIRECTIONS FOR USING A PRECISION NEPHELOMETER

ALMOST anyone can use a gravimetric balance for rough weighing, but the correct use of a precision balance for accurate weighing is not accomplished without considerable study and practice as well as the standardization of both balance and weights. Likewise, a rough matching of colors or turbidities can be practiced by the uninitiated, but the precise use of a photometric balance, a colorimeter or nephelometer, requires the consideration of many factors, of which the following are the most important alike for both instruments:

The Choice of Instrument.—In order to eliminate the errors arising from a meniscus, highest precision in nephelometry can be obtained only with a plunger type of instrument. This type of instrument is the only one thus far developed that measures the light between two parallel planes of glass, thus giving a very sharp differentiation to the heights of solution measured in the cells or cups. The plunger, with its mechanical and accurately adjustable stage, allows the measurement of the height to be made to any desired accuracy. In practice this accuracy has been limited to a tenth of a millimeter, which subdivision is usually obtained by means of a vernier. If the accuracy of observation warranted it, this reading of the scale could, of course, be made to a hundredth of a millimeter or less.

Specifications of the Instrument.—(*a*) *Good Optical Construction.*—One of the chief specifications of a precision nephelometer is good optical construction. This requirement is absolutely essential if accurate measurements are to be obtained. The parts must be made from flawless optical glass of minimum tint or having little or no absorption of light. Hollow plungers with

fused bottoms having parallel planes show much less absorption of light than do the best of solid plungers. The optical parts should be so mounted and incased as to eliminate stray reflections, and as much as possible to prevent deposition of dust and condensation of moisture. Even the best of instruments require from time to a time a thorough cleaning of all of the optical parts, which involves the complete taking apart of the eyepiece and prism house. The optical arrangement should be so constructed that, if necessary, readjustments or refocusing of the parts can be made. In addition to the glass parts of the optical system, suitable apertures and diaphragms are required. As a rule those instruments having small diaphragms are more sensitive than those with large diaphragms, but both extremes are to be avoided. Optical systems having little or no balsam in their construction are to be preferred to those having large surfaces balsamed.

(*b*) *Mechanical Construction.*—The accuracy obtainable with an instrument of good optical construction is often lost by the operation of a poor mechanical arrangement. Since the nephelometer, if it is sensitive, is like a precision gravimetric balance, the equality between the two sides is seldom maintained for a long time. It would not be a sensitive nephelometer if it did not respond to variations in temperature, dust, and moisture depositions. For this reason a good nephelometer must have its mechanical parts adjustable so that when necessary the instrument can be readjusted and a new balance of its sides obtained. Adjustable scales or verniers are important for accurate and convenient use of a precision nephelometer. In the moving parts the great requirement is the absence of lost motion, so that the eye can be trained to observe the slightest difference in light intensity. In instruments that have considerable lost motion the observer can never be sure of slight movements of cups or plungers and therefore does not know whether differences in observation really represent differences in the heights of the suspensions or not.

Where many determinations are to be made, convenience in reading the scale is a factor, for any operation which increases the fatigue or inconvenience during the determination will have its effect upon the sensitivity of the observer's eyes. Kober's

direct-reading scale (see p. 18) has a decided advantage in this respect. Furthermore, the instrument should be so constructed as to prevent glares and stray light from getting to the observer's eyes during the use of the instrument. In some of the nephelometers the eyepiece is placed in a horizontal position, enabling the observer to match the light intensity in a sitting position, while others have the eyepiece in a vertical position, which, unless the instrument is placed on a low stand or table, requires a standing position on the part of the observer. The nephelometers with a horizontal eyepiece have the disadvantage that unless the light source is carefully and completely shaded, stray light will get to the observer's eyes and make matchings more difficult and less sensitive. With nephelometers having a vertical eyepiece, the use of a black table top will practically exclude stray light from the observer's eyes. In short, if the proper arrangements are made with both types of eyepiece, there is no choice between them so far as convenience and accuracy are concerned.

(*c*) *The Light.*—Accuracy in nephelometry is dependent on the uniformity and constancy of the light thrown upon the cups or cells. Although errors due to uneven distribution of light may in most cases be eliminated by careful standardization of the light, the errors due to changes or inconsistency in distribution are beyond control. Attempts to develop an ideal nephelometric light, i.e., one in which the rays are both parallel and uniform, have thus far not been very successful, but the lamp shown in Fig. 11 (p. 15), is one of the best. The object in trying to get parallel rays is to eliminate as much as possible the zero light of the instrument.

The theoretical qualifications for such a light are: (1) a point source of light, that can be conveniently attached to ordinary lighting circuits; (2) an optical system, the simplest being the Ramsden ocular, for making the rays parallel. See Fig. 10 (p. 14).

Arc lights being excluded, the simplest form would be a filament lamp with a very compact filament. The compactness of the filament obviously depends on the length and thickness of the filament, but, as most filaments are very fine, the main

difficulty is in the length. If low voltage is available, say six volts (that of 2 to 3 storage cells), correspondingly shorter filaments may be used, making the task much easier. Although storage cells are in universal use, yet the introduction of them for nephelometric lights would almost make the price prohibitive.

In the light illustrated in Fig. 11 (p. 15), parallel rays are practically obtained, but for many purposes an ordinary powerful filament lamp, well screened and placed about 3 feet from the nephelometer, will give good results.

Care of a Precision Instrument.—(*a*) *When in Use.*—The necessity of caring for a delicate machine or instrument ought to be apparent to most workers, but it may not be amiss to point out some of the major precautions in handling a nephelometer. The cups and the plungers should, of course, be kept perfectly clean, and rinsed once or twice with the suspension to be used for determination. The cups must not be filled too high so that they overflow and thus stain and corrode the instrument. Care should also be taken that the plungers do not strike the bottom of the cups too hard while taking the so-called " zero-point " reading. Where long cups are used the zero-point must be measured by inserting into the cups a wooden or metal rod with parallel ends and of known length, usually called a " zero-gauge." The zero-point should be taken with each individual cup and the cup always used on the same side and in the same position. By making a small mark on the top of the cup this precaution can be easily carried out. This standardization of the instrument in relation to the cups is necessary, as it is seldom that the thickness of the glass used in the cups is uniform. It is, of course, obvious that the bottoms of the plungers and the sides of the cups should be kept free from scratches and cracks.

(*b*) *When Not in Use.*—The instrument should be kept covered to keep out the dust, but it is also important that the room containing the instrument be kept at a uniform room temperature and free from excessive moisture and fumes, particularly that of ammonia.

Precautions to be Taken Relative to the Observer.—The accuracy of photometric work greatly depends upon the care

taken with the observer's eyes. Although there are a number of persons whose sensitivity to differences in light intensity is very low and who are therefore incapable of making any accurate nephelometric determinations, most workers do not realize that the sensitivity of the eyes can be greatly increased by judicious use and training. The human eye is like some electromotive cells, in that it is easily polarized; that is, its greatest sensitivity or accuracy does not hold for a very great length of time, without many periods of rest. Therefore the period of observation should be as short as is conveniently possible. A good practice is to make a rough adjustment with one eye and the final setting with the other. A number of photometric observers have found it an aid to massage the eyes with hot or cold water several times a day, and it is a fact that the organ of sight, like other organs and tissues, can be strengthened and its value and accuracy greatly increased by proper use.

The best environment for photometric observation is that of a darkened but well-ventilated room whose walls and contents have a dark tint. It, of course, follows that the light source must be well screened so that at no time the observer's eyes are partially blinded by the glare. Moreover, the observer should remain in the dark for five or ten minutes before making a reading in order for the eyes to acquire their maximum sensitiveness.

For beginners, it is very important that a reasonable time be allowed for practice determinations, so that they become accustomed to the instrument and the environment of the instrument. Quietness and freedom from extraneous disturbances are also essential to accurate photometric work.

Method of Obtaining a Photometric Balance.—The light source must be adjusted with relation to the instrument, or the instrument must be adjusted to the light source, so that equal illumination on both sides of the instrument is produced. Once this is obtained it is best either to fasten the instrument and light source in position, or, if that is not possible, to mark on the table or support the exact position of each.

When everything is in proper adjustment, some standard suspension is put into the cups, after the operator has carefully

rinsed both cups of the instrument and both plungers first with distilled water and then with the standard suspension to be used; then one side, say the right side, is set at 20.0 or 30.0 mm. exactly. The observer now tries to obtain equal light intensity by operating the other side. A series of five or ten settings should give a good average of the reading on the right side. If they do not, it may be due to some poor adjustment of instrument or light source or to an idiosyncrasy on the part of the observer. In the former case, a readjustment will correct this inequality, or in both cases it may be taken care of as follows:

The plunger on the left side is set exactly on the average obtained above, and the standard suspension on the right side is discarded in favor of the next suspension, say one-half or one-third standard, and after making a series of settings using only the right side, the average is compared or used in calculation with 20.0 or 30.0, the original reading on the right side. In other words, the setting on the left side does not come into consideration, the suspension there acting only as a tare. If the suspension obeys Beer's law, the reading for one-half standard should read exactly twice that of the standard, and that of one-third standard should read exactly thrice that of the standard. This check on Beer's law should be made by those using a nephelometric reaction for the first time, as it enables the observer to check not only the instrument and his observations, but also the sources of error inherent in the reaction or the method of producing the reaction. If the solution does not obey Beer's law, a nephelometric curve must be constructed. (See p. 46.)

The precise matching of two faintly opalescent solutions is very difficult. The best way to obtain readings of satisfactory accuracy is to raise the left-hand cup until there is a shadow just distinctly visible on the right-hand side of the divided field, and then to lower it until a shadow of the same distinctness is visible on the left, and to take the mean of the extreme readings.

The number of readings necessary for accurate work varies with the type and concentration of the suspension to be determined. Other things being equal, the greater the number of readings the more nearly accurate is the result. Where many

determinations are to be made, many readings for each solution produce greater eye fatigue. Also, some nephelometric suspensions change fairly rapidly with time and are not constant for a long period. Moreover, the experience and the skill of the observer must be taken into account. As a rule, many readings are taken only when isolated analyses are to be made and the analyst has had no opportunity to keep in photometric practice. A trained and experienced observer usually varies the number of readings in accordance with their agreement. If three or four readings agree closely or check, further readings would hardly be of value for most analytical purposes.

Calibration or Correction Curves.—In the description of the Kleinmann nephelometer (Chapter III) it was pointed out that this instrument is so designed that it gives (when used with procedures developed by Kleinmann) readings in accord with Beer's colorimetric law, namely, that the concentrations of the solutions (or suspensions) are inversely proportional to the heights that give equal intensity of light. Other types of nephelometers may be used but they may require a calibration or correction curve. Once such a curve has been constructed for a given instrument and suspension, the instrument is capable of yielding accurate results. Kober and others have made use of nephelometric curves but their method of constructing them is not as simple as the one used by Lamb, Carleton and Meldrum [1] described as follows:

At the beginning of a series of analyses both cups of the instrument and both plungers are rinsed first in distilled water and then with the unknown suspension to be used. They are nearly filled with this solution and placed in position in the instrument. The position of the left-hand cup is adjusted until the scale reading is exactly 20.0; the position of the right-hand cup is then adjusted until the illuminations on both sides of the eyepiece are equal, this being determined as the average of several independent settings. This value is retained for the whole series of comparison with the standard, as the potential height of the right tube.

The solution in the left-hand cup is then discarded, the cup

[1] J. Am. Chem. Soc., **42**, 251 (1920).

and plunger rinsed with the standard solution to be used—which should be equal or slightly weaker in concentration than the unknown solution—and the cup nearly filled with the solution as before. The position of the left-hand cup is adjusted until the illuminations on both sides of the eyepiece are equal. The scale reading on the right side is recorded and the results computed, using the formula given by Kober:

$$20.0 = \frac{Y}{X} - \frac{(1 - X)YK}{X^2},$$

where Y signifies the scale reading, X the concentration, and K the constant for the suspension and instrument used.

If instead of using the above formula the results are plotted, a straight-line curve will be obtained, from which the concentrations can be read off directly. For illustrations of nephelometric curves see Nephelometric Research (Chapter VI).

CHAPTER V

THEORY OF NEPHELOMETRY

THE theory of nephelometry may be defined as an explanation of the scattered visible light coming at right angles from an illuminated column of suspended substance and of the relationship between the intensity of scattered or reflected light and the concentration of the suspended particles. As nephelometry in analytical chemistry is concerned primarily with the measurement of the concentration of suspended particles, the illuminating or incident light as well as all other factors are kept as constant as possible so that the concentration of the suspended particles can be gauged or determined by the amount or intensity of reflected light. The term "reflected light" used here includes and may actually be due to refraction or reflection or both.

The property of suspended substances scattering light is called Tyndall effect and has been carefully studied and measured. The measurement of the ratio of incident to reflected light is called Tyndallometry. The results of Tyndallometry will give us some insight as to the cause and also the amount and character of reflected light coming from different concentrations of suspended particles as well as the relationship of the size and form of the particles to the amount of light reflected.

THE TYNDALL EFFECT

A brief quotation from Tyndall's [1] own report will show the effect of the size of the particles on the color of the scattered light.

It has hitherto been my aim to render the chemical action of light upon vapours *visible*. For this purpose substances have been chosen, *one*, at least of whose products of decomposition under light shall have a boiling-point so high that as soon as the substance is formed it

[1] J. Tyndall, Proc. Roy. Soc., London, **108**, (1869).

shall be *precipitated.* By graduating the quantity of the vapour, this precipitation may be rendered of any degree of fineness, forming particles distinguishable by the naked eye, or particles which are probably far beyond the reach of our highest microscopic powers.

I have no reason to doubt that particles may be thus obtained whose diameters constitute but a very small fraction of the length of a wave of violet light.

In all cases when the vapours of the liquids employed are sufficiently attenuated, no matter what the liquid may be, the visible action commences with the formation of a *blue cloud.* I would guard myself at the outset against all misconception as to the use of this term. The blue cloud here referred to is totally invisible in ordinary daylight. To be seen, it requires to be surrounded by darkness, *it only* being illuminated by a powerful beam of light. This blue cloud differs in many important particulars from the finest ordinary clouds, and might justly have assigned to it an intermediate position between these clouds and true cloudless vapours. . . .

In all cases, and with all substances, the cloud formed at the commencement, when the precipitated particles are sufficiently fine, is *blue,* and it can be made to display a colour rivaling that of the purest Italian sky. In all cases, moreover, this fine blue cloud polarizes *perfectly* the beam which illuminates it, the direction of polarization enclosing an angle of 90° with the axis of the illuminating beam.

It is exceedingly interesting to observe both the perfection and the decay of this polarization. For ten or fifteen minutes after its first appearance the light from a vividly illuminated incipient cloud, looked at horizontally, is absolutely quenched by a Nicol's prism with its longer diagonal vertical. But as the sky-blue is gradually rendered impure by the introduction of particles of too large a size, in other words as *real* clouds begin to be formed, the polarization begins to deteriorate, a portion of the light passing through the prism in all its positions. It is worthy of note that for some time after the cessation of perfect polarization the *residual* light which passes, when the Nicol is in its position of minimum transmission, is of a gorgeous blue, the whiter light of the light of the cloud being extinguished. [This seems to prove that particles too large to polarize the blue, polarize perfectly light of lower refrangibility.] When the cloud texture has become sufficiently coarse to approximate that of ordinary clouds, the rotation of the Nicol ceases to have any sensible effect on the quality of the light discharged normally.

RAYLEIGH'S LAW

A theory of scattering of light by particles in suspension was developed by Lord Rayleigh [2] in 1871. P. V. Wells [3] has given the following excellent account of Rayleigh's work as it applies to turbidity measurements:

The simplest case is that of a single particle of infinitesimal size compared with the wave length of light. The particle then acts as an electric oscillator, performing forced vibrations in the direction of the impressed force with a certain amplitude, a. The oscillator, therefore, sends out scattered waves in all directions, the vibrations being, of course, in every case perpendicular to the direction of the light, since light waves are transverse. But the component of a normal to a line making an angle θ with the vibration is $a' = a \sin \theta$, so that the scattered intensity in this direction, measured by the square of the amplitude is

$$I_s = ka'^2 = ka^2 \sin^2 \theta \tag{1}$$

Here the incident light is regarded as plane polarized. By Eq. (1) the scattered intensity vanishes when $\theta = 0$, that is, normally to the incident ray, and in the direction of the incident vibration, in agreement with Tyndall's experiment.

If the light is unpolarized it is more convenient to consider, not the direction of vibration, but the direction of light propagation. If unpolarized light is incident along the axis of y, the incident vibration may be regarded as compounded of two vibrations of equal amplitude in the directions of the axes of x and z. If the particle is situated at the origin of coördinates, two vibrations of equal amplitude $-a$, along x and z—spread out in all directions from the origin as from a source. The components of these vibrations perpendicular to a direction r, defined by the angles α, β, γ, with the axes of x, y, z, are, respectively, $a \sin \alpha$ and $a \sin \gamma$. The resultant intensity I_s, of the scattered light along r, is

$$I_s = ka^2 (\sin^2 \alpha + \sin^2 \gamma) \tag{2}$$

but from geometry $\cos^2 \alpha + \cos^2 \beta + \cos^2 \gamma = 1$, and hence

$$I_s = ka^2(1 + \cos^2 \beta) \tag{3}$$

[2] Lord Rayleigh (J. W. Strutt), Phil. Mag., **41**, 107 (1871).

[3] P. V. Wells, Chem. Reviews, **3**, 331 (1927).

This gives the variation of the scattered intensity with the angle between the directions of the incident and scattered light. The intensity is a maximum in the direction of the incident light, decreasing to one-half normally, and zero in the opposite direction.

The amplitude of the vibration in the scattered light, on either the elastic solid theory or the electromagnetic theory of light, is proportional to the volume, V, of the small disturbing particle. At a distance r from the particle the amplitude must be inversely proportional to r, so that in order to be dimensionally correct, the ratio of the amplitudes a of the scattered light and a_o, of the incident light of wave length λ must be

$$a/a_o = kV/\lambda^2 r \tag{4}$$

These simple considerations may help to explain Rayleigh's expression for the intensity I_s of the light scattered from N particles each of volume V, the incident intensity being I_o, and the wave length λ. This is

$$\frac{I_s}{I_o} = \frac{n'^2 - n^2}{n^2} \cdot \frac{NV^2}{\lambda^4 r^2}(1 + \cos^2 \beta) \tag{5}$$

Here n is the refractive index of the medium, n' that of the particles. The particles are supposed to be contained in such a small volume that the distance r and the angle β between the scattered and incident beams are the same for all the particles. For particles of different size, all small compared with the wave length, a summation must be made, requiring the size distribution of the particles. When the particles are not small compared with the wave length, terms of higher order must be included, and again the expression becomes complicated.

No account is taken in Eq. (5) of secondary scattering. All of the light scattered by the particles in the direction β is supposed to reach the eye without loss. When the medium is densely filled with particles, this factor may become of first importance. The fractional decrease of the intensity I in traversing a thickness, $d\chi$, of the turbid medium is

$$dI/d\chi = - hI/\lambda^4 \tag{6}$$

where h is a constant independent of λ.

Integrating

$$I\chi = I_o \exp.\ (- h\chi/\lambda^4) \tag{7}$$

where I_0 is the intensity of the light when $\chi = 0$ and $I\chi$ is the intensity after traversing a thickness χ.

The most striking characteristic of Eqs. (5) and (7) is the occurrence of the factor $1/\lambda^4$, indicating that the scattered light increases rapidly as the wave length decreases. The scattered light is, therefore, much bluer than the incident light, while the blue is correspondingly absent in the transmitted portion, which contains a relatively large fraction of the red light. This was used by Rayleigh to explain the blue color of the sky, as well as the red colors of the sunset. The blue color may be used as a test of the size of the particles of any turbid medium. Thus the fine blue smoke from the end of a cigar is an indication that the smoke particles are much smaller than the wave length of light. Tyndall's test of complete polarization at right angles to the incident beam is still more sensitive.

The constant h in equation (7) has been evaluated by Schuster [4] from general considerations, independent of any particular theory of the mechanism of scattering. He obtains the same expression as did Lord Rayleigh, using the electromagnetic theory, namely,

$$h = 32\pi^3(n - 1)^2/3N \tag{8}$$

where n is the refractive index of the dispersion as a whole. If the blue color of the sky is due to air molecules alone, N is Avogadro's constant, and its value is known from other measurements. The experimental researches on the absorption of the atmosphere by Abbot [5] and Fowle [6] give good values for Avogadro's constant, and indicate quite definitely that the extinction coefficient is inversely proportional to the 3.9 power of the wave length, which is quite close to the inverse fourth power of Rayleigh's formula. Crova,[7] Zettwich,[8] and Boutaric [9] have obtained inverse powers approaching, and in some cases even exceeding, 4. Boutaric thinks that such excessive powers disagree with Rayleigh's theory, and suggest fluorescence as

[4] Theory of Optics, 2d ed., p. 326, Edwin Arnold, London, 1909.

[5] Ann. Astrophys. Obs., Smithsonian Inst., Vols. **2** and **3**; Astrophys. J., **23** (1906).

[6] Ann. Astrophys. Obs., **2** (1908) and **3** (1913); Astrophys. J., **40**, 435 (1914); *ibid.*, **42**, 394 (1915); Smithsonian Misc. Coll., **69**, No. 3 (1918); J. Opt. Soc. Am., **6**, 99 (1922).

[7] Ann. Chim. phys., **20**, 480 (1890); *ibid.*, **25**, 534 (1892).

[8] Phil. Mag., **4**, 199 (1902).

[9] La Radium, and J. chim. phys., **12**, 517 (1914); Ann. phys., **9**, 113 (1918); *ibid.*, **10**, 5 (1918); J. phys., **9**, 239 (1920).

a possible cause, but Rayleigh has shown that the " residual blue " observed by Tyndall can be explained by taking into account the second order of small quantities, the residual blue varying inversely as the *eighth* power of the wave length. The occurrence of powers higher than 4 is thus accounted for. Powers lower than 4 can be ascribed to larger particles, and Boutaric shows that from 100,000 particles per cubic centimeter of radius 0.05 micron, to 1000 of radius 0.1 micron, are sufficient to account for the excess of atmospheric absorption over that which can be ascribed to the air molecules. These numbers are not excessive for the persistent nucleation of the atmosphere. The changes in the polarization of sky-light indicate that the particles causing these changes are relatively large (approaching the wave length of light). Rayleigh's theory has received many other experimental verifications.

Rayleigh's theory assumes that the particles are dielectrics, and so does not apply to the metal sols, which show such beautiful and varied colors. A theory of scattering by small particles was worked out by J. J. Thomson,[10] assuming them to be perfect conductors; and Maxwell-Garnett,[11] Mie,[12] and others have extended the theory to larger particles, and imperfect conductors. A large number of researches on gold and silver sols have shown that the absorption band crosses the visible spectrum toward the red, with increasing size of submicroscopic particle, just as the theory demands. The spectral absorption data of Pihlblad [13] are most complete, showing but one absorption band, and confirming quantitatively the theory of Mie. As the particles approach molecular dimensions, the absorption spectrum approaches that of the molecular solution. These colors are so characteristic of the particular molecule, however, that no simple theory can apply. Rayleigh's theory owes its simplicity to the fact that the selective effect of scattering is not specific, all non-conducting particles looking alike when small, just as all coarse dispersions of these substances appear white. Our discussion of turbidity measurements will be limited to dispersions of such colorless transparent substances, the specific absorption of which can be neglected, except as represented by the ordinary index of refraction.

. . . The turbidity T is proportional to the concentration C and

[10] Recent Researches in Electricity and Magnetism, p. 437, Oxford, 1893.

[11] Phil. Trans., **203**, 385 (1904); *ibid.*, **205**, 237 (1906).

[12] Ann. Physik, **25**, 377 (1908).

[13] Z. physik Chem., **92**, 471 (1917); Inaug. Diss., Upsala, 1918.

to the depth χ as a first approximation. Its dependence on color is intimately bound up with size of particle. For particles small compared with the wave length of light, Rayleigh's law, Eq. (5), states that the Tyndall ratio is proportional to the cube of the particle size d and to the inverse fourth power of the wave length λ. But particles larger than a wave length merely reflect the light from their surfaces, and so the turbidity is proportional to their total surface. We may combine both these relations in a single formula for the turbidity

$$T = \frac{kC\chi d^3}{d^4 + \alpha\lambda^4} \tag{9}$$

where k and α are constants.

Formula (9) holds approximately for both very large and very small particles, but in the transition region, which is probably from a centimicron to a micron, it may depart from the facts more widely. It indicates a very sharp maximum in turbidity when $d/\lambda = (3\alpha)^{1/4}$, for a given concentration and depth. The constant k is specific both for the dispersion and for the method of measurement, but the constant α probably depends upon the method only. As a function of concentration and depth, Eq. (9) is of the same form as Beer's law for molecular absorption. For equal turbidities dilutions are proportional to depths, and since the relation is usually tested by diluting a standard dispersion, we shall call it for brevity the *dilution law.* When turbidity is the diffuse density ($T = D$), a comparison with the exact expression (12) shows that the rejectance factor has been neglected, so that formula (9) holds only for very thin layers, or for very dilute dispersions.

THE STUDY OF DIFFUSION

For deeper layers of suspension, the work of Channon, Renwick and Storr[14] and of Bloch and Renwick,[15] gives us valuable information and data. Their object was to explain why two layers of suspensions of equal density gave less than twice the density of a single layer. This fact had been observed by a number of workers but it remained for the above authors to study the phenomenon quantitatively and to propose an explanation for it.

[14] Phot. J., **58**, 121 (1918).
[15] Phot. J., **56**, 49 (1916).

Most of their work was done with very thin sheets of opal glass, cut into suitable circular disks, studied singly and also one upon another with or without cedar oil contact, using completely diffused light upon the disks. Channon, Renwick and Storr developed the following formula which P. V. Wells [16] has applied to turbidity measurments:

If I_o is the incident light intensity, I_r the intensity rejected by the diffusing layer, and I_t that transmitted, the rejectance R is defined by the ratio I_r/I_o, and the obstructance Ω by the ratio I_o/I_t. Superposing two layers (m) and (n), the intensity transmitted through them both, taking account of the successive inter-rejections, is

$$\frac{I_o}{\Omega_m\Omega_n}(1 + R_mR_n + R_m^2R_n^2 + R_m^3R_n^3 + - - -) = \frac{I_o}{\Omega_m\Omega_n(1 - R_mR_n)}$$

so that the combined obstructance is

$$\Omega_{m+n} = \Omega_m\Omega_n(1 - R_mR_n) \tag{10}$$

Since this is symmetrical, the obstructance is independent of the arrangement. The combined rejectance is similarly

$$R_{m+n} = R_m + R_n/\Omega_m^2(1 - R_mR_n) \tag{11}$$

The relative obstructance (O) of a layer superposed on a standard of rejectance ρ, is defined as $O \equiv \Omega(1 - R\rho)$, so that the proper expression for the diffuse density is

$$D \equiv \log O = \log \Omega + \log (1 - R\rho) \tag{12}$$

where log is the symbol for the logarithm to the base 10. When the rejectance of the sample is very small, as in the black silver deposit of photographic images, the rejectance factor can be neglected. The density is evidently never as great as it would be without the rejectance factor.

The general expression for the obstructance as a function of the thickness (χ) is

$$O_x = P \exp. (\alpha\chi) + (1 - P) \exp. (-\alpha\chi) \tag{13}$$

where P and α are constants, and exp. is the symbol for the exponential function. This is evidently the integral of $d^2O/d\chi^2 = \alpha^2O$.

[16] *Loc. cit.*

Schuster [17] has derived a similar equation for the radiation through a foggy atmosphere. The relation for Ω_x is of precisely the same form. It is evident from the form of this expression that the density increases more slowly than the thickness, and indeed Bloch and Renwick [18] have found an approximate formula for the density

$$D_x = D_1 \chi^n \tag{14}$$

to hold within the limits of experimental error over a five-fold range in thickness. The exponent for the opal glass samples measured was $n = 0.80$ with air contact, and 0.86 with cedar oil contact. When the thickness was expressed in millimeters, the value of P was 1.342 and $\alpha = 0.4392$, for opal glass with oil contact; whereas for air contact $P = 1.600$ and $\alpha = 0.3838$. The agreement between the approximate and the exact equations is shown in Table I.

TABLE I

Opal Glass Thickness (χ)	Densities (Air Contact)			Densities Oil (Contact)		
	$D\chi$ (Approx.)	Log $O\chi$ (Exact)	Difference	$D\chi$ (Approx.)	Log $O\chi$ (Exact)	Difference
Mm.						
1	0.282	Used for	0	0.270	Used for	0
2	0 492	constants	0	0 490	constants	0
3	0 680	0 675	−0 005	0.694	0 692	−0 002
4	0 856	0 848	−0 008	0 889	0 887	−0 002
5	1 023	1 015	−0 008	1 076	1 080	−0 004
6	1 185	1 180	−0 005	1 260	1 271	−0 011
7	1 340	1 344	−0.004	1 438	1 462	−0 024

The densities calculated from the approximate equation never differed from those observed by more than 0.007, and 0.0023 was the average difference This agreement is equivalent to a photometric precision of 0.5 per cent, quite up to the best standards of photometry. Richtmyer and Crittenden [19] give for the average

[17] Astrophys. J., **21**, 1 (1905).

[18] *Loc. cit.*

[19] J. Opt. Soc. Am., **4**, 371 (1920).

deviation of a single observation from the mean of its set, 0.3 per cent, in a study of the photometric precision of 20,000 readings by 15 observers.

Their general expression for the rejectance as a function of the thickness is

$$R_x = R^{\infty}[1 - \exp.(-2\alpha\chi)]/[1 - R_{\infty}{}^2 \exp.(-2\alpha\chi)] \quad (15)$$

where R_{∞} is the rejectance of an infinitely thick layer. Their measurements on opal glass gave 91 per cent for the maximum total rejectance of a thick solid block, which checks the values obtained on similar materials by A. H. Taylor [20] with his diffuse reflectometer, by an entirely different method. The laws of perfect diffusion, here briefly outlined, are therefore well established by this work of Channon, Renwick and Storr.[21] Notice that the theory is purely geometrical, and makes no assumptions except that the light is perfectly diffused, and that the diffusing media are large enough to neglect edge effects. It has nothing to do with the mechanism of scattering by the particles constituting the dispersed phase.

The most important consequence of the laws of perfect diffusion for turbidity measurements is that the density increases more slowly than the depth. It disposes once for all of the claim sometimes made that one instrument is better than another because it conforms to the "theoretical" linear relation between density and depth. If the calibration curve of an instrument is linear, which of course is an advantage, it can be due only to some empirical shape factor which compensates for the rejectance factor in Eq. (12).

We may apply the laws of perfect diffusion to obtain a complete expression for the intensity of a thick Tyndall beam, as used in tyndallmeters and nephelometers. The incident beam is assumed to be of uniform intensity, and so each layer scatters at right angles an increment which is proportional to its thickness. Before reaching the eye, this increment must traverse the depth χ of the dispersion, suffering an obstructance Ω_x. Moreover, the rejectances of the layers both above and below contribute their quota to the Tyndall intensity ratio T, and so the total increment dT due to the layer $d\chi$ is given by

$$\frac{dT}{d\chi} = \frac{k}{\Omega\chi} \frac{(1 + R_a - \chi)}{(1 - R\chi R_a - \chi)} \quad (16)$$

[20] U. S. Bur. Standards, Sci. Paper, **391** (1920); also, Illum. Eng., **13**, 265 (1920).
[21] *Loc. cit.*

where R_x is the rejectance of the layer above, R_{a-x} is the rejectance of the layer below $d\chi$, and k is a constant. Substituting the functions of χ from Eqs. (13) and (15) and integrating,

$$T = \frac{T_\infty}{1 - R_\infty^2 u^2}\left[1 - Au - Bu^2 + (E - Fu^2)ln\left(\frac{c + u}{c - u}\right)\right] \qquad (17)$$

where $u \equiv \exp.(-\alpha\chi)$, and A, B, E, F, c, T_∞, and R_∞ are constants, and χ now represents the depth of the Tyndall beam. It is quite obvious from Eq. (17) that tyndallmeters and nephelometers give calibration curves even more complicated than do turbidimeters which measure the density of a dispersion. It is only when the concentration of dispersed phase is so small that secondary scattering can be neglected, or when empirical shape factors compensate, with dispersions of a certain range of concentration, that anything like a linear calibration curve is obtained. This limitation must be carefully investigated for every instrument before the results can be interpreted.

THEORETICAL CONSIDERATIONS INVOLVED IN THE PRACTICE OF NEPHELOMETRY

(1) **Influence of Size and Shape of Cell.**—As nephelometric analysis is concerned with liquid suspensions or turbidities, a container—tube, cell or cup—must be used to hold the liquid. While in colorimetric work the diameter and shape of the walls of the cell have no influence upon the result, in nephelometry, where the incident or illuminating light strikes the cell walls at right angles, the shape and the diameter are important and have to be considered.

In the earlier forms of nephelometers the suspensions were put into suitable test tubes and the scattered or reflected light determined through the meniscus. How much of the total light reflected upward by all of the particles illuminated reached the eye, in such forms of nephelometers, is not known. Later forms of the instrument introduced an optical plunger which eliminated not only the meniscus but also restricted the measured light to that getting into or through the horizontal lower end of the plunger. In Fig. 23 are shown the types of cells.

While the use of the plunger, either stationary or movable in the solution, eliminated errors due to the meniscus, it introduced

a variable in the matter of the diameter of reflecting or measured column. As may be readily seen in Fig. 23, the amount of suspension actually measured and therefore the amount of light reaching the eyepiece is different in each type of arrangement.

Disregarding for the time being the errors due to the meniscus, it can be said that different instruments measure different

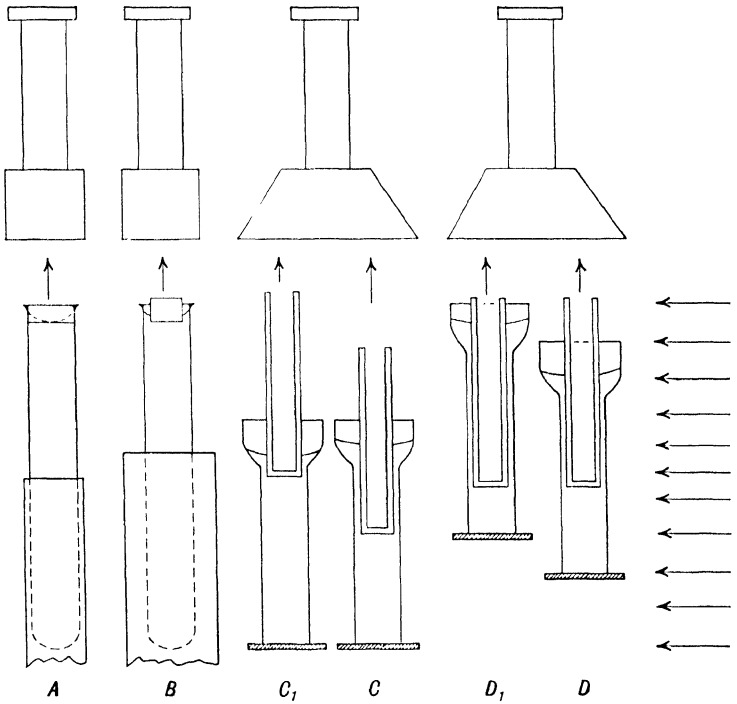


FIG. 23.—Constant Upper-End Type of Instruments. *A*, Richards; *B*, Bloor, Kleinmann; D_1, *D*, Kober; constant lower-end type, C_1; *C*, Kober's first nephelometer.

sections of the Tyndall cone, but each instrument always measures the same section; in other words, the measured portion of the illuminated column in each instrument has its own peculiar shape.

The differences in shape, however, in themselves do not involve any errors so far as actual analytical work is concerned. Careful standardization as shown on p. 46 will eliminate any shape factors.

In testing out the theoretical formula, these shape factors may, as will be shown, cause a marked difference in the results. This raises the question which type of arrangement will produce a shape of column closest to that demanded by the theoretical considerations in the formula developed.

Before discussing briefly the results with each of the types of instruments given in Fig. 23, another factor of importance requires consideration, which might be called the distance factor.

(2) **Influence of the Method of Changing Heights of Suspensions.**—All the instruments may be classified into two groups according to this distance factor. In one class, as shown in Fig. 23, the meniscus or upper surface of the two measured columns remains stationary and constant and the variations in the height of the illuminated column are brought about by moving the lower end of the illuminated column, or what amounts to the same thing, covering or uncovering the lower portion of the tubes by means of shutters or jackets. This type may be called the " constant-upper-end " type.

The second class of instruments is the reverse of the first type; the bottoms of the columns are at equal and constant heights and the upper portion of the column is subjected to change in height. This class may be called the " constant-lower-end " type.

In the first class of instruments the light from the particles in the longer of two illuminated and measured columns has a *greater* mean distance to travel to the eyepiece than the light from the particles in the shorter column. In the second class the light from the particles in the longer of two illuminated and measured columns has a *smaller* mean distance to travel to the eyepiece than those of the shorter column.

The influence that this distance factor may have upon the standardization of the instrument and upon the theory of nephelometry as applied in practice is not known. So far as is known to the author, no published work containing any study of this factor has appeared. It is therefore impossible now, in view of the lack of information upon the shape and upon the distance factors, to draw any conclusion whether or not nephelometric

determinations and results are in precise harmony with the above theoretical considerations.

To show the influence in a crude way, a review of the behavior of the better-known instruments in actual work is given.

(3) **The Behavior of Different Types of Nephelometers.** (*a*) *Richards Nephelometer.*—This instrument, as may be seen on p. 6, is of the constant-upper-end type.

The light coming from the illuminated particles passes through the meniscus. The length of the illuminated columns is varied by jackets operating on and from the lower end of the tubes.

Richards and his collaborators found that with silver-halogen clouds of very low concentrations the ratio of the heights to the concentrations did not follow the inverse proportion, as might be expected from the simple application of Rayleigh's law or by assuming that Beer's colorimetric law also holds for suspensions. It was for this reason that Richards and his collaborators used only the inverse proportion as a first approximation and then repeated their determination with a more suitable standard, so that the final comparisons were made with solutions whose concentrations were within 10 per cent of equality, thus reducing to a negligible amount any deviations from the law of inverse proportion.

(*b*) *Kober's First Nephelometer.*—Kober's first instrument was made by slightly changing the arrangement of a regular French Duboscq colorimeter. The instrument possessed excellent optical equipment and in previous work as a colorimeter had given colorimetric results completely in harmony with Beer's law. The only essential changes made were the substitution of nephelometric cups for colorimetric cups and coating the vertical sides of the plunger with asphaltum paint. As a nephelometer the instrument was used as a constant-lower-end type, but no data are available regarding shape or distance factors.

The results of Kober and his collaborators, who studied a large number of suspensions, showed considerable deviation from the law of inverse proportion, and the amount of deviation varied somewhat with the substance used or determined. These deviations, however, were uniform and followed a smooth curve,

so that they could be expressed by an empirical dilution formula.

Wells,[22] using a later Kober instrument of a constant-upper-end type in the study of very dilute silica suspensions (50 parts per million), found that the results also deviated from the law of inverse proportion in the same direction and to about the same extent as Kober found with his colloidal suspensions. Wells also studied this silica suspension, using the instrument as a turbidimeter, measuring the absorbed light, and found that the results followed the same dilution formula as the nephelometric results had.

Whether these experiments indicate that the shape and distance factors are of minor importance is difficult to say, since here too no data are presented either to exclude them or to show their influence upon the results obtained.

(*c*) *Bloor's Nephelometer.*—Bloor's instrument is also a Duboscq colorimeter converted into a nephelometer, but of a meniscus, constant-upper-end type, using movable jackets to secure variations in the heights of illuminated columns. Bloor found deviations from the law of inverse proportion, using fat suspensions; but since his suspensions were not very stable the amount of deviation, compared to that found by other workers, is not accurately known. Recently short stationary plungers were added to the instrument to eliminate the meniscus.

(*d*) *Kleinmann's Nephelometer.*—This instrument is also of a constant-upper-end type, but variations in illuminated heights are obtained by shutters. The Kleinmann nephelometer also eliminates the meniscus by means of short tapered plungers.

Kleinmann, who has developed some excellent nephelometric suspensions and methods, finds that all of his results follow the law of inverse proportion within the error of photometric measurement. The following [23] is Kleinmann's own discussion of his results:

The light coming from a turbidity at right angles to the incident light consists of refracted light, pure light of reflection, or a mixture

[22] P. V. Wells, J. Am. Chem. Soc., **44**, 275, (1922).

[23] Private communication to the author.

of these, depending upon the size of the particles composing the suspension. The total amount of scattered light certainly depends upon the size and the number of the particles.

The total amount of scattered light, however, does not emerge from the solution, but that part of it coming from the lower layers is in part absorbed by the supernatant layers. The light emerging from the column of suspension is the result of reflection and absorption. Both of these processes are again influenced by the form of the particles and by the index of refraction of the particles and that of the solvent. Finally, since the index of refraction and absorption of each wave length of light is different, the wave length of light plays a rôle.

The development of a law which includes all these factors represents an extremely difficult and heretofore unsolved problem of physical chemistry. In spite of a large number of published works upon the optical behavior of turbid media, a complete review of which is given by P. V. Wells,[24] the relationships in detail remain unexplained.

For pure Tyndall light, Rayleigh gave the formula,

$$I = \frac{n \cdot v^2}{y^4} \cdot K \qquad (1)$$

in which I, represents the intensity of the Tyndall light, n, the number of colloidal particles, v, the volume of the particles, and y the wave length of the light. K represents a constant.

Substituting the concentration of the particles $c = n \cdot v \cdot s$, in which s is the specific gravity of the particles, the formula,

$$I = \frac{c \cdot v}{y^4 \cdot s} \cdot K \qquad (2)$$

is obtained.

According to this formula, which holds only for definite conditions of turbidity, that is, for pure Tyndall light, and for a single point of the Tyndall cone, the amount of light is directly proportional to the concentration. If the conditions required by the theoretical considerations are changed, or if the total Tyndall light is considered, no direct proportion need exist between I and c. According to the

[24] P. V. Wells, Chem. Reviews, **3**, 331 (1927); see also, J. Am. Chem. Soc., **44**, 267 (1922).

formula which P. V. Wells has devcloped, it seems highly probable that for large thicknesses of suspension and considering the total Tyndall light, the scattered light is an exponential function of c.

Wells gives the following: " The portion dI of the reflected intensity coming from the layer dx at the depth x is

$$dI = kI_o \exp. (-\alpha\ x)dx \tag{3}$$

the exponential representing the attenuation in transmission. The coefficient k is probably more nearly constant than α, because of the similarity in the conditions at each layer. Integrating (3), the reflection R of a thickness, x, or ratio of reflected and incident intensities, is thus

$$R = m(1 - T), \tag{4}$$

where $m = k/\alpha$."

Here α represents the absorption coefficient. Since T or the ratio of " transmitted to incident light " is practically equal to

$$T = \exp. (-\alpha\ x) \tag{5}$$

it follows that

$$T = k/\alpha\ [1 - \exp. (-\alpha\, x)] \tag{6}$$

This formula agrees completely with that of Lednický [25] which he developed on the basis of Lambert's law relative to pure Tyndall light.

$$I = K \cdot \frac{v}{y^4 \cdot s} \cdot \frac{c}{k} \cdot e^{-Kh}(1 - e^{-KH}) \tag{7}$$

In this formula the letters of the first factor are the same as in (2), $e^{-K} = \alpha$ the absorption coefficient, K the absorption constant (Kayser's nomenclature), H is the height of the Tyndall cone and h is the height of layer between plunger and the upper end of the Tyndall cone, in the Kleinmann nephelometer, which is practically zero. Substituting

$$\frac{K \cdot v}{y^4 \cdot s} = K_1 \tag{8}$$

and

$$K = \beta \cdot c \tag{9}$$

[25] M. U. C. Al. Lednický, Kolloid Z., **32**, 12, (1923).

which means that the absorption constant K is directly proportional to the concentration for the range of measurement. It then follows that

$$I = K_1 \cdot \frac{1}{\beta}(1 - e^{-\beta \cdot c \cdot H}) \tag{10}$$

which corresponds completely with that of Wells' formula. The formula only indicates that in accordance with Lambert's law the light absorption goes into the reflected light, and that in addition the expression K_1 in accordance with the formula, indicates that the amount of reflected light depends upon the factors relating to the turbidity.

From the formula of Lednický for Tyndall light it follows directly that Beer's law holds. As both the solutions to be compared (left and right) having different concentrations and heights give equal illumination, then it follows that,

$$K_1 \cdot \frac{1}{\beta}(1 - e^{-\beta \cdot c_l \cdot H_l}) = K_1 \cdot \frac{1}{\beta}(1 - e^{-c_r \cdot H_r}) \tag{11}$$

and therefore

$$c_l \cdot H_l = c_r \cdot H_r \tag{12}$$

This deduction assumes in accordance with formula (9) that $K = \beta \cdot c$; that is, the absorption constant is directly proportional to the concentration.

However, for nephelometry it is unnecessary to go into these theoretical considerations, as the absolute measurement of the amount of light is not involved, but only the comparison of two turbidities of the same substance so that all variables fall away. It is only important for nephelometry to determine within what limits Beer's law holds, that is, that changes of concentration and heights of suspensions involve no change of functional relationship.

(*e*) *Weinberg's Nephelometer.*—Weinberg [26] gives the results with his instrument which is a plunger, constant-upper-end type. He found, with cups of a diameter 1 mm. larger than that of the plunger, that the results deviated from the inverse proportion law. With cups of a diameter 2 mm. larger than that of the same plunger, he found the results closely fitted those required by the inverse proportionality.

[26] A. A. Weinberg, Biochem. Z., **125**, 292 (1921).

Whether this influence of the diameter of the cups will make a corresponding change in the standardization of other instruments and suspensions is not known.

Summary.—P. V. Wells [27] makes the following comment upon the results obtained by others and those of his own:

It is therefore obvious that the only feature common to all the instruments for measuring turbidity is a rough approximation to the dilution law over limited ranges. The exact theory shows that the reason for this is inherent in the optical properties of dispersions themselves, and is not due to any "imperfections" in the instruments. To be sure, each instrument has its own shape factors and its own edge effects, but even if these could be eliminated, the dilution law would still be merely an approximation.

Therefore it is readily apparent that each instrument should be standardized in reference to the cups, light, and other working conditions with each suspension used. For details on standardization see Chapter IV.

[27] Chem. Reviews, **3**, 353 (1927).

CHAPTER VI

NEPHELOMETRIC RESEARCH

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The production of insoluble compounds for the purpose of identification and analysis of one or more constituents has heretofore been the object of most analytical research work. For gravimetric analytical work these insoluble compounds were studied in regard to: (1) their solubility or insolubility, (2) purity, (3) ease and completeness of filtration, (4) the amount and the effect of washing, (5) stability and composition on drying, and (6) accuracy in weighing the compounds, because it is on the regulation and the determination of these factors that the accuracy of gravimetric analysis depends.

The production of insoluble compounds suitable for nephelometric determination involves the first two factors, (1) solubility, and (2) purity or at least uniformity of composition. Since in nephelometry the mass of the insoluble compound is determined in the solution directly, considerations of filtration, washing, drying and weighing of the insoluble compound are eliminated. In nephelometry, however, other factors require attention and it is the object in this chapter to discuss in a general way the factors that are inherent in nephelometry, although some of them have their application in colorimetry too.

To those who have not used nephelometry for analytical purposes, and possibly a few whose experience has been limited to one or two crude nephelometric procedures, the question may arise whether or not nephelometry, after all, can be used with sufficient accuracy to place it alongside of accepted gravimetric and

volumetric procedures. There is no doubt now that quite a number of nephelometric procedures yield an accuracy comparing favorably with the older analytical methods. It is true that some nephelometric procedures are still somewhat crude, because the methods have not been carefully worked out or because some factors relating to these particular procedures remain hidden. Yet it is unsafe to draw general conclusions from one or two isolated instances.

We have been so accustomed to making precipitates to agglutinate and to settle out, that the reverse, the production of a stable suspension, which is a necessary condition for nephelometry, seems *a priori*, and at times difficult. An example will make this clear.

Nephelometry had its start, as is well known, through Richards in 1894, with the estimation of silver chloride precipitates that escaped with the filtrates, in atomic weight work. Richards and many workers subsequently for a period of twenty years studied the nephelometric behavior of silver chloride, and practically all came to the conclusion that nephelometry had a very low accuracy, believing that the difficulties in producing suitable silver chloride suspensions were inherent with suspensions generally.

After Kober had shown, in 1912 and in the following years, that for many insoluble compounds these difficulties did not exist and that nephelometry could be practiced with satisfactory accuracy, Lamb, Carleton and Meldrum (1918), of the U. S. Chemical Warfare Service, showed how silver chloride suspensions could be made accurately and uniformly. It was after all only a question of temperature and a suitable medium.

GENERAL CONDITIONS FOR PRODUCTION OF NEPHELOMETRIC SUSPENSIONS

Our knowledge regarding suspensions is still very meager. Much work remains to be done regarding their production and their behavior, especially from a theoretical point of view. Certain facts, however, have been gathered from the experience of those who have worked in this field.

Properties of Nephelometric Suspensions.—Compounds which have an appreciable solubility in the media in which they are produced are not suitable for nephelometric work. Nephelometric suspensions have thus far been produced in very dilute solutions, and a compound with an appreciable solubility, which may not be a large factor in ordinary gravimetric conditions, will, in the dilutions required for nephelometry, either not precipitate at all or only very slightly. Of course, the medium in which the reaction takes place may in some cases be changed so as to make the compound less soluble and nephelometrically suitable.

Therefore the *first requirement* of a compound for nephelometric determination must be great insolubility, or in other words the reaction producing the insoluble compound must be highly sensitive.

The *second requirement* is that of color, or rather the absence of color. Colored suspensions can be determined too, but as a rule they are measured by means of the absorbed light, that is, colorimetrically, especially if the absorption of light is at all marked.

The exact chemical composition of the suspension in many cases is not known, but this, although desirable and interesting, is not an essential matter, and does not enter into consideration in making a determination. Good nephelometric suspensions have been produced with almost all classes of chemical compounds: inorganic, organic, fat, oil, carbohydrate and protein suspensions have been produced. Therefore it is impossible to predict or limit the nephelometric behavior of any compound.

Thus far the term nephelometric suspension has been used rather freely and therefore before proceeding with the conditions governing its production it is well to define the term. By nephelometric suspensions are meant very insoluble suspensions produced in dilute solutions (100 mg. per liter or less), which show the same and constant amount of light on reflection, for a period sufficiently long to place them in a nephelometer and make the necessary number of settings with the instrument. This period varies with the substance; some suspensions show a change in the amount of light within ten to thirty minutes, others only after

one or two days. The change in the amount of light referred to is usually a decrease in the amount of light and is caused by the growth in size of the particles in suspension, until agglutination sets in, so that the particles become macroscopically visible and settle out. Before this change takes place, they are to the naked eye homogeneous and visible only with the microscope or ultramicroscope. In most cases this change amounts to only 2 to 3 per cent of the light reflected before. To the observer this change is apparent by a sudden jump in the readings which before that time were uniformly constant.

This change in the amount of light, or the breaking down of the suspension, does not affect the accuracy of well-developed nephelometric procedures, as the readings are easily made before this phenomenon occurs. Furthermore, the change can be delayed to any desired time by the use of a protective colloid, which subject is discussed on pages 72 and 76.

The *third requirement* for nephelometric suspensions is that the precipitation must be undertaken, as stated before, in a dilute solution. For most substances the concentration is 100 mg. per liter, some even requiring a concentration considerably less than this.

At first thought one would expect that nephelometric suspensions are obtainable only in one class of substances, namely, colloids, but this is far from the truth. Harry Jones [1] in his book, published before the development of nephelometric methods, states that "precipitation" (meaning settling out of solution) "is not the natural condition in chemistry" but that it "*is one of the most important phenomena in all chemistry*. We are so familiar with precipitation from our analytical days that we are accustomed to look upon it as the natural condition when a solid is formed as a result of a reaction between two solutions. We see from above [meaning some previous discussion] that such is not at all the case. A moment's thought will show why this is true. When substances react, they react, we believe, molecule for molecule. The solid when first formed either has molecular dimensions or there are only a few molecules of the solid aggre-

[1] "New Era of Chemistry," D. Van Nostrand Co., New York, 1913.

gated." He then concludes that "*the colloidal solution or at most the colloidal suspension is the natural condition of solid matter when first formed as the result of a reaction.*"

In ordinary gravimetric precipitation the concentration is so great and the conditions are made such that the precipitate is in the form of a suspension only for a short period of time, so short that in most cases it escapes observation. But as stated before this period is greatly prolonged by dilution, by the character of the solvent, and by the presence of protective colloids or other stabilizers. The widespread application of nephelometry to so many different types of substances fully affirms Harry Jones' statement that the colloidal suspension is "the natural condition of solid matter when first formed as the result of a reaction."

Nephelometric Precipitants.—Precipitants for the production of nephelometric suspensions are as varied as the substances precipitated: inorganic and organic, of both synthetic and biological origin. Nephelometric precipitants possess four great advantages over gravimetric reagents:

(1) Since in many cases the amount used for a determination is very small, the cost of a reagent, even though the cost of the material may be appreciable, is very small and seldom a factor.

(2) The nature and composition of the precipitant and precipitate, as stated before, need not be known and do not affect the accuracy or the usefulness of a procedure.

(3) Since the reaction is carried on in a very dilute solution, a more complete control over the media of precipitation is obtained. On account of the great dilution necessary in nephelometry, color and other interfering substances of a sample are so reduced in concentration that they seldom play a rôle.

(4) Nephelometric precipitants are and can be found in many instances among organic substances. In gravimetric analysis this would not be an advantage, since it is necessary as far as possible to have the precipitate in relatively large masses for filtering and in a condition easy to dry. In many cases the occlusion and adsorption in gravimetric precipitates are very appreciable. In nephelometry, however, occlusion and adsorption are of no consequence, inasmuch as the standard, having practically the same

occlusion or adsorption, would cancel the error. Of course, it does not follow that occlusion or adsorption in nephelometric precipitates causes any difference in the amount of reflected light, as the occlusion may be in a dissolved phase, and would not reflect light. Furthermore, since organic reactions as a rule are more specific and therefore more accurate, nephelometry and colorimetry enable us to make use of organic substances; this means that our sources of reagents and possible methods are almost unlimited.

Medium of Precipitation.—Although most nephelometric determinations have been developed by using aqueous solutions, nephelometry is by no means confined to aqueous solvents. Organic solvents, either alone or in combination with other solvents, can be used when they are suited to the production of nephelometric suspensions, since the amount of solvent occluded or combined as a liquid phase with the precipitate does not as a rule interfere with the accuracy of the determination. For example, a medium of 50 per cent ethanol in water was found a very suitable medium for the production of silver chloride suspensions.

A study of the most suitable hydrogen-ion concentration is often necessary as it has been found to influence the speed of reaction, solubility, and stability of suspensions. In short, any medium that is colorless, clear, and of the proper hydrogen-ion concentration, may be used.

Nephelometric Dilution Flasks.—Standard and unknown solutions requiring dilution involve the use of volumetric flasks. When the highest accuracy is not required, the substance may be weighed in very small amount and dissolved and diluted to the desired volume in one flask—a 500- or a 1000-cc. flask. If large amounts are weighed off, the material may be dissolved and diluted first in a 50- or 100-cc. flask, and then by means of an accurately calibrated burette or pipette, a portion may be taken and diluted further in a 500- or a 1000-cc. flask.

Protective Colloids or Stabilizers.—Protective colloids or substances acting like protective colloids are a source of annoyance, delay, error, and occasionally a complete hindrance to ordinary

analytical procedures. In nephelometry they are an aid to accuracy. Just how a colloid or stabilizer exerts its protective action is not known. Several theories have been proposed but none of them seem as yet to account for all the facts. The envelope theory which assumes a sort of mechanical buffer effect does not explain why one colloid protects suspensions and another colloid does not. On the other hand, the theory that the colloids exert their protective action through union with the suspension by means of secondary valences requires elaboration to explain the many anomalies encountered. All stabilizers seem to have either hydroxy or nitrogen groups or both.

The precise rôle of the stabilizer, the effect of dilution, and the effect of a change of hydrogen-ion concentration upon nephelometric suspensions are subjects for further research.

For a discussion of colloids and colorimetric stabilizers see Chapter VI in Volume I.

DEVELOPMENT OF NEPHELOMETRIC PROCEDURES

While detailed directions for the development of nephelometric procedures covering many different substances cannot be given empirically, nevertheless a discussion of the methods used heretofore will serve as a guide and may indicate the avenue of approach to not a few new procedures.

Search for Precipitants.—Precipitants used in gravimetric or even in colorimetric analytical work can be quite often adapted for the production of nephelometric suspensions. In a few cases the amount and concentration of the precipitant used in previous work needs merely to be reduced considerably; or in addition, a suitable protective colloid or stabilizer may be necessary. In other applications, the chemical constitution of the precipitant can be varied; exchanging one or more of the constituents for some substitute may produce, in place of a precipitant that yielded only a quickly settling precipitate, a more suitable solution that tends to remain in suspension.

Many precipitants can undoubtedly be found among reagents usually listed as qualitative and heretofore used as aids in identi-

fication of a compound, although their reaction products did not and could not become a gravimetric procedure either owing to their unknown constitution, their colloidal condition, or instability or variation on isolation, drying, and weighing.

In addition to the above-mentioned possible nephelometric precipitants, a large number are found among the synthetic and natural compounds of organic and inorganic derivatives that are available in the market and in the laboratory but have as yet not found use in analytical chemistry.

Testing Out of Possible Nephelometric Precipitants.—A quick test to see whether a substance can be used for the production of a nephelometric suspension with a given compound consists in adding it in varying concentrations to very dilute solutions of the compound to be precipitated. The compound may be in a dilution of 1 part in 100,000 to 1 part in 1,000,000. If no marked cloud is obtained under these conditions and especially after varying the medium by using another solvent or a mixture of solvents for the compound, then the reagent as used is not suitable for nephelometric work. It may be possible however to obtain a more suitable reagent by some change in its composition, if there are indications that this might improve matters.

After it has been shown that a precipitant is very sensitive with respect to the compound it precipitates, then further study with the aid of a nephelometer is necessary in order to determine quantitatively its behavior under such conditions as are likely to be met in actual analytical work. Prior to the days when nephelometry had been developed into an accurate analytical procedure, criticism against nephelometry was based upon the assumption that where there were variations in the amount of reflected light as a result of adding a precipitant to a dissolved substance, it was due to variations in the size or light reflecting properties of the particles; that is, the precipitate or insoluble substance must be there regardless of its light-reflecting properties. *On the basis of nephelometric measurements covering many compounds of the greatest variety it may now be concluded that where there are marked changes in the amount of light reflected (except a few per cent due to the agglutination referred to above) these changes*

are due to variations in the amount of substance precipitated out of solution.

The study of the efficacy of nephelometric precipitants can be accomplished with the aid of a nephelometer in a number of ways. In essence these methods involve the same principle, namely, whether the amount of light reflected by the precipitate over a range of dilutions suitable for actual analytical work is the same or not. In terms of the instrument it means whether or not the readings of the instrument with precipitates made over a range of dilutions follow a dilution law and curve.

The most convenient method of making a dilution curve is described on p. 46 and need not be elaborated here. Kober and his collaborators used the dilution curves as a means of determining whether the precipitation was complete or not. By taking a certain strength of solution as a standard, and precipitating, they compared the amount of light reflected by it to a half-standard solution similarly precipitated. By use of their dilution formula

$$Y = \frac{S}{X} - \frac{(1 - X)SK}{X^2},$$

they drew a curve or calculated the constant K, which applied to that particular suspension, the instrument they used, and conditions under which they were working. Then they took a standard solution and precipitated it, first, after which they diluted it with the reagent solution to one-half standard value, and redetermined the curve or constant K. This last curve or constant they designated as the theoretical curve or constant, on the assumption that the standard and half-standard in this case contain the same proportion of precipitated and dissolved compound. If the actual curve or constant was close to or identical with that of the theoretical, they assumed that the precipitation was practically complete. They were aware that the suspension in the weaker solution might redissolve or hydrolyze slightly, until the same equilibrium was reached as in the one precipitated after dilution, but realized that the speed of solution near the saturation point and the speed of hydrolysis near the equilibrium point are

extremely slow, as is well known, and that any error due to this was considered negligible.

With the method of using the instrument and making a curve described (see Chapter IV) it is not necessary actually to draw a curve each time or to determine the nephelometric constant. Since the readings of the instrument have always the same values when the unknown solutions are kept at a constant height, the deviations from the theoretical will be directly proportional to the differences in the heights of the standard with that of the one-half standard, precipitated before and after diluting, respectively.

Another method of studying the behavior of nephelometric suspensions is the employment of a fixed standard suspension such as that of silica whose behavior is known, or the use of etched or ground-glass reflectors, of one shape or another, put in one side of the instrument as a standard and constant source of reflected light. These devices do not always have the same tint as the light reflected by the suspensions and although this may be overcome by suitable filters or by the use of color in the reflectors, yet the results achieved by their use are no better than the method used by Kober just described.

STUDY OF NEPHELOMETRIC PROTECTIVE COLLOIDS OR STABILIZERS

The term "protective colloid" used here is employed in a broad sense and means any soluble substance added to the solvent that acts like a protective colloid by aiding to keep the suspension from agglutinating. Some of the protective colloids found useful are solutions of soluble starch, egg albumen, serum albumen, or gelatine; and such substances as ammonium nitrate and triolein have been used as stabilizers. Owing to our lack of knowledge concerning their action, however, no principle concerning them can be deduced that will be helpful in finding new colloids for other procedures. This search must remain as yet empirical, a hit or miss affair.

The study of the efficacy, however, is, as a rule, well defined and comprises: (1) Testing the effect of the solvent and precipi-

tant upon the stabilizer, and (2) testing the effect of the stabilizer upon the suspension.

If it has been found that a newly developed precipitant produces a suspension that is very sensitive but is not stable long enough to permit leisurely settings of the instrument, the problem is to add the smallest amount of stabilizer that will suffice to delay the agglutination for an adequate period without disturbing the sensitivity of the suspension.

Believing that a concrete example of the testing out of a precipitant and the adaptation of a stabilizer will be more useful than general suggestions and directions, the following research by Graves[2] on a precipitant for ammonia will serve as a good example of well-developed procedure.

REAGENT

Mercuric Chloride Complexes.—When a solution of mercuric chloride is made alkaline, immediately the yellow or red oxide forms, but it was found that by first adding sodium chloride a complex was formed which was stable except in the presence of a large excess of alkali. As will be seen from the following equation, two molecules of sodium chloride unite with one of mercuric chloride:

$$2\ NaCl + HgCl_2 \rightleftarrows Na_2HgCl_4$$

This complex is readily soluble in water and while it has an appreciable dissociation into $HgCl_2$ and NaCl,[3] with large amounts of sodium chloride the dissociation is practically zero. It has the further advantage that chlorine compounds are more stable than those of the other halogens. Thus, the ammonium complexes of mercuric chloride are more stable than those of the bromide or iodide. The neutral iodine complex has a noticeable vapor tension of ammonia, which the analogous complex of chlorine has not.

[2] J. Am. Chem. Soc., **37**, 1171 (1915).

[3] T. W. Richards and E. H. Archibald, Z. Physik. Chem., **40**, 385 (1902); M. Le Blanc and A. A. Noyes, *ibid.*, **6**, 393 (1890).

Components of Reagent.—The reagent used consisted of mercuric chloride, sodium chloride, and lithium carbonate. The last was chosen because of the low atomic weight of the cation and, therefore, but slight tendency to cause agglutination of suspensoids. Each component of the reagent was varied, in order to ascertain the most favorable conditions for the quantitative precipitation of ammonia.

Fifty cubic centimeters of a cold saturated solution of mercuric chloride and an equal quantity of water were chosen arbitrarily and the sodium chloride and lithium carbonate varied to the points at which mercuric oxide formed—with the results given in Table II.

TABLE II

PRECIPITATION POINTS OF MERCURIC OXIDE

Saturated Solution of $HgCl_2$, Cc.	H_2O, Cc.	NaCl, Grams	Saturated Li_2CO_3 Solution, Cc.
50	50	5	5
50	50	10	30
50	50	15	50 plus
50	50	20	50 plus

Fifty cubic centimeters of mercuric chloride solution, 15 g. of sodium chloride, 35 cc. of saturated lithium carbonate solution, and 65 cc. of water were found to produce a satisfactory reagent. Experiments showed that it is fully as sensitive as Nessler's reagent, and it seemed likely that, owing to the sensitiveness of nephelometry, very small amounts of ammonia could be determined quantitatively more easily as a cloud than as a very faint color. The reagent precipitates ammonia from ammonium sulfate solutions as dilute as 1 part per million, producing a bluish white cloud which agglutinates in stronger solutions after a few minutes.

Before studying the reagent further it was necessary to find a protective colloid, to keep the precipitate in suspension long

enough to make nephelometric readings (10–30 minutes). Soluble starch, when very dilute, proved satisfactory.

Two sets of experiments were accordingly carried out, using 10 cc. of ammonium sulfate solution (10.0 mg. per liter), 15 cc. of starch solution (5.0 mg. per liter) and 5 cc. of reagent, in which the mercuric chloride and lithium carbonate were varied. The clouds formed were compared with a fixed standard, in the nephelometer.

(1) **Mercuric Chloride.**—The mercuric chloride was varied from 25 to 75 cc. of saturated solution per 150 cc. of reagent. There was no appreciable change in the readings of the different solutions tested.

(2) **Lithium Carbonate.**—The amount of saturated solution of lithium carbonate (about 1 per cent) was varied from 12.5 to 75 cc. Sixty-five to seventy-five cubic centimeters produced mercuric oxide on standing. Solutions containing less than 65 cc. showed no marked difference in nephelometric readings.

Protective Colloid.—The effect of starch upon salt was studied in two connections: (1) When the reagent contained varying amounts of sodium chloride; (2) when the ammonia solutions contained varying amounts of potassium sulfate, thus allowing for any reasonable variation in the amount of ammonia as compared to salt found in micro-analysis.

(1) To 150 cc. of reagent, which already contained 15 grams of sodium chloride, more sodium chloride was added in varying amounts of 10, 20, 25, and 35 grams, respectively. It was found that the solutions containing 20, 25, and 35 grams additional salt all allowed the use of a fairly large amount of starch. Therefore, 25 grams was decided upon, making a total of 40 grams of sodium chloride in 150 cc. of reagent. Later experiments show the effect of sodium chloride upon the constants of the reagent.

(2) The ammonium sulfate solutions were of three types: *A*, pure; *B*, with the amount of potassium sulfate found in normal Kjeldahl work; *C*, with four times the amount of salt found in normal Kjeldahl work.

Solution A contained 1.0 mg. of ammonium sulfate in 100 cc. or 2.57 mg. of ammonia per liter.

Solution B contained 1.0 mg. of ammonium sulfate, and 10 cc. of potassium sulfate solution (75 grams per liter), in 100 cc.

Solution C contained 1.0 mg. of ammonium sulfate, and 40 cc. of potassium sulfate solution (75 grams per liter), in 100 cc.

To solutions A_1, B_1, and C_1 (10 cc.) were added various amounts of starch, always diluting to 25 cc. at this point, and the time was determined from the moment the reagent (5 cc.) was added, until agglutinations were visible to the naked eye. At the same time it was observed that the nephelometric readings became slightly higher. The results obtained are seen in Table III, the minutes indicating the time during which the solutions are constant and suitable for nephelometric work, and the concentration of starch indicating the strength of the final solutions.

TABLE III

TIME OF AGGLUTINATION

Concentration Starch, Mg. per Liter	A_1, Min.	B_1, Min.	C_1, Min.
0	14	11	5
2.5	27	15	6
5	37	25	10
10	48	40	14
15	60	60	20
20	. . . . .	. . . . .	28
25	. . . .	. . . . .	32
40	. . .	. . . .	50
50	. . . .		60

It may be seen that the suspensions are fairly stable even without starch.

Completeness of Precipitation.—It was necessary to determine whether or not the completeness of precipitation was affected by the colloid.

To do this, solutions A_2, B_2, and C_2 were prepared containing 0.500 mg. of ammonium sulfate instead of 1 mg. as in A_1, B_1, and

C_1. To 10 cc. of solutions A_2, B_2 and C_2 were added definite amounts of starch, diluting at this point to 25 cc. and finally 5 cc. of reagent were added with shaking, and the solutions compared with solutions A_1, B_1, and C_1, respectively, in the nephelometer. From the results the nephelometric constants (K) were calculated, using the formula[4]

$$Y = \frac{S}{X} - \frac{(1 - X)SK}{X^2},$$

where Y = the reading of A_2;
S = the reading of A_1;
X = the ratio of A_1 to A_2 or 0.500.

Using the reagent containing 40 g. of sodium chloride per 150 cc., constants were obtained as given in Table IV.

TABLE IV

EFFECT OF STARCH ON NEPHELOMETRIC CONSTANTS

Concentration of Starch, Mg. per Liter	A/K	B/K	C/K
50	0 033	0 140	0 138
35	0.075	0 135	0 132
25	0.130	0.136	0 136
15	0.133	0 138	0 136
5	0 140	0 132	0 133

The above results show that in starch solutions containing 35 and 50 mg. per liter the precipitation of the pure solutions A is retarded, but that in solutions containing less than 35 grams per liter the precipitation of the three solutions is apparently complete.

Table V shows the effect of a reagent containing only 15 grams of sodium chloride per 150 cc. under similar conditions.

[4] For details as to nephelometric instrument, formula and manipulations, see Kober, J. Biol. Chem., **13**, 490 (1913).

TABLE V (Continuation of IV)

Concentration of Starch Mg. per Liter	A/K	B/K	C/K
15.0	Negative	Negative	0 077
5.0	0.090	0.180	0.173
2.5	0.164	0.154	0.166

Here it is evident that the precipitation is delayed in a starch solution containing 15 mg. per liter. The slightly higher constants with this reagent might be explained by the following equation:

$$2Na^+ + 2Cl^- + HgCl_2 \rightleftarrows HgCl_4^{--} + 2Na^+$$
$$+$$
$$NH_4^+$$
$$\Updownarrow$$
$$\text{Complex}$$

According to the Law of Mass Action the increased amount of sodium chloride would have a tendency to shift the equilibrium from the ammonium complex formation and therefore the reagent containing less sodium chloride might give nearer complete precipitation and thus slightly higher nephelometric constants.

RESULTS WITH REAGENT

To test the precipitability of ammonia by the modified mercuric chloride reagent, three sets of ammonium sulfate solutions A, B, and C were made, as previously described, in which the amount of ammonium sulfate was varied from 10 to 5 mg. per liter.

Starch was added, the ammonia precipitated by the reagent, and the solution compared with a known standard in the nephelometer. From the readings (usually two) the accompanying curves were plotted. They are compared with the hypothetical

or colorimetric curves.[5] Curve in Fig. 24, with solutions *A*; curve in Fig. 25 with solutions *B*; curve in Fig. 26, with solutions *C*.

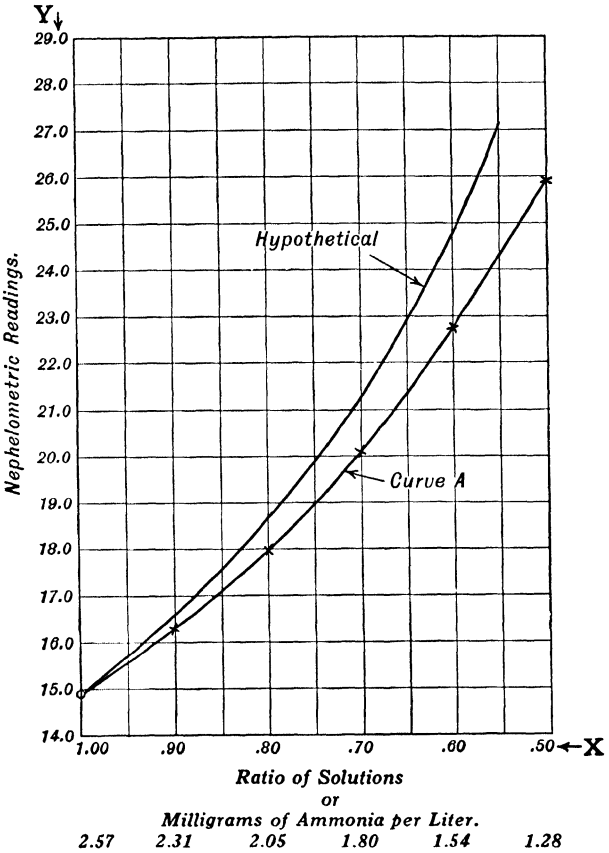


FIG. 24.

Solutions *A* contained 10 cc. of ammonium sulfate solutions containing 100, 90, 80, 70, 60, 50 mg. per liter, respectively, and each made up to 100 cc. with ammonia-free water.

Solutions *B* contained ammonium sulfate in the same amounts as solutions *A*, and in addition 10 cc. of potassium sulfate solution (75 grams per liter); the volume was then made up to 100 cc. with ammonia-free water.

[5] The hypothetical or colorimetric curve is obtained by assuming the readings to be inversely proportional to the concentration of the substance.

TABLE VI

NEPHELOMETRIC READINGS

$(NH_4)_2SO_4$, Mg. per Liter	Solutions *A*		Solutions *B*		Solutions *C*	
	Readings, Mm.	Constant, *K*	Readings, Mm.	Constant, *K*	Readings, Mm.	Constant, *K*
10 0	14 90	..	14 90	..	14 90	....
9 0	16 30	0 140	16 30	0 140	16.30	0 140
8 0	17 95	0 141	17 90	0 152	17.93	0 146
7 0	20 05	0 140	20 06	0.139	20 00	0.145
6 0	22 75	0 131	22 80	0 128	22 77	0 136
5 0	25 80	0 134	25 85	0 132	25 85	0 132
	Average,	0 137		0 138		0 140

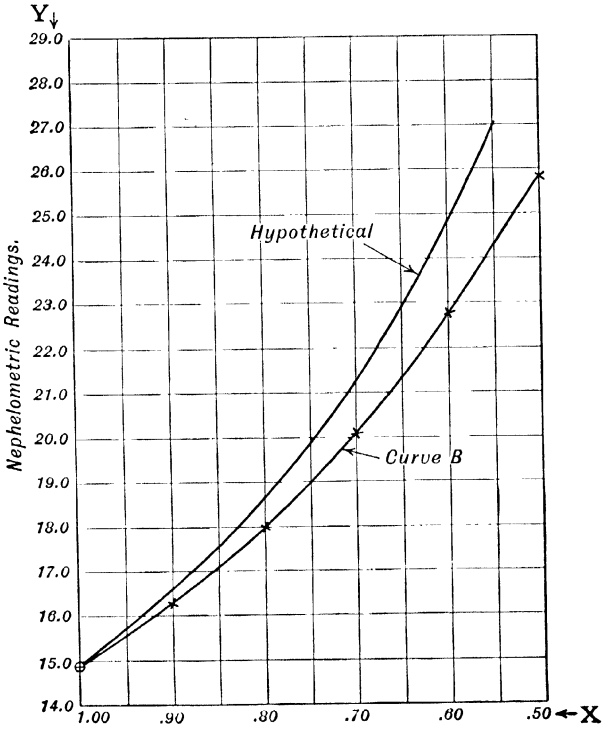


FIG. 25.

Solutions *C* were like solutions *B* except that they contained 40 cc. instead of 10 cc. of potassium sulfate solution (75 g. per liter).

To 10 cc. of solution were added 15 cc. of starch solution (30 mg. per liter) and finally, while shaking in a rotary fashion,

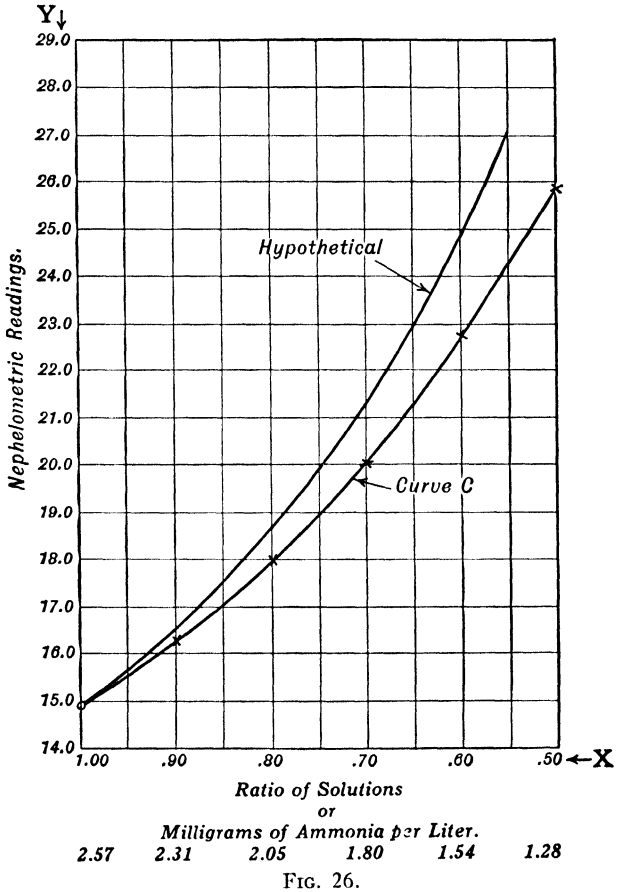


FIG. 26.

5 cc. of reagent were run in from a pipette. The strongest solution (10 mg. per liter) of a series was used as a standard and the others of a series compared with it in succession. Each reading in Table VI is the average of two or three readings.

From these results it seems probable that the precipitation is nearly complete, since:

(1) The curves are well below the hypothetical.
(2) The readings are consistent.
(3) The nephelometric constants are in close agreement.

DIRECTIONS

A. Solutions: Ammonia-free Water.—Ammonia-free water must be used in making up all solutions which enter into the standard solution, just as with the Nessler standard. The usual recommendation is water which contains not more than 0.005 mg. of ammonia in 100 cc. This may be easily obtained by distilling tap water which has been made slightly acid with sulfuric acid.

Reagent.—To 80 grams of sodium chloride are added 130 cc. of water and 100 cc. of a cold saturated solution of mercuric chloride, with shaking. When the salt is practically all dissolved, 70 cc. of a saturated solution of lithium carbonate (1 per cent) is added slowly while shaking, so that no mercuric oxide forms on the sides of the flask. The solution is usually cloudy, owing to the ammonia in the reagents, but if well shaken with talcum powder (3–5 grams) it is easily filtered clear. The solution may be used at once or if carefully stoppered may be kept almost any length of time.[6] Filtering the reagent does away with errors due to impurities in its constituents.

Protective Colloid.—A stock solution of starch is freshly made each day by boiling 1 gram of starch in ammonia-free water until clear and making up to 100 cc. with ammonia-free water.

Standard Solution.—Ammonium sulfate prepared and purified according to Folin's[7] directions is used for the standard. The standard solution is made by taking 10 cc. of an ammonium sulfate solution (100 mg. per liter), adding 10 cc. of potassium sulfate solution (75 grams per liter) and making up to 100 cc. in a volumetric flask with ammonia-free water. The amount chosen for a standard cloud is usually 10 cc. of this solution, ammonium sulfate (10 mg. per liter) to which is added 15 cc. of 0.003 per cent starch and 5 cc. of reagent. Half or even one-fourth of the above amount is ample, the object in choosing a fairly strong standard being to

[6] Solutions have been kept several weeks without noticeable change.
[7] *Loc cit.*

lessen the percentage of error due to traces of ammonia in dust or water. In all experiments controls should be made on the water as well as on the reagents.

Potassium sulfate is added to the standard solutions in order to reproduce as far as possible the conditions found in all Kjeldahl work. Preceding data show that large or small amounts of salt do not affect the precipitation of ammonia with the reagents as given.

B. KJELDAHL NITROGEN ESTIMATION

Catalyst.—It is necessary to use mercuric oxide instead of cupric sulfate as a catalytic agent, owing to the blue color developed by copper in alkaline solution.

Dilution.—After the usual heating, the solutions containing the substance are diluted to 500 cc. and the blanks to 250 cc. These solutions may be designated as solutions A_x and A_y.

Neutralization.—Five cubic centimeters or more of solution A_x and 50 cc. or more of solution A_y are neutralized with sodium hydroxide, using litmus paper as indicator, and each is made up to 100 cc. in a volumetric flask with ammonia-free water. Designation: solutions B_x and B_y.

To 5 cc. of solution A_x is added about 5 cc. 1 N sodium hydroxide and then 1 N sodium hydroxide drop by drop to the neutral point. To 50 cc. of solution A_y is added about 30 cc. of 15 per cent NaOH and then 1 N NaOH drop by drop to the neutral point.

Care must be taken not to add a considerable excess of sodium hydroxide or else mercuric oxide will form upon addition of the reagent. The small amount of ammonia in the control can be readily estimated, if the control solution is not more than one-twentieth as dilute as the solution containing the substance. If the solutions B are cloudy because of the precipitation of alkaline earths, etc., they should be filtered through a dry filter after making up to volume.

Precipitation.—To 10 cc. of solution B are added 16 cc. of starch solution (30 mg. per liter) and 5 cc. of reagent with shaking, and the cloud produced compared with a standard ammonium sulfate cloud by means of the nephelometer.

Calculation of Results.—From the readings of the standard solution and the unknown, by means of the formula

$$Y = \frac{S}{X} - \frac{(1 - X)SK}{X^2}$$

the ratio of the concentrations of the solutions can be found and the amount of ammonia or nitrogen in the substance taken for analysis readily calculated, subtracting the amount of ammonia in the control. A more convenient form of the equation when solving for X is

$$X = \frac{S + SK + \sqrt{(S + SK)^2 - 4SKY}}{2Y}$$

in which $(S + SK)$ is constant throughout any series of determinations. For this reagent K is about 0.138.

Applications

The preceding method of making nitrogen estimations was tried on ammonium sulfate alone, and on ammonium sulfate with organic matter (filter paper), as well as on uric acid and urine, with satisfactory results—which attest to the sensitivity of the reagent and its ability to precipitate ammonia quantitatively.

(1) First 0.1001 gram of ammonium sulfate was digested with 5 grams of potassium sulfate and 20 cc. of sulfuric acid in the usual way. Duplicate estimations as well as duplicate controls were made. The solutions were diluted directly *without the usual distillation*, and the ammonia was determined according to the preceding directions, the standard solution containing 0.1001 gram of ammonium sulfate.

Solution	Y	S	X	Nitrogen Found, G.	Percentage Nitrogen Found	Percentage Nitrogen Theoretical
I.....	13.75	13.96	1 016	0.02150	21.24	21.15
II.....	13.72	13.96	1.018	0.02155	21.29	21.15
Control [8].	25.43	$^1/_2S$ 13.96	0.483	0.00024	.....	...

[8] See solutions under *neutralization* marked A_y and B_y, $^1/_2 S$ indicates one-half as strong as standard solution (which see).

(2) The above experiment was repeated with the difference that one sheet of purest filter paper (7 cm.) was digested in each flask in lieu of organic matter.

Solution	*Y*	*S*	*X*	Nitrogen Found, G.	Percentage Nitrogen Found	Percentage Nitrogen Theoretical
I.....	13.82	14 00	1.014	0 02145	21.18	21.15
II... ..	13.8C	14.00	1.015	0.02150	21.23	21.15
Control [8].	24.00	$\frac{1}{2}S$ 14.00	0.503	0.00025		

(3) Then 0.100 gram uric acid was digested in each of four experiments with 10 grams of potassium sulfate and 20 cc. of concentrated sulfuric acid in the usual way. Two controls were made at the same time.

Two of the uric acid digestions and one control were distilled by the aeration method[9] and the ammonia was estimated by titrating the distillate. The other two digestions and a control were treated as in Experiments (1) and (2).

PERCENTAGE OF NITROGEN IN URIC ACID

Number	Found by Aeration Method	Found by New Method	Theoretical
I.....	32.62	32.90	33.33
II.....	32.60	32 95	33.33

No attempt was made to purify or dry the substance, which may well have contained 1 per cent of moisture.

(4) Experiment (3) was repeated, replacing the uric acid by 5 cc. of urine, with the following results (urine was diluted with an equal volume of 0.2 per cent tricresol):

[9] P. A. Kober and S. S. Graves, J. Am. Chem. Soc., **35**, 1594 (1913)

PERCENTAGE OF NITROGEN IN URINE

Number	Found by Aeration Method	Found by New Method
I.....	0.960	0.952
II.....	0.976	0.948

By using the method of making a calibration curve described on p. 46 the Kober nephelometer formula,

$$Y = \frac{S}{X} - \frac{(1 - X)SK}{X^2},$$

becomes

$$S = \frac{Y}{X} - \frac{(1 - X)YK}{X^2}.$$

Using the constants, K, obtained by Graves for the points on the curves A, B, and C, taking the weakest solution ("one-half standard") as a standard of comparison, and setting the standard as "unknown" at a fixed height of 15.00 mm. (Graves used 14.90 mm.) the data and curves (lines) of Table VII are obtained. See Figs. 27, 28 and 29.

TABLE VII

Ratio X	Curve A		Curve B		Curve C		Curve D	
	K	Y	K	Y	K	Y	K	Y
		Mm.		Mm.		Mm.		Mm.
2.00	0.134	28.11	0.132	28.14	0.132	28.14	0.260	26.55
1.80	.131	25.52	.128	25.54	.136	25.47	.260	24 20
1.60	.140	22.80	.139	22 81	145	22.76	.260	21 87
1.40	.141	20.19	.152	20.12	.146	20.15	.260	19.54
1.20	.140	17.57	.140	17 58	.140	17 58	.260	17.33
1.00	.....	15.00	.....	15.00	.....	15.00	...	15 00

As will be observed, Graves' points fall fairly close to a straight line, which lies below the hypothetical, that obtained by the law of proportionality. This method of calibrating a suspension and of determining the completeness of precipitation is decidedly simpler than that used by Kober, Graves and others. The curve marked "theoretical" (D) having a constant of 0.260 was obtained by diluting the solutions after precipitation as described and discussed earlier in this chapter, and shows the effect of dilu-

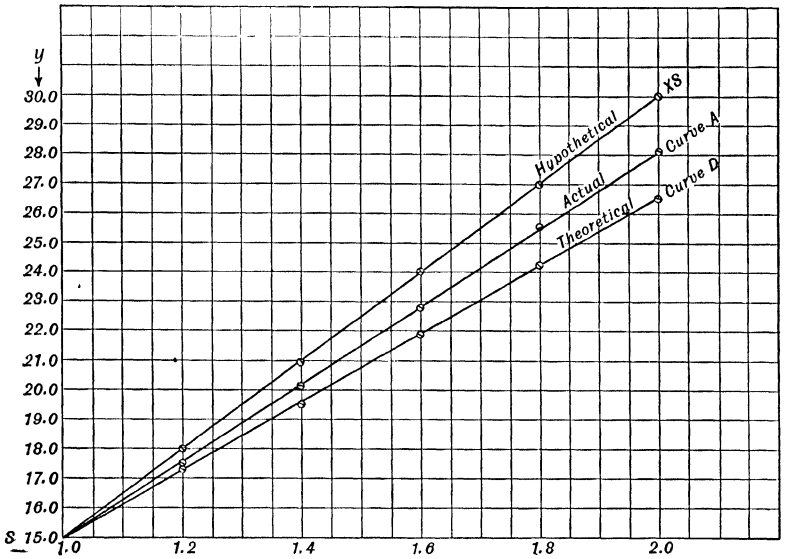


FIG. 27.

tion upon the completeness of precipitation, due either to solubility, hydrolysis, or the shifting of the equilibrium of the reaction described on p. 82. By further adjustment of the constituents of the reagent or by modifying the solvent, say by the addition of alcohol or some other water miscible solvent, this effect of dilution might possibly be decreased until the actual curve coincided with or fell closer to the theoretical curve.

With the fixed height of the "unknown," S, at 15.00 mm. and 20.00 mm., respectively, and varying heights of the standard, Y, and at a concentration ratio X of 2.00, the following heights of Y solution and deviations ($SX - Y$) from the hypothetical

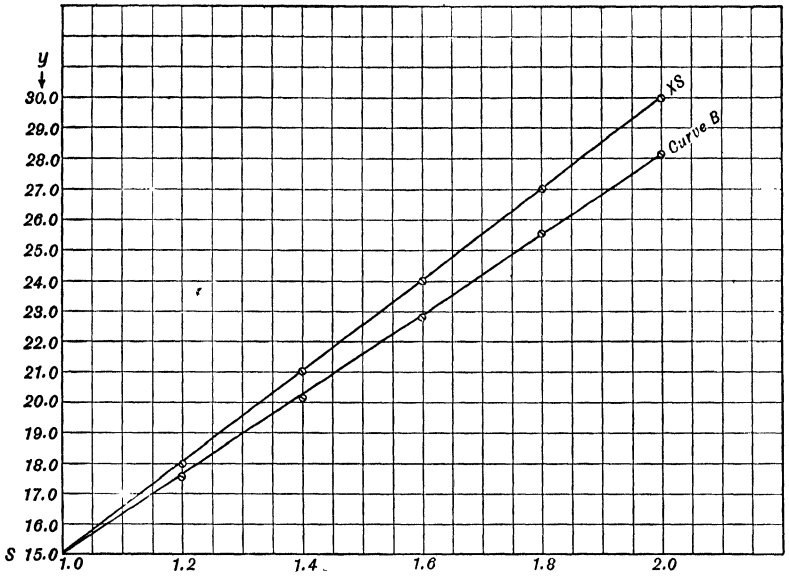

y
30.0
29.0
28.0
27.0
26.0
25.0
24.0
23.0
22.0
21.0
20.0
19.0
18.0
17.0
16.0
S 15.0
1.0
1.2
1.4
1.6
1.8
2.0
XS
Curve B

FIG. 28.

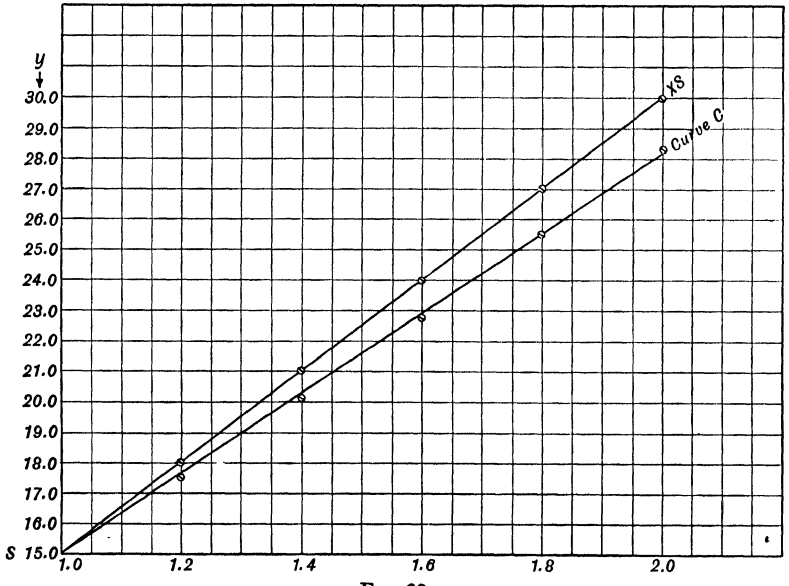

y
30.0
29.0
28.0
27.0
26.0
25.0
24.0
23.0
22.0
21.0
20.0
19.0
18.0
17.0
16.0
S 15.0
1.0
1.2
1.4
1.6
1.8
2.0
XS
Curve C

FIG. 29.

(SX) with constants, K, will always be obtained as in Table VIII.

TABLE VIII

K	Curve F		Curve G	
	$S=15.00$ Y	$30-Y$	$S=20.00$ Y	$40-Y$
0.30	26.09	3.91	34.78	5.22
.28	26.31	3.69	35.09	4.91
.26	26.55	3.45	35 40	4.60
.24	26 78	3 22	35 71	4.28
.22	27.03	2 97	36 04	3.96
.20	27.27	2 73	36.36	3.64
.18	27.52	2.48	36' 70	3.30
.16	27.77	2 23	37 04	2 96
.14	28.03	1.97	37.38	2.62
.12	28 30	1.70	37 73	2.27
.10	28 57	1.43	38 09	1.91
.08	28 84	1.16	38.46	1.54
.06	29 12	0 88	38 83	1.17
.04	29 41	0 59	39 21	0.79
.02	29 70	0.30	39 62	0.38
.00	30 00	0.00	40.00	0.00

The results are plotted in Fig. 30. Curve F shows the deviations using S at 15.00 mm. and curve G the deviations using S at 20.00 mm. By use of these tables or curves, providing of course the same method of handling the unknown and standard is employed, one can compare deviations and draw nephelometric curves without using the actual formula, the basis of these calculations. An illustration will help to make this clear:

Let us assume a standard solution of a given substance under investigation, and a solution of twice standard value are prepared. The concentration ratio is 2.00. The twice-standard solution is put into both cups and one side (let us say the right side) set at 20.00 mm., represented by S. The left side is then adjusted until equality of light in the eyepiece is obtained and the reading of the scale taken. When a suitable number of such

readings are obtained, the average is taken and the left side cup is placed exactly on this average.

The solution on the right side is now emptied and, after rinsing and filling with the standard solution, the right side is moved up

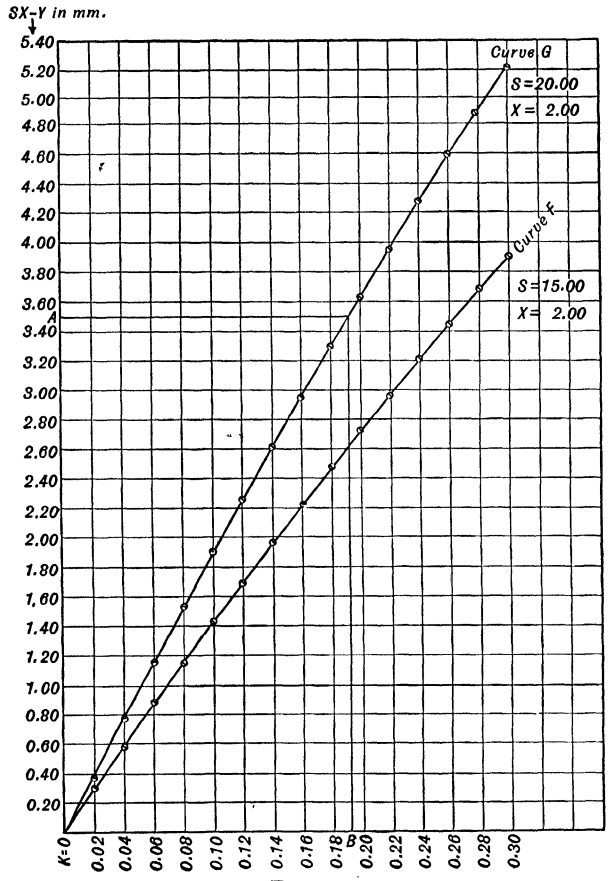


FIG. 30.

and down until equality of light in the eyepiece is obtained. The average of these readings is then taken and represents Y. Since X is 2.00 and S is 20.00, SX equals 20.00 times 2.00 or 40.00 mm. If we assume that Y in this case came out 36.50, what is the deviation and the nephelometric constant K? $SX - Y$ equals 40.00

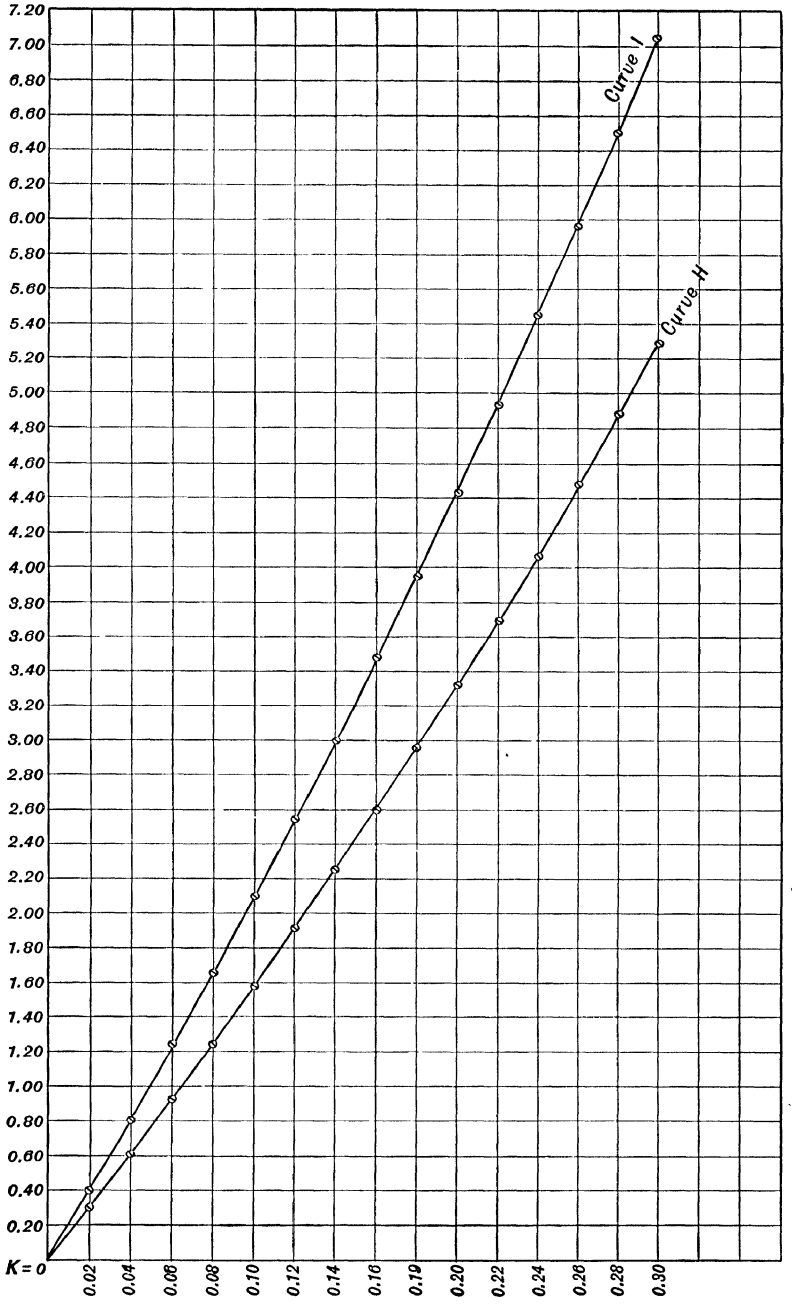

7.20
7.00
6.80
6.60
6.40
6.20
6.00
5.80
5.60
5.40
5.20
5.00
4.80
4.60
4.40
4.20
4.00
3.80
3.60
3.40
3.20
3.00
2.80
2.60
2.40
2.20
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
K = 0
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
0.20
0.22
0.24
0.26
0.28
0.30
Curve I
Curve H

FIG. 31

minus 36.50 or 3.50 mm. deviation. In Fig. 30, 3.50 mm. is marked off and the horizontal line run until it intersects curve G, and a vertical line B at this point intersects the scale of constants k at 0.192.

While most of the nephelometric curves (lines) lie below that of the hypothetical (proportionality) curve and the sign of K is positive, yet a few curves lie above it and therefore have a negative sign. In Table IX the values for similar data with a negative value for K are given and the curves for K are plotted in Fig. 31.

TABLE IX

$-K$	Curve *II*		Curve *I*	
	$S=15.00$ I'	I'−30.00	$S=20.00$ I'	I'−40.00
0.30	35 29	5.29	47 06	7.06
.28	34.88	4 88	46 51	6.51
.26	34.48	4.48	45 97	5.97
.24	34 09	4 09	45 45	5.45
.22	33 70	3 70	44 94	4 94
.20	33 33	3 33	44 44	4 44
.18	32 96	2 96	43 95	3.95
.16	32.61	2 61	43 48	3 48
.14	32.25	2.25	43.01	3.01
.12	31.92	1.92	42.55	2.55
.10	31 58	1 58	42 11	2.11
.08	31 25	1 25	41.66	1.66
.06	30.93	0 93	41 24	1 24
.04	30 61	0 61	40 81	0 81
.02	30.30	0.30	40.40	0 40
.00	30 00	0 00	40.00	0 00

PART II

INORGANIC

CHAPTER VII

AMMONIA

DETERMINATION OF AMMONIA BY MEANS OF GRAVES' REAGENT[1]

NESSLER'S reagent for ammonia, developed in the early part of the last century, was applied to water analyses in 1867. It has stood the test of time and has come to be used extensively; but with the development of colorimetry its disadvantages as well as its value have become apparent and innumerable modifications of the reagent have resulted. Its instability and tendency to produce a cloud in dilute solutions are the chief difficulties.

Recently efforts have been made to apply the reagent in micro-Kjeldahl work, without previous distillation, with varying degrees of success; the precipitate due to salts makes the matching of colors, however, extremely difficult.

A probable explanation of why the colored solution produced by Nessler's reagent becomes cloudy, especially in the presence of salts, may be found in the following consideration:

(1) Only the iodide complex of mercury and ammonia is highly colored.

(2) The other complexes of mercury and ammonia, like the chloride, Na_2HgCl_4, are colorless insoluble compounds.

[1] Sara S. Graves, J. Am. Chem. Soc., **37**, 1171 (1915); see also, P. A. Kober, J. Ind. Eng. Chem., **10**, 558 (1918).

Therefore, in the presence of the other salts the colored iodide complex is probably partially changed to and in equilibrium with the colorless complexes such as the chloride or sulfate.

On this basis Miss Graves has developed a sensitive nephelometric reagent for ammonia, consisting of mer curic chloride, sodium chloride, and lithium carbonate. The lithium carbonate was chosen on account of the low atomic weight of the cation and, therefore, but slight tendency to cause agglomeration of suspensoids.

The reagent will detect 1.0 part of ammonia in 160 million parts of water and is useful in various tests and Kjeldahl nitrogen determinations. Moreover, this method saves considerable time as well as eliminates the expense, attention and errors connected with a battery of Kjeldahl stills.

Reagents.

1. Sulfuric acid, sp. gr. 1.84.
2. Sodium hydroxide, 1 N.
3. Mercury or mercuric oxide.
4. Potassium sulfate.
5. Ammonia-free water. Ammonia-free water must be used in making up all the solutions. The water should not contain more than 0.005 mg. of ammonia in 100 cc. Tap water slightly acidified with sulfuric acid and distilled gives a satisfactory grade of water.
6. Graves' reagent. Dissolve 80 grams of sodium chloride in 130 cc. of water, add 100 cc. of a cold saturated solution of mercuric chloride, with shaking, and when the salt is practically all dissolved, add 70 cc. of a saturated solution of lithium carbonate (1 per cent), a little at a time while shaking, so that no mercuric oxide forms on the sides of the flask. The resulting solution is usually cloudy due to ammonium salts in the reagents. This may be overcome by shaking the solution with 3 to 5 grams of talcum powder and filtering. The filtered solution may be used at once, or if tightly stoppered may be kept for a long time. Solutions several weeks old were without noticeable change.
7. Protective colloid. Boil 1 gram of starch in ammonia-free

water until clear and make up to 100 cc. with ammonia-free water. This stock solution should be made up fresh each day.

8. Standard ammonium sulfate solution. (See Note 7.) Prepare pure ammonium sulfate according to the following method:[2] A good grade of an ammonium salt is decomposed with sodium hydroxide and the ammonia gas passed into pure sulfuric acid by means of an air current. The salt thus obtained is precipitated by the addition of alcohol, filtered, washed with a little alcohol, dissolved in water and again precipitated with alcohol, filtered, washed as before, and then dried in a desiccator over sulfuric acid.

Dissolve 94.3 mg. of the purified ammonium sulfate in ammonia-free water, dilute to a liter with the water and mix thoroughly. To 10 cc. of this solution add 10 cc. of potassium sulfate solution (containing 75 grams K_2SO_4 per liter—see Note 2), dilute to 100 cc. with ammonia-free water and thoroughly mix. This solution contains 9.43 mg. of ammonium sulfate, which is equivalent to 2.43 mg. of ammonia, or 2.00 mg. of nitrogen, per liter.

Manipulation of Nephelometer and Calculation of Results.—Suppose we add 15 cc. of the 0.003 per cent starch solution to 10 cc. of the standard ammonium sulfate solution (containing 2.0 mg. of nitrogen per liter) and then add 5 cc. of Graves' reagent, with gentle shaking. The resulting suspension is then put into both cups of the nephelometer. If we put the left cup at any convenient height, say 20 or 25 mm., and move the other cup up or down until the light in the eyepiece, coming from both tubes, is equal, we find that the height on the right is rarely, if ever, equal to the height set on the left side. It is in this respect like the zero-point of the analytical balance; it must be determined from time to time, and is seldom constant for a long time. The reading on the right side we will denote as the standard reading, S, but the actual value on the left is of no consequence and may be considered as a tare, so long as it is constant. If in place of the standard on the right we now put another solution of ammonium sulfate, say 0.900 as strong as S, we find a higher reading. If we then put

[2] O. Folin and C. J. Farmer, J. Biol. Chem., **11**, 496 (1912).

in 0.800, 0.700, 0.600 and 0.500 standard strength, we obtain, respectively, a series of corresponding readings. All of these readings except that from the standard S are indicated as Y in the curve and formula.

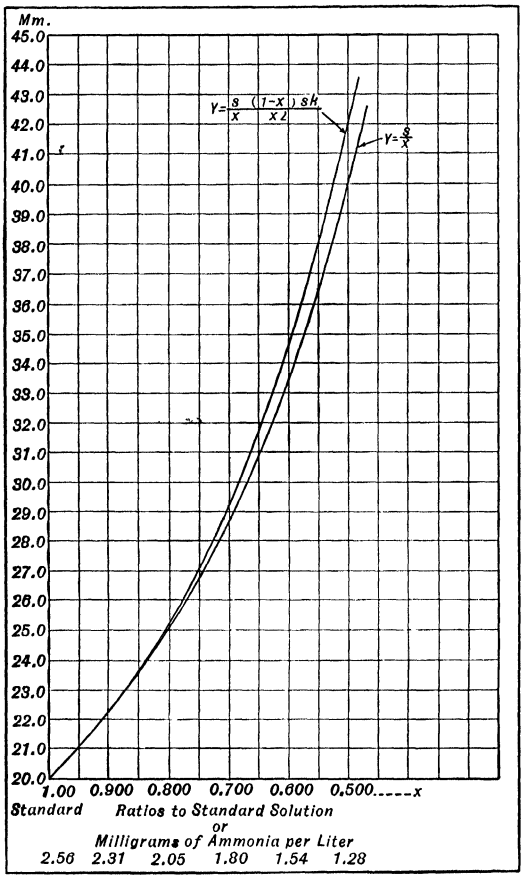


FIG. 32.—Showing a nephelometric curve (upper) and a colorimetric curve (lower). The abscissas give the concentrations of substance, in this case of ammonia; coordinates give the readings, i.e., the heights of solutions. Greater accuracy can be obtained by drawing the curves to a larger scale.

If we plot these readings on cross-section paper we obtain a curve which will be very useful in practical work. In Fig. 32 have been plotted the readings obtained from such a series of

graded known solutions of ammonium sulfate and a curve has been drawn through the points. Algebraically the curve is expressed by the formula

$$Y = \frac{S}{X} - \frac{(1 - X)SK}{X^2},$$

where $K = 0.052$ and $S = 20.0$ (see Note 6). The lower curve, shown here, is the colorimetric curve where the readings are inversely proportional to the concentrations.

When the instrument changes so that a restandardization is necessary, the nephelometric formula obviates considerable work, especially the readings for and the drawing of a new curve. However, by using the method of Lamb, Carleton and Meldrum (see p. 46) a single curve will serve for all subsequent determinations.

Since the formula is complicated and many prefer to do without mathematical calculations, the following scheme can be used: The instrument is standardized as before, but the curve is used alone in getting the amount of substance equivalent to the readings. When the value of the standard readings changes, due to any change in the instrument, the height of the solution on the left side, the one used as a tare, is adjusted so that the original reading for S is obtained and therefore the original curve is applicable. It is equivalent to changing the zero-point of a balance by adjustment so as to avoid calculation.

In Fig. 33, the same curve is used for practical work, i.e., in expressing the results in per cent of nitrogen, obtained in Kjeldahl nitrogen determinations, using 0.1000 gram of substance or sample.

Procedure.—Weigh out exactly 0.1000 g. of the sample and carefully transfer it to a Kjeldahl flask. Add 20 cc. of sulfuric acid, sp. gr. 1.84, 10 grams of potassium sulfate, a few tenths of a gram of mercury or mercuric oxide, and digest as usual by heating. (If all the nitrogen is in the form of ammonium nitrogen and no organic matter is present, digestion is of course omitted.) Dissolve the melt in ammonia-free water and make up to 500 cc. with ammonia-free water, thoroughly mix, and pipette off an

aliquot part of 5 cc. at the same temperature. Although the solution will be quite warm due to the heat of dilution of the sulfuric acid, by taking an aliquot portion at the same temperature

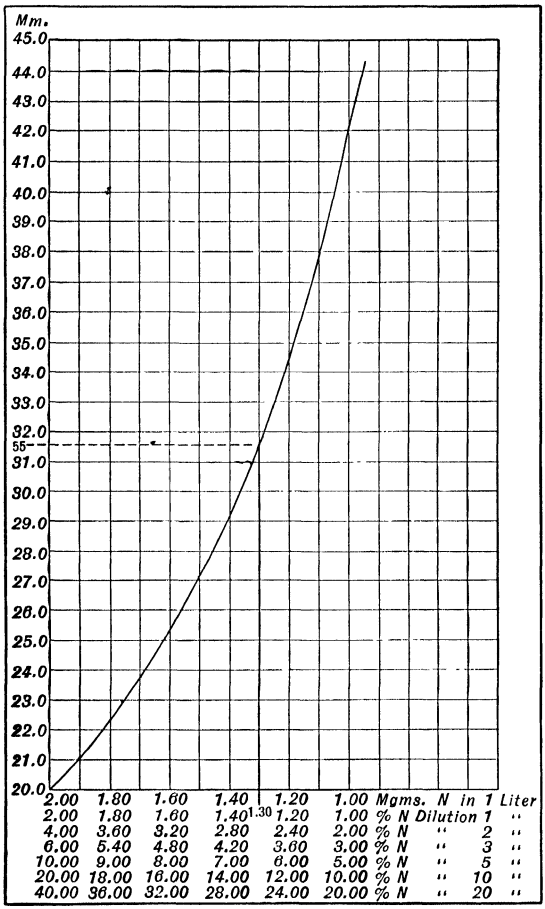


FIG. 33.—Showing a nephelometric curve similar to the one in Fig. 32 but with the abscissas giving the per cent of nitrogen per 0.1000 g. of sample. Greater accuracy can be obtained by drawing curve to a larger scale.

no error will be introduced in the determination and it will not be necessary to cool to room temperature.

To the 5 cc. aliquot part add about 5 cc. of 1 N sodium hydroxide and then drop by drop of the same solution until neutral to

litmus paper. (See Note 5.) Dilute the solution to 200 cc. with ammonia-free water. To 10 cc. of this solution add 15 cc. of 0.003 per cent starch solution and then 5 cc. of Graves' reagent, with gentle shaking. Match the cloud thus formed against 10 cc. of standard ammonium sulfate solution (containing 2.0 mg. of nitrogen per liter) similarly treated. From the nephelometer reading and the curve the per cent of nitrogen in the sample is obtained at once.

Suppose the sample had 0.1000 gram of ammonium sulfate. In the first step we diluted to 500 cc.; and in the second step, when 5 cc. was diluted to 200 cc., we diluted forty times, thus giving a total volume of 20,000 cc., or 20 liters. If the standard reading S were 20.00 mm., we would get a reading of 39.10 mm. for the unknown, which would give us 21.36 per cent of nitrogen in ammonium sulfate. (See Note 3.)

Notes.

1. When a solution of mercuric chloride is made alkaline, immediately the yellow or red oxide forms, but it was found that by first adding sodium chloride, a complex was formed which was stable except in the presence of a large excess of alkali. As will be seen from the following equation, two molecules of sodium chloride unite with one of mercuric chloride:

$$2NaCl + HgCl_2 \rightleftarrows Na_2HgCl_4$$

This complex is readily soluble in water and while it has an appreciable dissociation into $HgCl_2$ and NaCl,[3] with large amounts of sodium chloride, the dissociation is practically zero. It has the further advantage that chlorine compounds are more stable than those of the other halogens. Thus, the ammonium complexes of mercuric chloride are more stable than those of the bromide or iodide. The neutral iodine complex has a noticeable vapor tension of ammonia, which the analogous complex of chlorine has not.[4]

[3] T. W. Richards and E. H. Archibald, Z physik. Chem., **40**, 385 (1902); M. Le Blanc and A. A. Noyes, *ibid.*, **6**, 393 (1890).

[4] Sara S. Graves, *loc. cit.*

2. Potassium sulfate is added to the standard ammonium sulfate solution in order to reproduce as far as possible the conditions found in all Kjeldahl work. Experiment has shown that large or small amounts of salt do not affect the precipitation of ammonia with the reagents as given.

3. In all experiments, controls should be made on the water as well as on the reagents. For the control estimation the solution should not be more than one-twentieth as dilute as the solution containing the sample. This estimation will give a correction, i.e., a certain fraction of a per cent, which must be subtracted from the per cent of total nitrogen obtained in the determination.

4. Mercury or mercuric oxide instead of cupric sulfate must be used as a catalytic agent, on account of the blue color developed by copper in alkaline solution.

5. A considerable excess of sodium hydroxide would cause mercuric oxide to form upon the addition of the reagent.

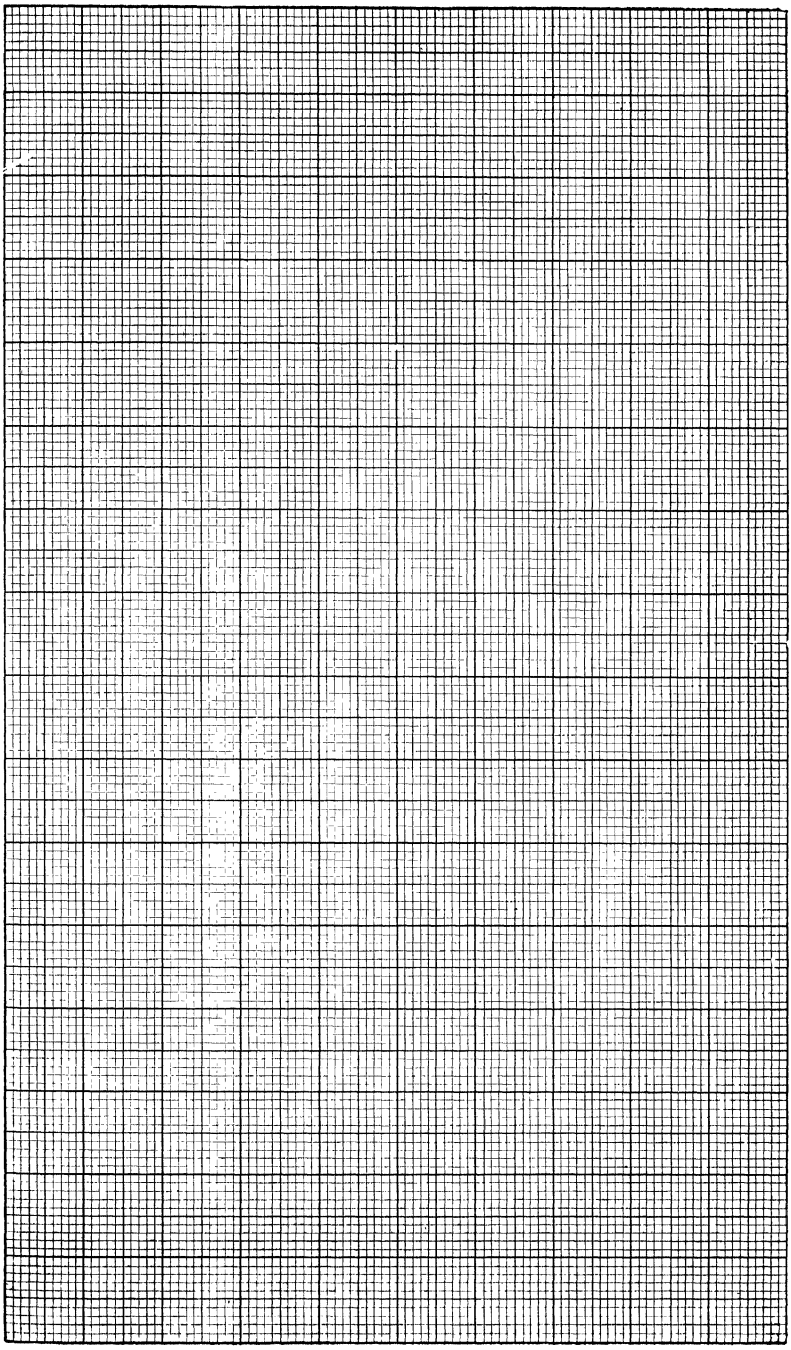
6. A more convenient form of the equation is obtained by solving for X which gives,

$$X = \frac{S + SK\sqrt{(S + SK)^2 - 4SKY}}{2Y},$$

where $(S + SK)$ is constant throughout any series of determinations.

7. Folin and Farmer [5] were at first unable to obtain satisfactory results in the determination of nitrogen in urine because their standard ammonium sulfate solutions were not trustworthy, notwithstanding the fact that they gave practically theoretical results when their ammonia content was determined by distillation and titration. They found other ammonium salts even worse than the sulfate. Their results were too high and they suspected the presence of ammonia or other nitrogenous products in their reagents. However, the error was due to pyridine bases present in the ammonium salts. These bases titrate like ammonia but do not give the reaction with Nessler's reagent.

[5] *Loc cit.*



CHAPTER VIII

ARSENIC

DETERMINATION OF ARSENIC

By Hans Kleinmann and Fritz Pangritz [1]

This method depends upon the production of a cloud in a pure, almost neutral solution of arsenic acid, in the presence of a definite amount of salt, by means of a cocaine-molybdenum reagent.

The solution of arsenic acid is prepared from any arsenic containing substance by digesting with sulfuric and nitric acids and then separating the arsenic as $AsCl_3$ from the solution of the digestion mixture.

The arsenic-cocaine-molybdenum cloud is compared with a standard prepared in a similar manner from an arsenic standard solution. When a Kleinmann nephelometer is employed, the turbidity is exactly proportional to the arsenic content.

Reagents.

(*a*) *Solutions for digesting arsenic-containing organic substances.*—As the nephelometric arsenic determination measures extraordinarily small amounts of arsenic, the amount of impurities in the reagents necessary for digesting the organic material play an important rôle. It is therefore necessary to purify free from arsenic all reagents which are used in larger amounts. Reagents (Kahlbaum's) for which no directions for purification are given are used in such small quantities that their arsenic content is not appreciable.

1. Sulfuric acid. Forty cubic centimeters of concentrated sulfuric acid (Kahlbaum's or similar grade) are necessary for the digestion of 20 grams of dry organic powder. This amount when distilled off leaves a residue (invisible) in which, by means of the

[1] Biochem. Z., **185**, 14 (1927).

arsenic reagent, a small amount of arsenic can be shown. Therefore a large amount of concentrated sulfuric acid is purified until free from arsenic. The method is as follows: Fill a large flat porcelain dish with sulfuric acid and add 10 per cent by volume of the described KCl-$FeSO_4$ mixture (see below) and 0.5 per cent by volume of potassium bromide with constant stirring and boil for one or two hours with frequent stirring. By this method the arsenic, even in smallest traces, is removed as $AsCl_3$ in accordance with the principle of the so-called Schneider distillation.[2]

2. Nitric acid. Sodium nitrate (Kahlbaum's reagent, certified) and sulfuric acid as prepared under (1) are put into a ground-joint distillation apparatus (Liebig) in the proportion of 2 moles of $NaNO_3$ and 1 mole of H_2SO_4, and the fuming nitric acid generated and distilled over. There will be obtained a yield of 80 per cent of fuming nitric acid, which contains only about 0.001 to 0.002 mg. of arsenic in 150 cc. (This figure is obtained by carefully evaporating and testing the residue.) As seldom the complete amount of nitric acid is used, the purity is a satisfactory one. Merck's purest fuming nitric acid contains 0.008 to 0.010 mg. of arsenic in 150 cc.

3. Copper sulfate solution, 10 per cent.

4. Potassium chloride. Mix 100 parts by weight of powdered KCl (Kahlbaum's reagent) with 10 parts of KBr and 5 parts of $FeSO_4$ (both finely powdered), in order to preserve the proportions in the Schneider distillation. Then mix the salts with distilled water to a thin paste and treat with a few cubic centimeters of hydrochloric acid and evaporate to dryness on the water-bath. If necessary at the end use a small flame. Repeat the procedure.

5. Potassium bromide (Kahlbaum's reagent).

6. Ferrous sulfate, cryst.

7. Perhydrol (Merck).

8. Hydrochloric acid, sp. gr. 1.19.

9. Sodium hydroxide, 1 N.

[2] According to the directions of G. Bodmar and L. E. Roth (Z. angew. Chem., **37**, 1101, 1026) the presence of a small amount of copper is necessary for the complete removal of the hydrochloric acid. Therefore, 2 mg. of copper in the form of $CuSO_4$ (not more) are added to every 10 cc. of sulfuric acid.

(*b*) *Solutions for the nephelometric determination.*

1. Potassium-molybdate solution, 1 per cent.
2. Hydrochloric acid, 1 N.
3. Cocaine hydrochloride solution, 2 per cent.
4. Nephelometric reagent. This reagent is prepared by taking 1 volume of (1), 2 volumes of (2), shaking and while continuing the shaking adding 1 volume of (3). The reagent keeps for weeks. Just prior to using, it is filtered free from a slight sediment, using a quantitative filter paper.
5. Standard arsenic acid solution. Dissolve 1.000 gram of purest arsenic acid (powdered, Kahlbaum) in distilled water, make up to a liter and mix thoroughly. The solution contains 1 mg. of arsenic acid per cubic centimeter. From this stock prepare standard solutions containing 0.1 mg. to 0.01 mg. and 0.001 mg. of arsenic acid per cubic centimeter. These dilute standard solutions are employed for the preparation of standard comparison solutions.

Digestion of Organic Arsenic-containing Material.—The substance to be digested, which must be very finely divided and thoroughly dried upon a water-bath (up to 20 grams of dry powder), is put into a Kjeldahl flask and treated with an amount of arsenic-free fuming nitric acid depending upon the nature of the material. In some cases a violent reaction takes place with a great deal of foaming and efficient cooling in a water-bath is required. As a rule, however, the reaction does not begin until after the lapse of a few minutes and then proceeds so quietly that no cooling is necessary. In every case the amount of material and the amount of nitric acid can be so chosen that after twenty-five minutes, at the most thirty minutes, the whole mass, with the exception of any fats present, is dissolved. The completely cleared solution has a dark brown color. After cooling the flask, 20 to 25 cc. of concentrated, arsenic-free sulfuric acid and 10 to 12 drops of a 10 per cent cupric sulfate solution, to serve as catalyzer, are added. The object now is to bring about the strongly exothermic reaction of the mixture at such a rate that, with the smallest amount of extraneous heat and the constant presence of a certain amount of excess fuming nitric acid, the liquid in the flask is

brought to a gentle boil and the development of too large amounts of nitrous oxide vapors is avoided. Often the reaction starts in when the flask is shaken for a while, but for the most part it is necessary to heat the flask over a low Bunsen flame. Then the reaction is always so vigorous at the beginning that one must remove the flask from the flame and cool it.

When the reaction has died down, which is shown by a decrease in the amount of nitrous oxide vapors, the flask is heated again with a micro-burner and the flask so set up that while continuing the heating, fuming nitric acid can be added drop by drop. For this purpose á graduated funnel with ground stop-cock outlet is set up which has a long delivery tube bent twice at an obtuse angle, so arranged that the stop-cock can be well regulated and so the nitric acid can drop directly into the sulfuric acid in the upright Kjeldahl flask. At first 10 to 15 drops of fuming nitric acid are added in somewhat rapid succession by which the reaction in the flask is for the first moment reduced. Then the height of the flame is so reduced or regulated that 1 drop every 10 to 12 seconds will bring about a gentle boil with a moderate evolution of nitrous oxide gas through the constant presence of nitric acid. This arrangement is important.

In the course of the digestion the addition of external heat and the amounts of nitric acid added are gradually increased so that at the end of three to five hours the full effect of a strong Bunsen burner is applied and the rate of addition of fuming nitric acid is 1 drop every four to six seconds. Under these conditions the digestion of 20 grams of dry substance is finished in about seven to eight hours. If the contents have taken on a yellow color the addition of nitric acid is stopped and the flask heated with full flame twenty minutes longer. The digestion is complete and is stopped when the liquid in the flask gives off sulfuric acid fumes and remains a bright yellow unchanged. Should there be the slightest dark coloring due to the formation of carbon from the mixture, the flame is withdrawn and the flask is thoroughly and quickly cooled with the aid of a stream of water. After cooling the flask, fuming nitric acid is again added and for at least fifteen minutes continued as described above. If after this the sulfuric

acid is heated for ten minutes and does not change color, then the digestion is ended. Residues of nitric acid and nitro-sulfuric acid remaining in the solution are removed by repeated boiling with water.

This general form of digestion must be varied in accordance with the character of the material to be analyzed and this variation concerns essentially only the preliminary treatment of the material and its solution in fuming nitric acid. Organs, parts of corpses, meat, etc., must be finely divided and thoroughly dried upon the water-bath, then powdered; paper and tapestry are finely cut up; blood, serum and other liquids are likewise dried upon the water-bath, before they are treated with fuming nitric acid.

Twenty cubic centimeters of fuming nitric acid are necessary for dissolving 20 grams of finely divided and thoroughly dried material; and for complete digestion in accordance with the above scheme, 70 to 90 cc. of fuming nitric acid (with fatty substances considerably more is necessary) are required.

When working with urine and other solutions strongly acid and containing sodium chloride, it is necessary or advisable to make the solution alkaline before evaporation, and then to add a little distilled water, finally dissolving the material by carefully adding fuming nitric acid dropwise.

The Treatment of Metals.—Metals (like zinc, etc.) in small pieces are put into a Kjeldahl flask containing a highly diluted solution of fuming nitric acid. A large volume of the acid must be used. Solution takes place with a quiet evolution of hydrogen gas, which is oxidized by the solution.

A safer way with zinc is to dissolve it in a closed system, in such a manner that the evolved hydrogen gas is bubbled through a few cubic centimeters of bromine because this, according to Lockemann, absorbs arsine quantitatively. The bromine is cautiously volatilized in order to avoid losing mechanically some of the arsenic. The residue is added to the zinc solution and treated further. On the addition of concentrated sulfuric acid, zinc sulfate precipitates out and must be brought into solution again on subsequently distilling to eliminate the arsenic, before one heats with a full flame.

Separation of the Arsenic from the Digestion Liquid.—The arsenic acid is reduced to arsenous acid by the addition of ferrous sulfate and then through the addition of KCl, in the presence of some KBr, is distilled off as $AsCl_3$ into a receiver containing sodium hydroxide solution.

Distillation Apparatus.—The distillation flask is a 500-cc. Jena or Pyrex distillation flask with a long side tube. The top of the flask is closed with a rubber stopper cleaned by boiling in hydrochloric acid, and the tube connected to a ball-type condenser set to slope downward. The end of the condenser dips into a safety receiver. (See Fig. 34.) The receiving liquid obviously conforms to the pressure variation during the distillation, without, however, rising up into the condenser, because as soon as the receiving liquid is drawn up into the receiver, the tube is sucked empty and air enters into the condenser. When the pressure is raised again the liquids return to their normal position again, so that no loss of arsenic trichloride occurs. It is an advantage to have the diameter of the suction flask as small as possible so that the tube *b* dips deeply into the receiving liquid. With such an arrangement, very little attention is necessary during the distillation.

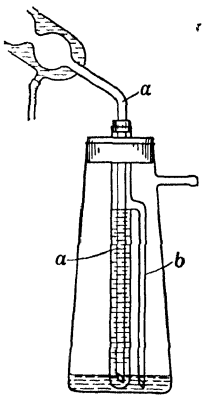


FIG. 34.

Process of Distillation.—The cooled digestion mixture is poured into the distillation flask and the Kjeldahl flask rinsed with a little distilled water. The 2 grams of KCl with 0.2 gram of KBr and about 2 grams of ferrous sulfate are added as described above. The distillation flask is closed immediately and about 30 cc. of 1 N NaOH are put into the receiver.

These amounts of reagents are used for arsenic in amounts above 0.01 mg. H_3AsO_4. If the amount of arsenic to be determined is smaller, the final liquid obtained must be smaller and since this increases the concentration of the salt formed, the following quantities of reagents are used for smaller amounts of arsenic:

For amounts of arsenic less than 0.01 mg., use 0.5 gram KCl, a trace of KBr and 0.5 to 1.0 gram of ferrous sulfate in the distillation flask, and use 7.5 cc. of 1 N NaOH in the receiver.

The distillation of $AsCl_3$ in this apparatus must be carried out as follows: During the first five minutes heat cautiously until the air bubbles slowly through the receiving liquid. Only when the gas or air stream slows up and the receiving liquid begins to back up, is the full flame applied and the distillation is stopped after twenty-five to thirty minutes.

After the distillation, the connection between the condenser and receiver is loosened and the condenser and tube of the distilation flask are carefully rinsed with distilled water into a porcelain dish of 200 cc. capacity. The receiver is taken apart and the receiving liquid also put in the dish. Then the test tube with side tube, as well as the suction flask, are carefully rinsed with water, which is added to the combined liquids in the dish.

The total distillate should be distinctly alkaline to litmus paper. If this is not the case, it is possible that not enough 1 N NaOH was put into the receivers, but there is also the possibility that in the long distillation the ferrous sulfate reduced some of the sulfuric acid to sulfurous acid, which was then distilled off. However, this does no harm. The distillate must be made alkaline immediately with a few drops of 1 N NaOH. The total arsenic is therefore in the form of sodium arsenite. Thereupon one adds 10 drops of Perhydrol (Merck) to the alkaline distillate and warms the dish upon the water-bath. The evaporation of the solution is continued until a volume is reached which is judged smaller than that to which one desires to finally measure it. Through this evaporation the oxidation is finished. The dish is removed from the water-bath and one drop of phenolphthalein added. The neutralization is made with dilute hydrochloric acid. A few drops are sufficient to bring the solution to the end-point. The dish is warmed again for a short time on the warm water-bath (no longer heated as with strong heating arsenic may be lost). After a slight carbon dioxide evolution the red phenolphthalein color comes back again. Now a few additional drops of acid are sufficient for complete neutralization. It is

necessary that the neutralization be made in the warm and not in the hot solution. A filtration of this solution is not necessary if the final volume is 50 to 100 cc. If a slight sediment (silicates from the alkalis) is found, the solution can be decanted. If cruder impurities are found, especially if the total distillate was evaporated to 10 cc., it is necessary to filter through a glass filter (Schott, Jena). This is cleaned by allowing it to stand in hydrochloric acid and rinsing thoroughly with distilled water. After filtering into the graduated flask, the filter is washed with a few drops of distilled water which are added to the flask. When made up to the mark, the solution is ready for measurement.

The making of the solution up to a definite volume and the use of a portion of this solution is to be undertaken only if a sufficient amount of arsenic is present. As shown below, the amount needed for a nephelometric determination is 0.06 to 0.0025 mg. of arsenic. This amount must be contained in 10 cc. of liquid, or at least in 7.5 cc., which is the amount of arsenic solution required for the macro-nephelometer.

The concentration of the arsenic may be gauged by means of a preliminary trial. If the amount is too small one can, if 30 cc. of NaOH was used in the receiver for neutralization, evaporate to 15 cc. without going beyond the range of concentration of salt in which the determination can be made.

When measuring an amount of arsenic less than 0.01 mg. and using 7.5 cc. NaOH in the receiver, the distillate is concentrated, after the treatment described above, to 3 cc. in a small porcelain or platinum vessel. Then by taking 2.5 cc. with a micro-pipette and adding 2.5 cc. of reagent, one is in a position to make measurements in a micro-nephelometer such as Kleinmann's, using a standard as low as 0.001 mg. of H_3AsO_4 (0.0005 mg. arsenic). Under these conditions the requirements for measurement remain fulfilled.

Making the Measurements.—For measurement a desired volume of the solution to be analyzed is taken and treated with an equal volume of clear, filtered reagent. For the Kleinmann macro-nephelometer this volume must be at least 7.5 cc. The amount of arsenic present in this volume can be between 0.06 and 0.0025 mg.

With the Kleinmann micro-nephelometer, for which a total volume of only 5 cc. is necessary, 2.5 cc. of the test solution is used in which the arsenic content can go as low as 0.0005 mg.

The arsenic solution and the reagent are best mixed in a small glass-stoppered flask because this permits more thorough mixing. The best clouds are made with 0.05 to 0.005 mg. of arsenic in the 7.5 cc. used.

The adding of the reagent to the unknown and standard solutions should be done as nearly simultaneously as possible. The standard solution should be so chosen that it is as close to the unknown in concentration as possible, because this makes the measurements more accurate. It is recommended that several concentrations of standard solution be made ready for comparison.

Clouds with arsenic concentrations down to 0.01 mg. of H_3AsO_4 in the final volume (solution plus reagent) of 15 cc. are allowed to stand for twenty minutes before measuring. Clouds with less than 0.01 mg. of H_3AsO_4 in this volume are allowed to stand thirty minutes and can be measured within the next half hour. It is necessary to keep within these time limits in order to make certain that the maximum turbidity is obtained and that flocculation of the precipitate is avoided. Large flocculations cannot be measured. The error of the method, including the digestion process, down to amounts of 0.005 mg. of arsenic is 2 to 3 per cent. Below this amount of arsenic the percentage of error is somewhat greater.

The calculation follows the principle that the turbidities are inversely proportional to the nephelometric heights, when a Kleinmann nephelometer is employed. Other instruments will probably require a calibration curve.

If C is the concentration of the standard solution,
X_1 is the concentration of the unknown solution,
H is the height of the standard solution, and
H_1 the height of the unknown solution, then

$$X_1 = \frac{C \times H}{H_1}.$$

The result is in the same terms in which the concentration of C is expressed.

A correction for arsenic in the reagents is obtained by a " blank " test, in which the same amounts of reagents are used as in the determination. The correction is subtracted from the amount of arsenic found in the determination.

Notes.

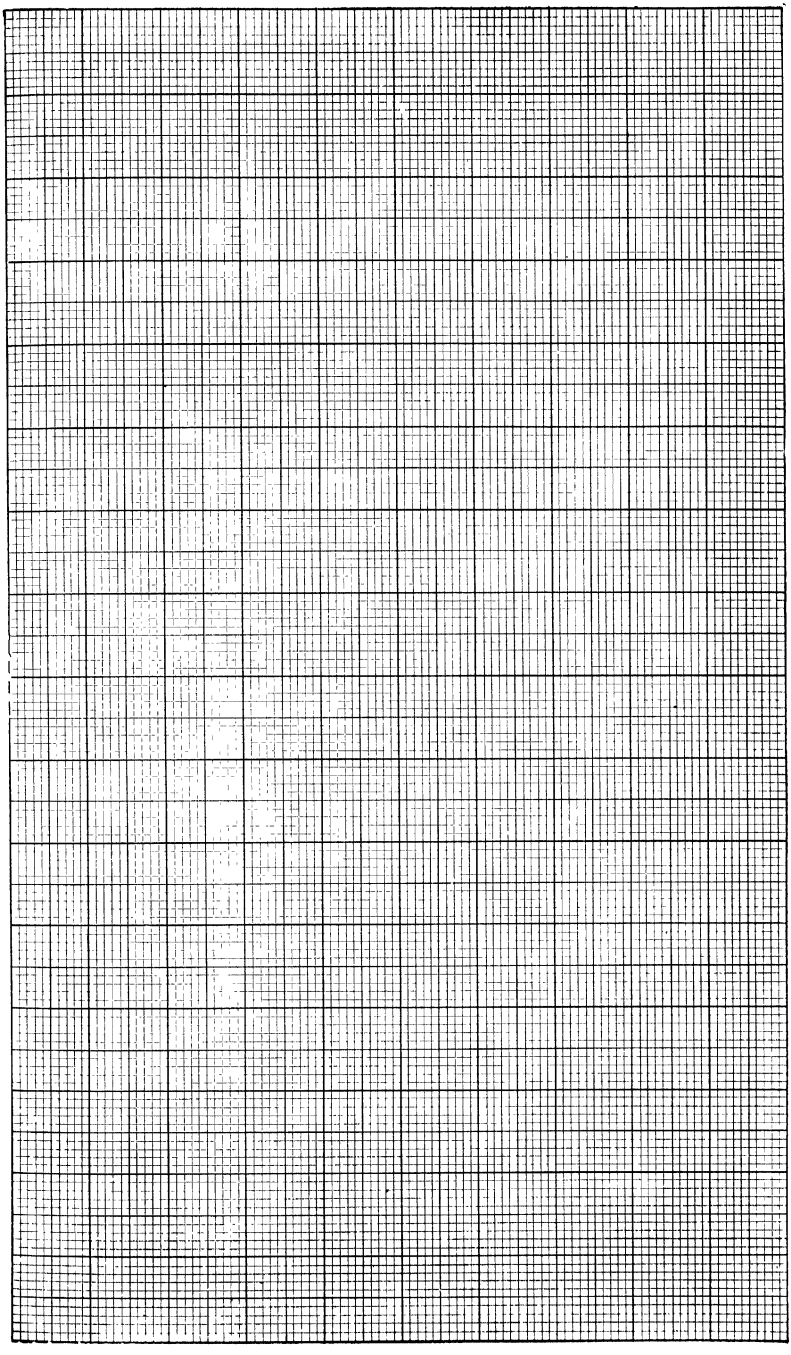
1. The method permits the measurement of extremely small amounts of arsenic (down to 0.0005 mg.) with a small percentage of error. In determining the arsenic in solutions directly (in such as require no digestion and further treatment) colored solutions (as for example solutions containing colored salts such as cupric sulfate) must not be used as otherwise precautions must be taken in regard to color filters, etc., in order to make nephelometric measurements.

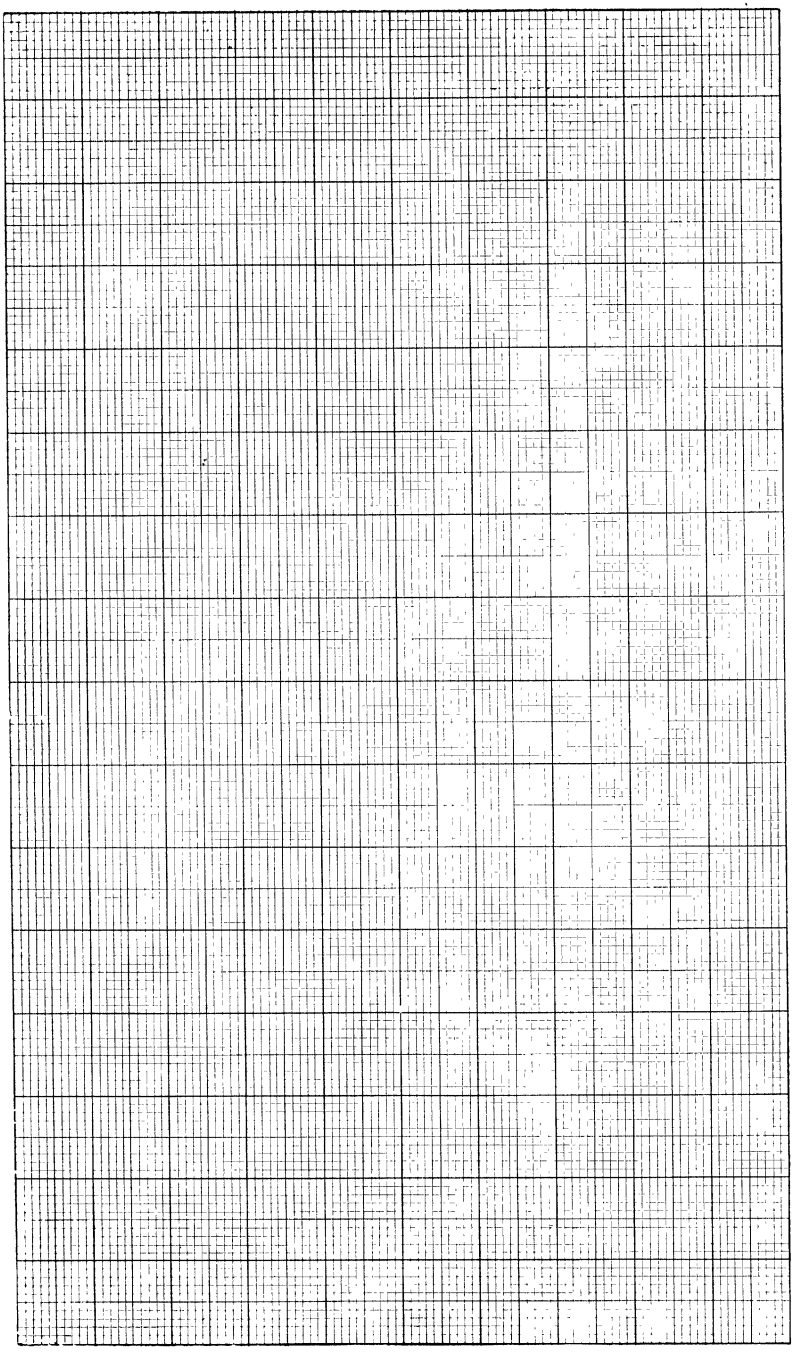
Furthermore, it is necessary to remove completely any phosphoric acid that is present. Phosphoric acid, i.e., phosphates, gives with the cocaine-molybdate reagent a very sensitive cloud which has approximately the same limits as those obtained with arsenic and this reagent.

2. Antimony compounds do not disturb in the method described above. Disregarding that in the distillation of $AsCl_3$ (boiling-point 130° C.) any antimony as $SbCl_3$ (boiling-point 223° C.) could come over only in traces, these would in the weak solution be precipitated as insoluble compounds. Such amounts of this substance which might remain after filtration of the insoluble substance do not form any cloud with the reagent for arsenic, as experiments show, and the arsenic cloud remains completely uninfluenced.

3. The duration of the digestion amounts to one day at the most. The treatment of the digestion fluid requires three hours. The method is much simpler than the accurate description of the method would make it appear.

4. This determination requires no larger amounts of arsenic than are required in the most refined method based upon the Marsh test and is much more exact. It can, therefore, be applied to all micro-determinations of arsenic.





CHAPTER IX

CALCIUM

DETERMINATION OF CALCIUM

METHOD OF LYMAN [1]

THIS nephelometric method is based essentially on the same principles used by Lyman in his turbidimetric method for the determination of calcium in urine and feces (see Volume I, pp. 143 and 146), the chief differences being that a solution of ammonium stearate is substituted for the castor oil soap and the clouds produced are matched in a nephelometer instead of a colorimeter.

The method is short, two hours being required for a set of four determinations as against three full days by the ashing and gravimetric procedure. Moreover, it requires only 5 cc. of blood instead of 300 cc. to 500 cc. and the results are accurate to within less than 1 per cent. The reagent will easily detect 1.0 part of calcium in 5 million of water and is useful in various tests and calcium determinations in blood, milk, water, etc.

A. DETERMINATION OF CALCIUM IN BLOOD

Reagents.

1. Nitric acid, 0.1 N and 0.05 N. These concentrations need not be exact, provided acid of the same strength is used in all steps of the process, including the making up of the standard. A 2 N stock solution will be convenient for preparing more dilute solutions as needed.
2. Oxalic acid, 4 per cent.
3. Trichloroacetic acid, 6.5 per cent.

[1] H. Lyman, J. Biol. Chem., **29**, 169 (1917).

4. Ammonium hydroxide, 2 N. Dilute 13.5 cc. of ammonium hydroxide, sp. gr. 0.90, with 86.5 cc. of water.

5. Ammonium oxalate, 0.5 per cent.

6. Ammonium stearate reagent. Dissolve 4.0 g. of stearic acid and 0.5 cc. of oleic acid in 400 cc. of hot alcohol (95 per cent). Add 20 grams of ammonium carbonate dissolved in 100 cc. of hot water, allow the solution to boil a minute or two, cool, add 400 cc. of alcohol (95 per cent), 100 cc. of water, and 2 cc. of ammonium hydroxide, sp. gr. 0.90. Filter. The filtered solution should be water-clear and colorless. If well stoppered it will keep indefinitely.

Before using the solution, test it as follows: Into two flasks pipette respectively 10 and 5 cc. of the calcium oxalate standard and to the 5 cc. add 5 cc. of nitric acid, 0.05 N. Treat both with 25 cc. of the ammonium stearate reagent and read on the nephelometer. If they do not read exactly 2 to 1 there is some impurity present in the chemicals used.

If the alcohol has stood in a wooden barrel it will give a yellow coloration with ammonia and will contain suspended particles which reflect light in the nephelometer. It should be redistilled with a little calcium carbonate. The stearic acid may be purified by recrystallizing from boiling alcohol. Purify the ammonium carbonate by resubliming.

7. Sodium acetate, 20 per cent. Dissolve 20 grams of crystallized sodium acetate in 100 cc. of water.

8. Standard calcium oxalate solution. Dissolve 72.9 mg. of pure calcium oxalate, $CaC_2O_4 \cdot H_2O$ in 25 cc. of 2 N nitric acid, and dilute to 1000 cc. with water. Thoroughly mix. This solution contains 0.02 mg. of calcium per cubic centimeter and is 0.05 N in nitric acid.

9. Methyl orange indicator, 0.1 per cent. Dissolve 0.1 gram of methyl orange in 10 cc. of alcohol and dilute to 100 cc. with water.

10. Filter paper. Use a good grade of quantitative filter paper (washed in hydrochloric and hydrofluoric acids) for filtering the coagulated blood or milk. The reagents may be filtered through absorbent cotton which has been washed first with 3 N

hydrochloric acid, and then with water until the wash water no longer reacts acid to litmus, and finally dried.

Procedure.—The specimen of blood is drawn according to the method of Folin and Denis,[2] using a paraffined pipette attached to a hollow needle by a piece of rubber tubing. The potassium oxalate is omitted. Five cc. of the blood are run into a small flask containing 15 cc. of 6.5 per cent trichloroacetic acid. The flask is agitated while the blood is being added. Mix the contents of the flask, allow to stand for a few minutes, and then filter through a folded calcium-free filter paper. Transfer, by means of a pipette, 10 cc. of the filtrate to a 50-cc. Erlenmeyer flask, add 1 drop of methyl orange indicator and run in 2 N ammonium hydroxide, drop by drop, until the solution becomes just yellow. Next add 0.05 N nitric acid until the solution turns pink, and then 1 cc. more. Add 1 cc. of 4 per cent oxalic acid, 1 cc. of 20 per cent sodium acetate, cool under a water tap until a faint cloud appears and shake ten minutes (or allow to stand over night). Rinse the stopper with a few drops of 0.5 per cent ammonium oxalate, pour the mixture into a centrifuge tube and centrifuge. Draw off the supernatant liquid with a pipette. Rinse the flask with 5 cc. of 0.5 per cent ammonium oxalate, pour into the centrifuge tube, stir, rinse the rod with a little of the ammonium oxalate solution, and again centrifuge. Draw off the supernatant liquid, dissolve the precipitate in 5 cc. of 0.1 N nitric acid with the aid of a stirring rod, and pour the solution into the original flask. Shake the flask so as to dissolve any precipitate adhering to the walls. Rinse the stirring rod and centrifuge tube with 5 cc. of water, allowing the washings to run into the flask.

Place 20 cc. of the standard calcium oxalate solution in a 100-cc. flask and 50 cc. and 25 cc., respectively, of the ammonium stearate reagent in two clean dry beakers. Pour the standard solution into the 50 cc. of reagent and the unknown into the 25 cc. Mix the solutions by pouring back and forth twice. Stopper the flasks and allow to stand ten minutes. Then fill both nephelometer tubes with the standard, set the left side at 32 mm., and take

[2] J. Biol. Chem., **11**, 527 (1912).

a reading to be sure the two sides of the instrument are balanced. Replace the standard in the left tube with the unknown and read. Any gas bubbles adhering to the walls of the tubes must be removed with a glass rod before making a reading.

With the unknown set at 32 mm., the reading of the standard divided by 4 will equal the number of milligrams of calcium (not CaO) in 100 cc. of blood.

Notes.

1. Be certain all reagents are calcium-free.

2. The trichloroacetic acid is used for the coagulation of both blood and milk, as suggested by Greenwald.[3] It gives a water-clear filtrate, free from protein, and holds the calcium salts in solution.

3. The very large excess of oxalic acid is used in order that the calcium oxalate in such low concentrations will precipitate promptly.

4. Nitric acid is used as a solvent throughout the procedure instead of hydrochloric acid, since chlorides affect the solubilities of calcium soaps.

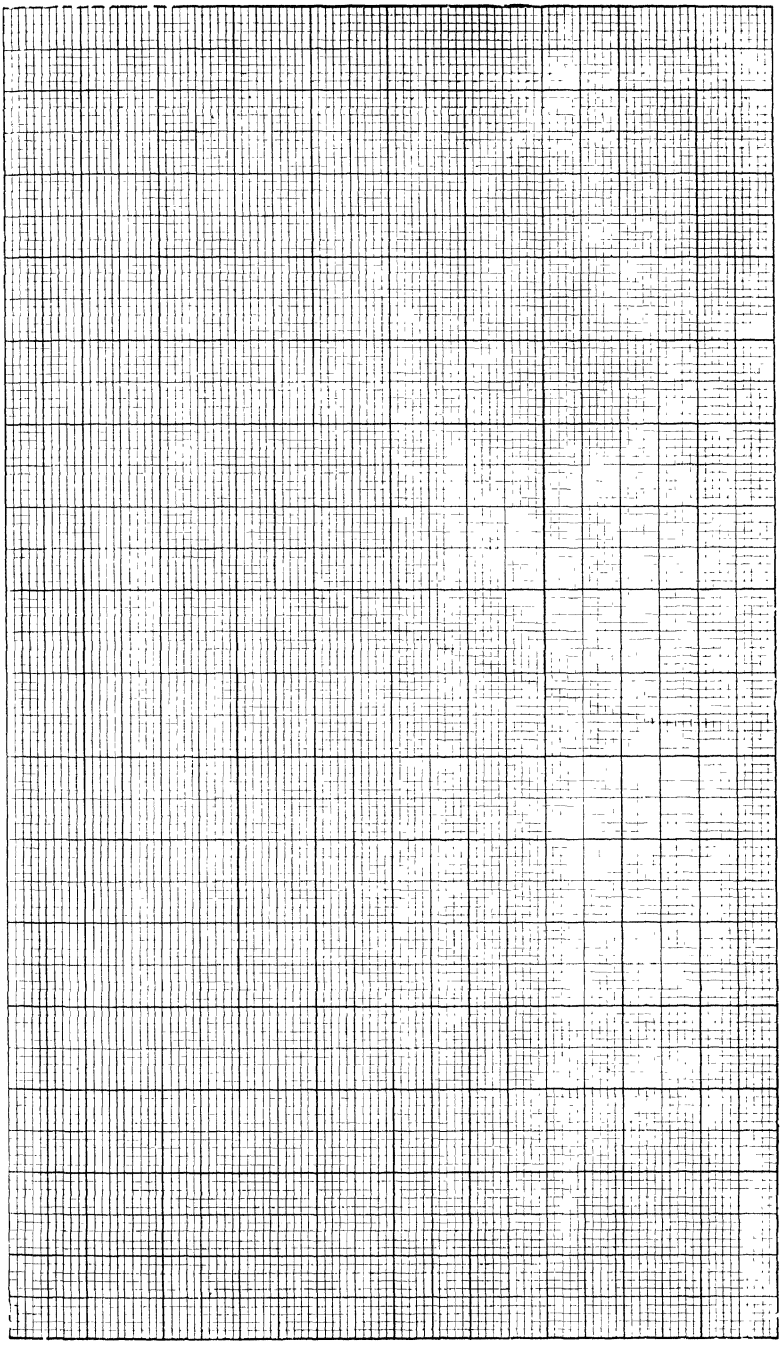
5. The oleic acid added to the reagent acts as a protective colloid in the precipitation. A small amount delays the agglutination for hours.

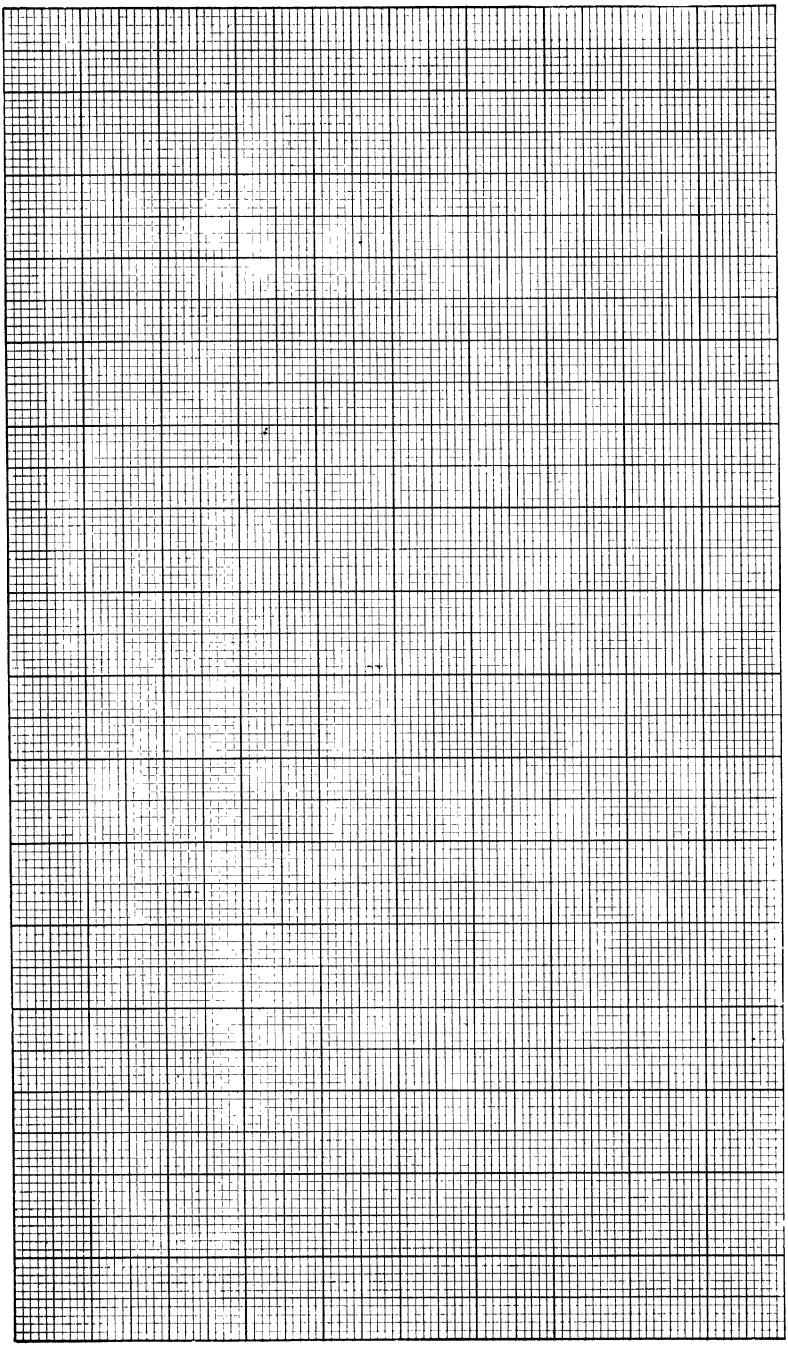
B. DETERMINATION OF CALCIUM IN MILK

After suitable dilution, the calcium content of the milk is determined exactly as in the case of blood.

Procedure.—Pipette 10 cc. of the well-mixed sample into a volumetric flask, a 200-cc. flask for cow's milk or a 100-cc. flask for human milk. Make up to volume with distilled water and mix thoroughly. The mixture is now of approximately the same per cent calcium content as blood. Pipette 5 cc. of this mixture into 15 cc. of trichloroacetic acid, 6.5 per cent; let stand, filter, and proceed as with blood. To find the number of milligrams of calcium per 100 cc. of milk, the nephelometer reading is multiplied by

[3] J. Biol. Chem., **21**, 61 (1915).





5 or by 2.5, according to whether the milk was originally diluted to 200 or 100 cc.

DETERMINATION OF CALCIUM

By P. Rona and H. Kleinmann [4]

This method for the nephlometric determination of calcium depends upon the production of a cloud in a neutral calcium solution of definite salt content, by means of a reagent, which is prepared by dissolving sodium sulforicin [5] in sodium hydroxide.

The solution of calcium chloride is prepared by dry ashing the substance to be analyzed for calcium, and dissolving the ash in a definite amount of hydrochloric acid. A standard calcium solution is used for comparison. The turbidities vary in proportion to the content of calcium and the nephelometric measurement gives the results directly in per cent of the solution.

Reagents.

1. Hydrochloric acid, 1 N.
2. Ammonium chloride, 1 N.
3. Conductivity water.
4. Nephelometric reagent. Ten cubic centimeters of sodium sulforicin according to Berlioz-Heryng (Merck) are measured out in a graduate. If this substance is too viscous or if there are crystals present, it is previously warmed slightly in a water-bath until it is quite fluid and clear. To this is then added 112 cc. of 1 N sodium hydroxide, which should be free of shreds and optically clear, and if necessary, filtered. The solution is then made up to 125 cc. with distilled water and stirred vigorously with a glass rod until it is clear and homogeneous. It is preserved in a glass-stoppered bottle. The reagent is a clear, homogeneous, but decidedly yellow solution. It will keep indefinitely. The color of the reagent does not disturb the measurement, since only 0.4 cc. of it is used in a dilution of 25 cc. and this solution is perfectly

[4] Biochem. Z., **137**, 157 (1923).

[5] This reagent is listed by Merck as sulforicinate—Berlioz-Heryng.

colorless. This concentration of reagent in conductivity water does not produce a cloud and on long standing shows itself to be a water-white solution, optically clear.

5. Standard calcium solution. Pure calcium carbonate is dried in a desiccator to constant weight. Then 0.4995 gram is weighed off, rinsed into a 100-cc. beaker, and covered with conductivity water; 23.0 cc. 1 N hydrochloric acid are added, keeping the vessel covered with a watch-glass during the treatment in order to avoid loss due to spray. After the calcium carbonate has dissolved, the solution is rinsed into a 2-liter volumetric flask and made up to the mark with conductivity water. The solution contains 0.1 mg. of calcium per cc.

The calcium standard solution must be preserved in a paraffined flask since on long standing calcium is dissolved from the glass. The paraffining of the flask is done as follows: A sufficient amount of solid paraffin is put into the flask and melted by placing the flask in a hot water-bath. After the paraffin is melted the flask is rolled on its side upon a table, until the flask is uniformly covered and the paraffin has solidified. The flask is then rinsed out with conductivity water and filled with standard solution. The calcium standard solution should be used as fresh as possible.

Procedure.—The organic material to be analyzed for calcium is ashed dry. For this purpose the substance (for example 1 cc. of serum) is dried first on the water-bath and then in an oven at 100° C. to complete dryness and finally ashed with the Bunsen burner.

As a rule the ashing goes easily if the crucible is kept covered. Acid substances (particularly hydrochloric acid) are neutralized before ashing and made slightly alkaline (using an indicator), because $CaCl_2$ is slightly volatile. Usually the ashing is completed in a few minutes and the residue should be pure white.

The ash is dissolved in hydrochloric acid. This solution of the ash should be done by a few drops of relatively strong acid rather than by larger amounts of dilute acid. The reason for this lies in the fact that the preservation of such a solution in glass flasks results in calcium being taken up from the glass. Therefore the

volume is kept as small as possible. The dilution of the solution is always done with conductivity water.

For analysis, about 0.04 to 0.4 mg. calcium is necessary. If apparently the ash contains no more than this amount of calcium the whole of it is used for analysis. For this purpose the ash is treated with 4 to 5 drops of 1 N hydrochloric acid. The crucible is uniformly turned so that the solution rinses the sides clear to the edge. Then the solution is drawn up in a micro-pipette and transferred to a glass-stoppered flask, preferably one holding 50 cc. but having a mark indicating 5 cc. and one indicating 25 cc.

After the solution of the ash has been transferred, add to the crucible an equal portion of hydrochloric acid and repeat the process. Finally rinse with 2 to 3 cc. of conductivity water, the crucible and the capillary pipette, into the flask, and add conductivity water to bring the volume up to 5 cc. Now add to the solution the same number of drops of 1 N ammonium hydroxide as 1 N hydrochloric acid was added. Use the same burette or pipette used in dropping in the acid, in order to have the same size of drops. It is suitable to use, instead of drops, a measured volume by means of a small calibrated pipette.

Not more than 0.5 cc. of hydrochloric acid and 0.6 cc. of ammonium hydroxide are used. In every case the test solution should be as nearly neutral as possible after the ammonia addition. Excess of ammonia produces flocculation of the cloud.

If not all the ash is used for analysis, it may be dissolved in a corresponding increase of acid, diluted to a definite volume with conductivity water, and from this an aliquot portion taken for analysis, seeing to it that the volume used for analysis does not contain more than about 0.5 to 0.6 cc. 1 N ammonium chloride.

To the 5 cc. of test solution are now added 0.4 cc. of the filtered clear reagent. The measurement of 0.4 cc. must be done accurately with a pipette graduated in 1/100 cc., or more conveniently with a Bang micro-burette.

The standard solution is treated in the same manner. Standard and test solution should be treated with reagent as closely following each other as possible. The turbidity is allowed three

minutes to develop and then the solution is made up to 25 cc. with conductivity water.

The solution should be compared in the nephelometer within half an hour.

Notes.

1. The method effects the rapid determination of calcium in amounts of 0.04 to 0.4 mg. when the Kleinmann macro-nephelometer is used. By using the micro form of the instrument it is possible to determine one-fifth of this amount, i.e., 0.025 mg. of calcium can be determined. This makes it possible to use with the macro form of instrument 1 cc. of blood or serum, and 0.25 cc. of blood when the micro-nephelometer is used.

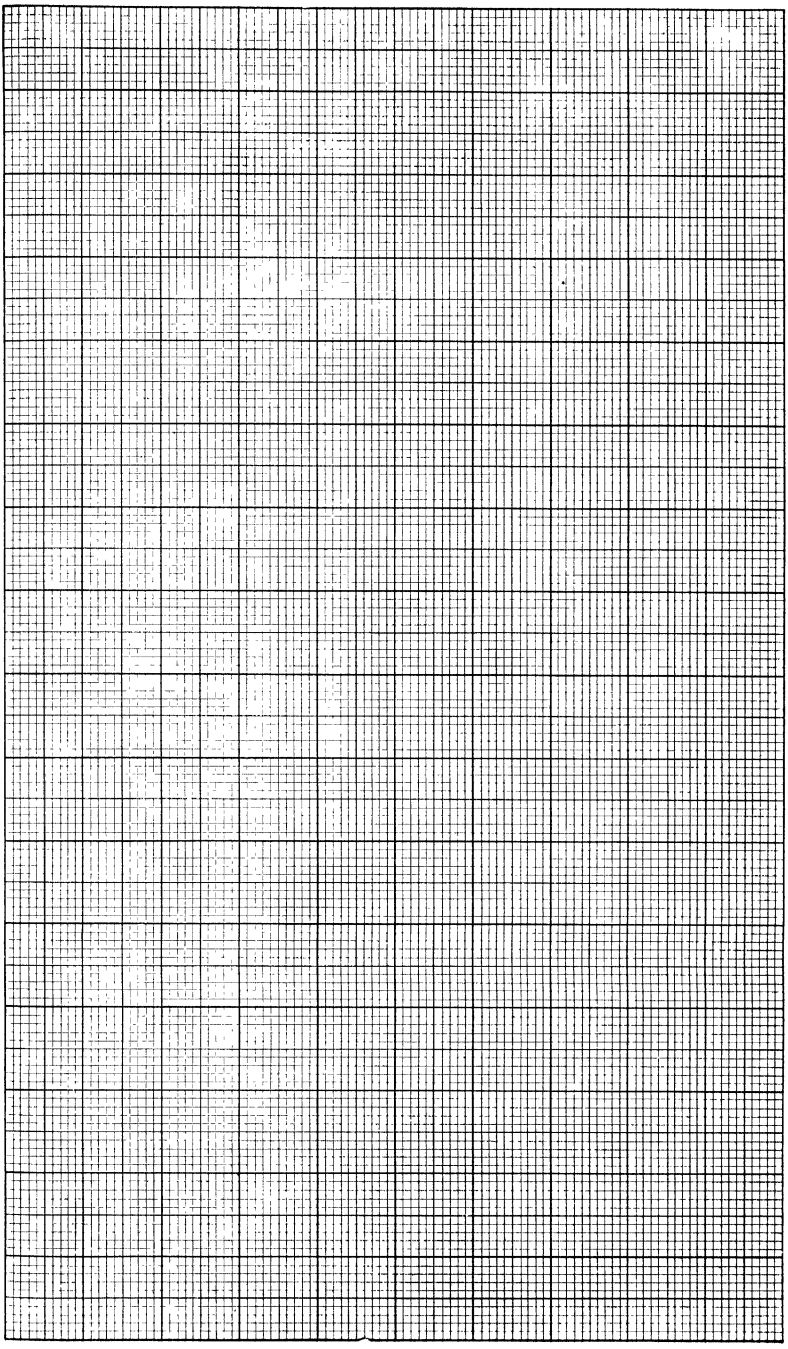
2. The error of the method amounts to about 1 per cent. It is however advisable, on account of slight variations, to make duplicate determinations. The determination is very convenient and can be carried out in a few minutes.

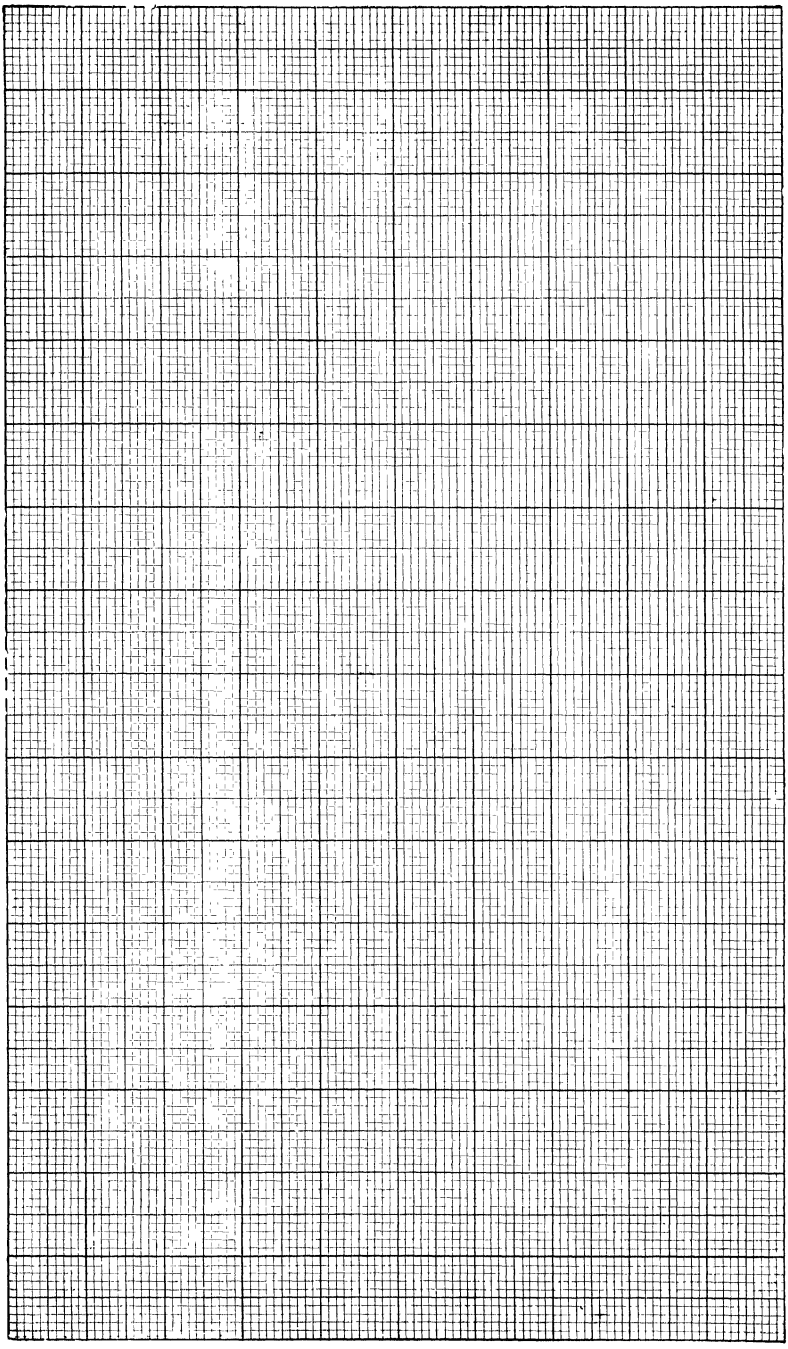
3. The analysis of blood requires the removal of the iron by means of ammonium hydroxide in slight excess to the acid solution of the ash. After filtering through quantitative filter paper and diluting to 25 cc., 10 cc. are used for analysis.

4. According to Leonia Kriss[6] the reagent gives no cloud with magnesium salts if sufficient ammonium chloride is present. For the determination of calcium in the presence of magnesium, it is necessary to add for every gram-ion of magnesium ten moles of ammonium chloride. The standard solution is treated likewise.

The method is suitable for the direct determination of hardness in water, as well as for all micro-chemical calcium determinations.

[6] Biochem. Z., **148**, 203 (1925).





CHAPTER X

CHLORINE

DETERMINATION OF CHLORINE (AS CHLORIDE)

Method of Richards and Wells

The opalescence obtained by adding silver nitrate solution to a solution containing a small amount of chloride ions is made the basis of this method. It was first used by T. W. Richards [1] in connection with some atomic weight determinations and was designed to estimate precipitates in amounts under 1 or 2 mg. per liter of suspending solution.

In general, the method of Lamb, Carleton and Meldrum (see p. 46) is recommended for this determination. Richards' method is of historical interest since it is the first real nephelometric method to be developed. It was applied only to aqueous solutions. Silver chloride suspensions thus formed are not very satisfactory for precise nephelometric measurements. A maximum and more constant opalescence can be developed by the technique of Lamb, et al.

Reagents.

1. Nitric acid, 0.025 N, chloride-free.
2. Potassium nitrate, 0.025 N. Purify the salt by recrystallization until free from chlorides.
3. Silver nitrate, 0.000040 N. Purify the salt by recrystallization.

[1] Proc. Am. Acad. Arts Sci., **30**, 385 (1894); Z. anorg. Chem., **8**, 268 (1895); Richards and Wells, Am. Chem. J., **31**, 235 (1904); J. Am. Chem Soc., **27**, 484 (1905); Wells, Am. Chem. J., **35**, 99, 508 (1906); Richards, *ibid* , **35**, 510; J. Chem. Soc., **99**, 1204 (1911); Orig. Com. 8th Intern. Congr. Appl. Chem., **1**, 423 (1912); *ibid.*, **27**, 24.

4. Standard potassium chloride solution. The potassium chloride is purified by recrystallization and then fused in a platinum dish. Dissolve 0.2103 gram of the fused salt in distilled water, dilute to a liter and mix thoroughly. One cubic centimeter of this solution contains 0.10 mg. of chlorine. Prepare more dilute standards as needed.

5. Distilled water. The water used in preparing the above solutions must be doubly distilled. A good quality of distilled water should be redistilled into a large bottle, through a special adapter for excluding dust. When the adapter is removed, a tube containing soda lime should replace it so that no trace of hydrochloric acid from the air can enter the bottle when water is withdrawn through a siphon.

Procedure.—The unknown and standard solutions must be prepared under as nearly identical conditions as possible. Place the unknown solution (a few cubic centimeters) in a nephelometer tube. To a second nephelometer tube add a quantity of standard potassium chloride solution approximately equal to the amount of chloride in the unknown. To each tube add silver nitrate solution in excess. During precipitation each solution must be stirred thoroughly and uniformly, usually 20 strokes, with a platinum stirrer, made by sealing a propeller-shaped piece of platinum foil upon the end of a glass rod. Add nitric acid or potassium nitrate in 8-fold excess, i.e., 8 moles per mole of silver chloride. Read in four minutes.

The order of mixing the above solutions may be varied, provided, of course, the same order is followed with both the unknown and the standard. The following proper treatment for estimating various silver chloride opalescences was used by R. C. Wells[2] and will serve as a guide:

Intense Opalescences (0.00005 N)

Silver nitrate added to potassium chloride: Add nitric acid or potassium nitrate. Use 8-fold excess. Read in four minutes.

[2] Am. Chem., J. **35**, 113 (1906).

Silver nitrate added to silver chloride already containing potassium nitrate. Read in twenty minutes.

Potassium chloride added to silver nitrate: Add electrolyte. Use 50-fold excess. Read in thirty minutes.

Potassium chloride added to silver chloride, already containing potassium nitrate. Read in twenty minutes.

Weak Opalescences (0.000005 N)

Silver nitrate added to potassium chloride: Add electrolyte. Use 100-fold excess. Read in twenty minutes.

Silver nitrate added to silver chloride, already containing electrolyte. Read in thirty minutes.

Potassium chloride added to silver nitrate: Add electrolyte. Use 70-fold excess. Read in thirty minutes.

Potassium chloride added to silver chloride, already containing electrolyte. Read in thirty minutes.

Notes.

1. The precipitation of the silver chloride should be made in the dim light of a small electric light bulb in a dark room. All tubes should be kept in absolute darkness when not under direct observation.

2. The room must be as free as possible from dust. Great care must be taken to keep all apparatus, vessels, and the operator's hands thoroughly clean with large quantities of redistilled water.

3. In order to avoid variations in the standard, Wells [3] used ground-glass plates of ordinary window glass cut into pieces about 1 inch square and ground by fine emery until a series was obtained which ranged from opacity to transparency. Some were ground on both sides. Each piece of glass was covered by black paper having a round hole in the center about 16 mm. in diameter. The plates were placed in the nephelometer in a *definite and reproducible position* and " had exactly the appear-

[3] Am. Chem. J., **35**, 100 (1906).

ance of an opalescent precipitate." The glasses were carefully standardized.

Richards,[4] commenting upon Wells use of ground-glass plates as standards, points out that many of his (Wells) solutions were about four times as concentrated as a saturated silver chloride solution and, hence, showed a rapid rate of deposition. Such precipitates should be collected and weighed, not determined nephelometrically. Richards goes on to say that " while the ground-glass plate employed by Dr. Wells, when properly and steadily illuminated, undoubtedly affords an excellent means of studying the rate of change in a given mixture, I cannot believe that it provides the best standard of reference for analytical purposes, because it is unaffected by the many variables which affect the mixture to be estimated. In my opinion, if even moderately accurate analytical results are to be had with the nephelometer, the one essential point to be heeded is this: *the unknown solutions to be estimated must be treated in exactly the same way as the known standard solutions, which serve as the basis of comparison.* If this precaution is adhered to, the changes of temperature, the presence of electrolytes, the concentration of the solutions and all the other variables, affecting each precipitate in like manner, are eliminated from the comparison."

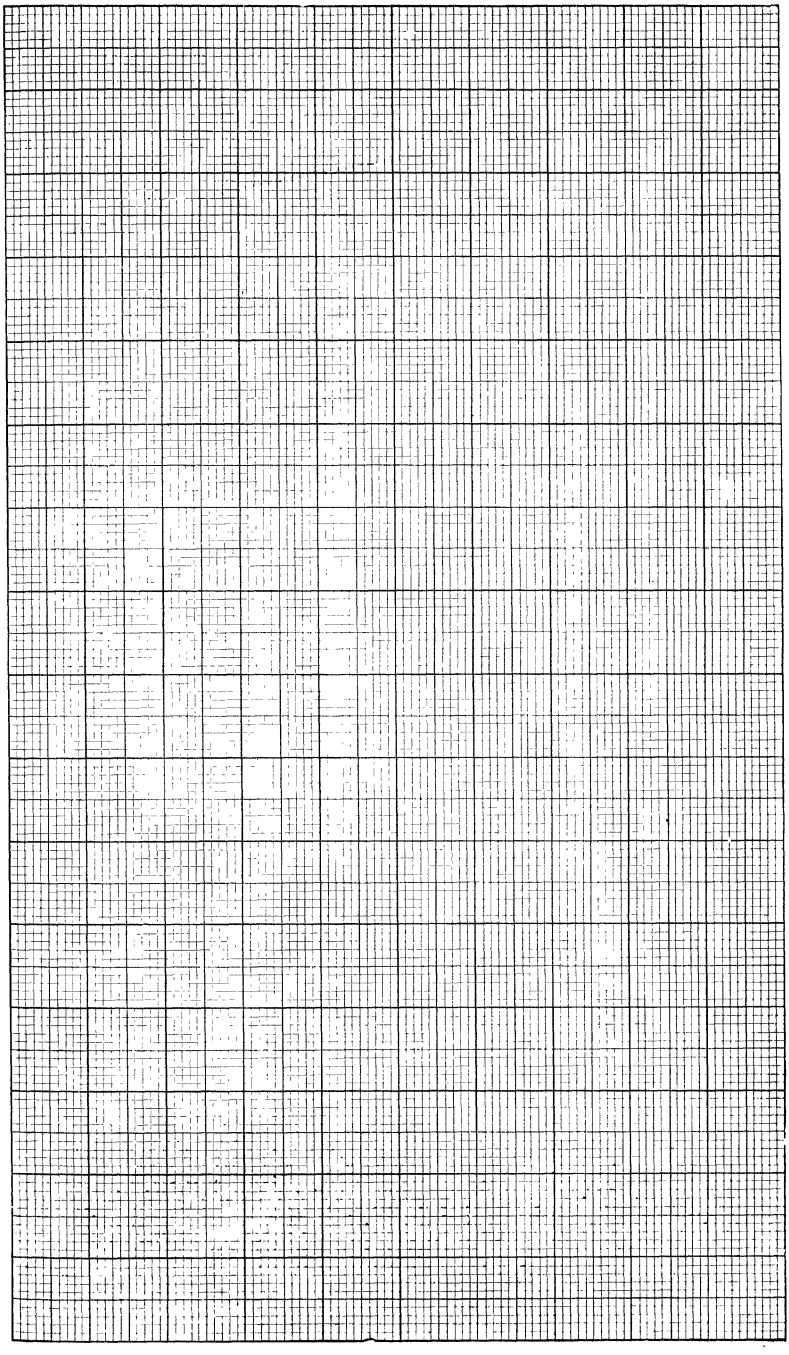
4. Strong electrolytes aid in producing maximum opalescence and also hasten the speed of its formation. It is for this reason that potassium nitrate or nitric acid is added in the above procedure recommended by Wells.

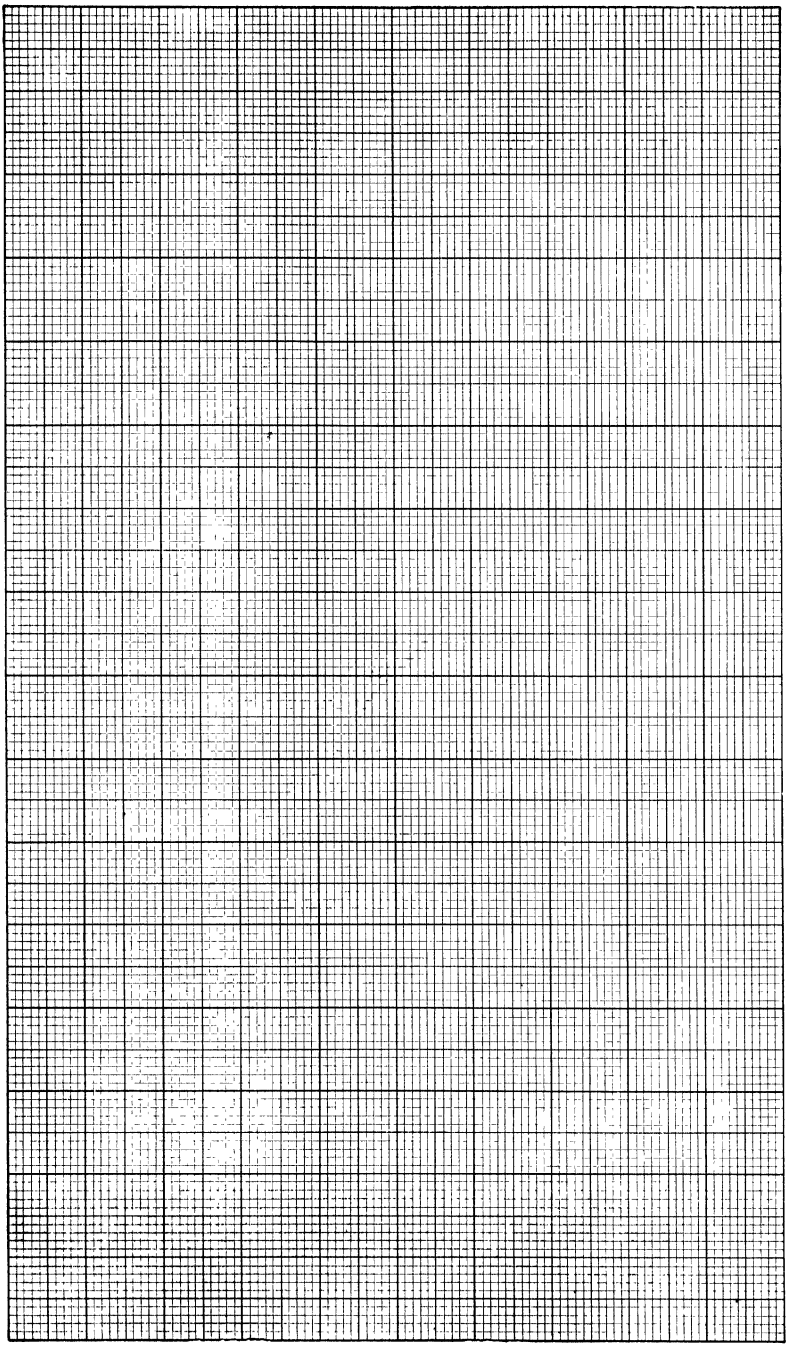
5. The time factor must be carefully regulated for intense opalescences, but ceases to be a major variable with weak ones.

6. Richards and Wells [5] state that with their instrument " the average of many results is within 1 or 2 per cent of the truth, so that the error with 2 mg. of dissolved silver chloride is probably less than 0.04 mg."

[4] Am Chem. J., **35**, 511 (1906).

[5] J. Am. Chem. Soc., **27**, 484 (1905).





DETERMINATION OF CHLORINE (AS CHLORIDE)

METHOD OF LAMB, CARLETON AND MELDRUM[6]

This method is identical in principle with the original one developed by Richards and Wells (see p. 131) but differs in technique from the latter and appears superior in two respects: (1) it develops a maximum and more constant opalescence (especially in the case of more concentrated solutions), and (2) it is simpler in that the same procedure is applicable over a wide range of concentration. The chloride in solutions ranging in concentration between 4 and 300 $\times 10^{-6}$ M can be determined with an average deviation of about 3 or 4 per cent.

The method was developed in connection with the study of " war gases " so very toxic that extremely minute amounts are significant. Even when large samples of the air-gas mixtures were collected (13 liters), it was necessary to estimate, quantitatively, chlorine as small as a few thousandths of a milligram.

The method of Richards and Wells had been applied only to aqueous solutions. In the analysis of toxic gases it was necessary to absorb them in alcoholic solutions and it was desirable to apply the method directly to this solution. After an extensive experimental study of the various factors involved, a procedure was finally obtained which satisfied all the requirements.

Reagents.

1. Nitric acid, 0.1 N, chloride-free. Prepare from acid redistilled in such a manner as to avoid contamination with dust.

2. Alcohol, chloride-free. Redistill commercial 95 per cent alcohol from sodium or potassium hydroxide, using about 2 grams of alkali to 700 cc. of alcohol.

3. Silver nitrate, 0.005 N. Use silver nitrate purified by recrystallization.

4. Standard potassium chloride solution. Prepare as directed on p. 132.

5. Distilled water. Prepare as directed on p. 132.

[6] J. Am. Chem. Soc., **42**, 251 (1920).

Procedure.—Lamb, Carleton and Meldrum used a Kober type nephelometer and observed the following procedure in its use:

At the beginning of a series of analyses both cups of the instrument and both plungers were rinsed first with distilled water and then with the standard suspension (silica or silver chloride) to be used. They were nearly filled with this solution and placed in position in the instrument. The position of the left-hand cup was adjusted until the scale reading was exactly 20.0; the position of the right-hand cup was then adjusted until the illuminations on both sides were equal, this being determined as the average of several independent settings. This setting was retained for the whole series of comparisons with the standard.

The solution in the left-hand cup was then discarded, the cup and plunger rinsed with the solution next to be used, and the cup nearly filled with the solution as before. It was placed in position in the instrument and the height adjusted until illuminations on both sides were again equal. The scale reading was recorded and the results computed using the formula given by Kober:

$$Y = (20/X) - (1 - X) \times 20 \times (0.052/X^2).$$

Where Y signifies the scale reading, X the concentration, and 0.052 is the nephelometric constant.

The chloride sample of a few cubic centimeters is diluted to 20 cc. with alcohol, 10 cc. of 0.1 N nitric acid added, followed by a large excess (10 cc.) of 0.005 N silver nitrate solution, making a total volume of 40 cc. (The resulting solution, therefore, contains approximately 50 per cent alcohol). The solution is placed in a water-bath immediately after precipitation and kept at 40° for thirty-five minutes. It is then removed, rapidly cooled to room temperature and within thirty minutes is compared in a nephelometer with suitable standards similarly prepared.

Notes.

1. A very appreciable error may result from a variation in the light conditions in successive comparisons, unless the position of the cup in its holder is always the same. It is advisable to mark the cup for this purpose.

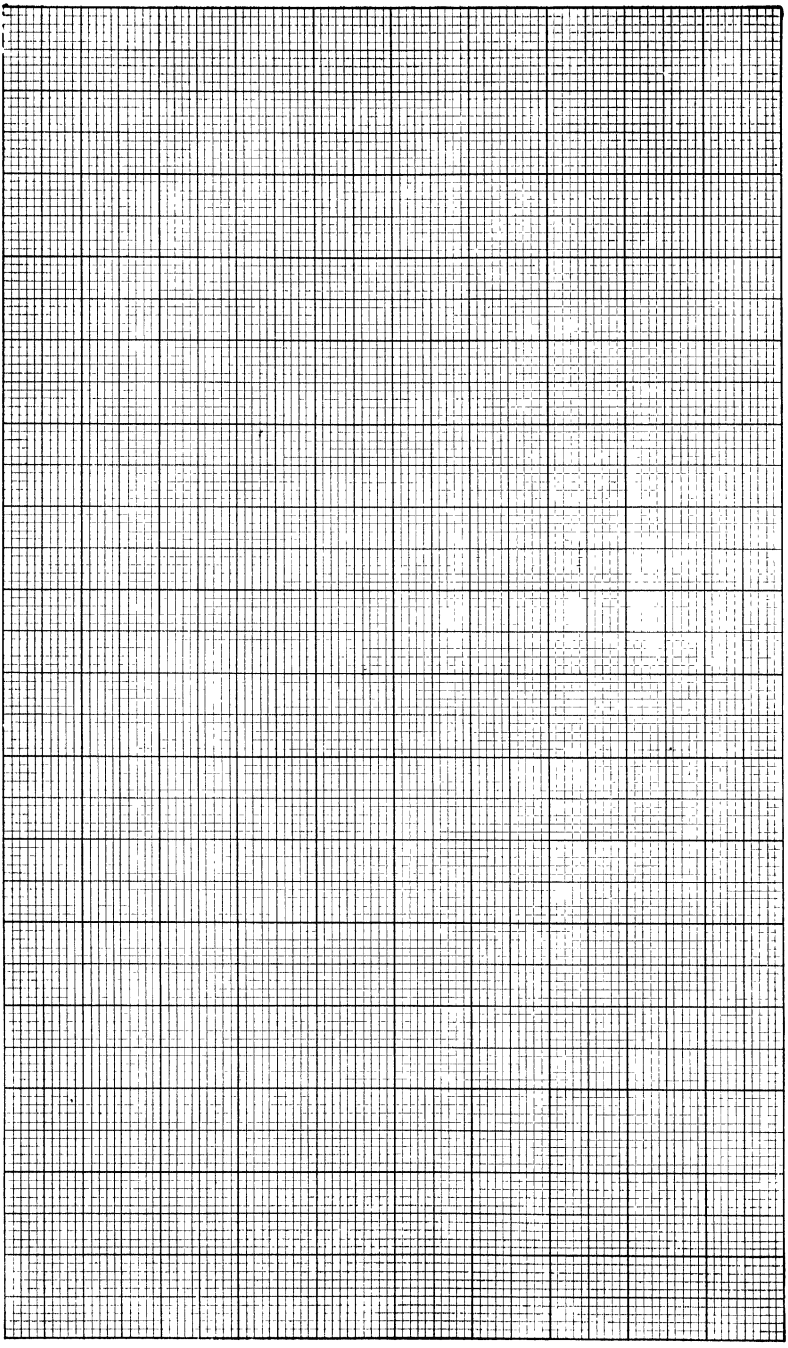
2. The precise matching of two faintly opalescent solutions is difficult. The only practical method of attaining readings of satisfactory accuracy is to raise the left-hand cup until there is a shadow just distinctly visible on the right-hand side of the divided field, then to lower it until a shadow of the same distinctness is visible on the left, and to take the mean of the extreme readings. Moreover, with these faint opalescences satisfactory results can be obtained only when the eyes have acquired their maximum sensitiveness by remaining in the dark for five or ten minutes. Similarly, too long continued observation tires the eyes sufficiently to produce a marked decrease in the accuracy of the settings. See Chapter V in Volume I.

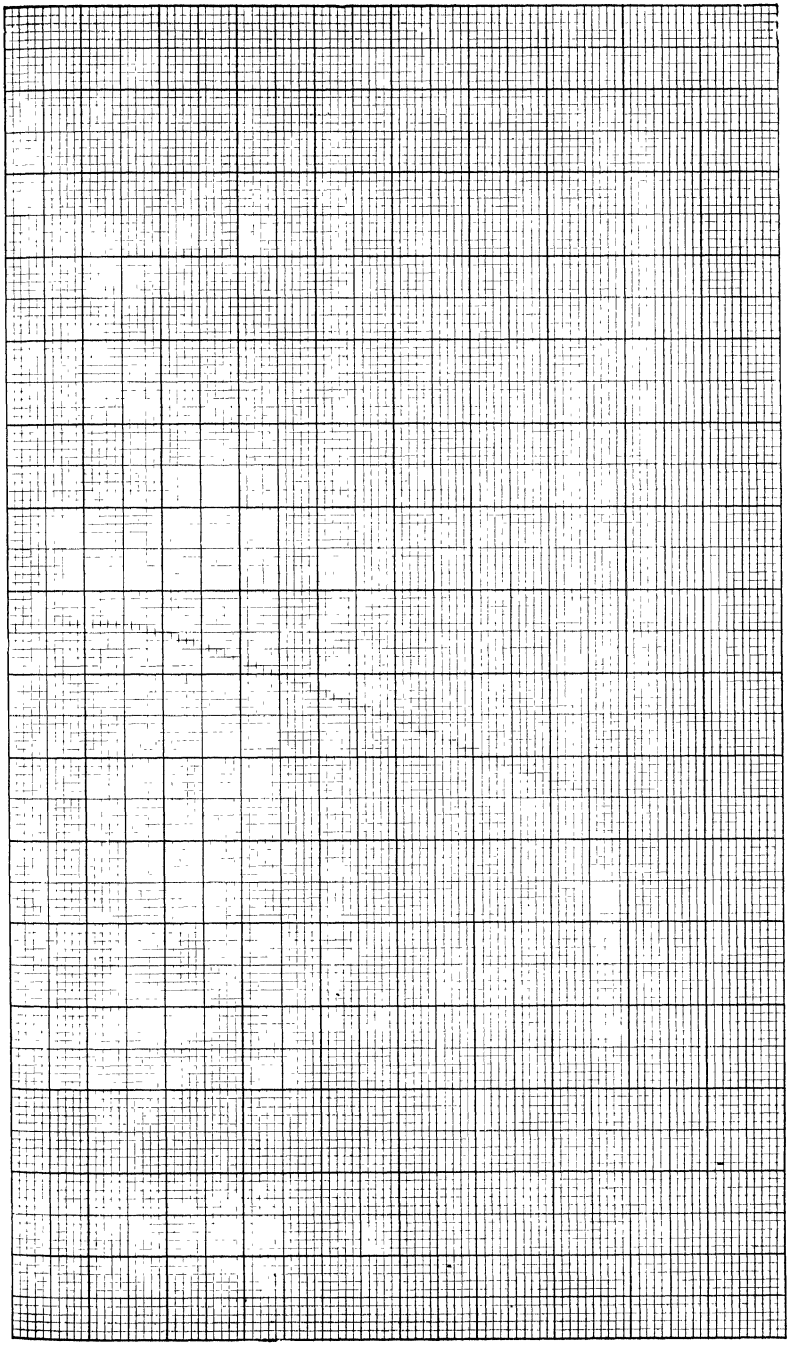
3. Lamb, Carleton and Meldrum also tried water and glacial acetic acid as solvents and found the opalescence in 50 per cent acetic substantially the same as in 50 per cent alcohol, but somewhat greater than in water. No effect of concentration was noted.

4. In a 50 per cent alcohol-water solution, heating silver chloride suspensions of widely differing concentrations to 40° for thirty minutes after precipitation, produces a more intense and constant opalescence than can be attained at room temperature; further heating for thirty minutes at 40°, or standing at room temperature for an hour, produces no perceptible change in this opalescence.[7]

5. Such opalescences decrease more rapidly in diffuse daylight than in the dark.

[7] Lamb, et al., *loc. cit.*





CHAPTER XI

PHOSPHORUS

DETERMINATION OF PHOSPHORUS

METHOD OF KOBER AND EGERER [1]

THIS determination is based upon the strychnine-molybdic acid colorimetric reagent of Pouget and Chouchak,[2] except that the reagent is made up in hydrochloric acid instead of nitric acid. The nephelometric reagent remains practically colorless for an indefinite length of time, is stable, and gives quantitative and constant results. It will detect 1.0 part of phosphorus in 333 million parts of water and 0.005 mg. of phosphorus in 10 cc. of solution, i.e., 1 part of phosphorus in 2 million parts of water can easily be estimated quantitatively with the nephelometer.

The reagent is useful in various tests and in phosphorus determinations in urine, iron, steel, etc.

A. DETERMINATION OF PHOSPHORUS IN URINE.

Reagents.

1. Sulfuric acid, sp. gr. 1.84.
2. Hydrochloric acid, 1 : 1 and 0.5 N.
3. Ammonium hydroxide, 6 N.
4. Potassium sulfate.
5. Sodium molybdate. The usual commercial grades of molybdic acid or sodium molybdate are not satisfactory. Merck's sodium molybdate may be used or a satisfactory molybdate may be prepared in the laboratory.

[1] P. A. Kober and G. Egerer, J. Am. Chem. Soc., **37**, 2373 (1915).
[2] Bull. soc. chim., **5**, 104 (1909); **9**, 649 (1911).

Boil 35.5 grams of molybdic acid (C. P. free from ammonia, " 100 per cent ") for about an hour with 50 cc. of sodium hydroxide solution (containing 400 grams of 96 per cent NaOH per liter). Dilute the mixture to 84 cc. with distilled water and shake until practically all the solid sodium molybdate is dissolved. Add several grams of pure talcum powder, thoroughly shake, and filter. In some preparations the residue may be very dark. The filtrate should be clear and should not have more than a slight yellowish tint. It is evaporated almost to dryness on a water-bath, being stirred occasionally to break up the crust of sodium molybdate which forms as the solution becomes concentrated. The residue is first washed by grinding with 40 cc. of 95 per cent ethyl alcohol, filtering, and then washed on the filter with several 20-cc. portions of alcohol. The residue should be perfectly white. It is dried in an oven at about 50° C. or in a vacuum desiccator.

6. Strychnine sulfate solution, 2.0 per cent. Place 2 grams of pure strychnine sulfate in a 100-cc. volumetric flask, add 80 cc. of water and heat to about 90° C. As soon as the salt has dissolved, the solution is cooled and diluted to the mark with distilled water.

7. Strychnine-molybdic acid reagent. Dissolve 150 grams of sodium molybdate in 250 cc. of distilled water and add slowly, with shaking, 100 cc. of 1 : 1 hydrochloric acid. The precipitate which appears at first redissolves in the excess of acid. Next add, with shaking, 150 cc. of the 2 per cent strychnine sulfate solution, allow the solution to stand over night and then filter. (See Note 3.) The filtered solution should be perfectly clear and practically colorless.

8. Standard phosphate solution. Dissolve 0.1000 gram of potassium dihydrogen phosphate, KH_2PO_4, in a little water, dilute to 2 liters, and mix thoroughly. Keep as a stock solution. Dilute 100 cc. of the stock solution to a liter and thoroughly mix. One cubic centimeter of this solution contains 0.005 mg. of $KH_2PO_4 \simeq 0.00114$ mg. P.

Procedure.—Digest 10 cc. of urine, as in a Kjeldahl nitrogen determination, with 20 cc. of sulfuric acid, sp. gr. 1.84, and 10

grams of potassium sulfate until a clear solution is obtained. Cool the melt, dissolve it in water and make up to 500 cc. Pipette out 25 cc. of the solution, neutralize with ammonium hydroxide, filter through a dry, acid-washed filter paper, and dilute the filtrate to 200 cc.

Add 30 cc. of distilled water, 5 cc. of 0.5 N hydrochloric acid, and 5 cc. of the strychnine-molybdic acid reagent to each of two flasks and shake thoroughly. To one flask add 10 cc. of the diluted sample solution and to the other add 10 cc. of the standard phosphate solution (containing 0.00114 mg. of phosphorus per cubic centimeter). The solutions are added slowly with a pipette, with gentle rotation of the flasks. After standing three minutes, the suspensions are ready for reading in the nephelometer. The amount of phosphorus in the original sample may be obtained from the nephelometer reading and a curve made similarly to the one for ammonia on p. 100.

B. DETERMINATION OF PHOSPHORUS IN IRON [3]

Dissolve 2.00 grams of cast-iron borings in 100 cc. of 1 : 2 nitric acid by boiling. Cool under the water tap and dilute to 100 cc. Pipette off 5 cc., mix with 5 cc. of sulfuric acid, sp. gr. 1.84, boil for two minutes to eliminate the nitric acid, dilute to 100 cc. and filter through a dry, acid-washed filter paper. Twenty-five cubic centimeters of this solution are diluted to 100 cc.

Place in each of two flasks 35 cc. of 0.5 N hydrochloric acid and 5 cc. of the strychnine-molybdic acid reagent and shake thoroughly. To one flask add 10 cc. of the diluted sample solution and to the other add 10 cc. of the standard phosphate solution (containing 0.00114 mg. of phosphorus per cubic centimeter). The solutions are added slowly with a pipette, with gentle rotation of the flasks. After standing three minutes, the suspensions are ready for matching in the nephelometer. The percentage of phosphorus in the original sample may be obtained from the nephelometer reading and a curve made similarly to the one for ammonia on p. 102.

[3] P. A. Kober, J. Ind. Eng. Chem., **10**, 561 (1918).

Notes.

1. Kober and Egerer[4] on studying the reaction between phosphate solutions and the strychnine-molybdic acid reagent of Pouget and Chouchak, found that (*a*) it was not constant and quantitative and (*b*) the reagent gradually became yellow and deteriorated, probably due to the action of the nitric acid. Pouget and Chouchak realized this and directed that the constituents must be mixed just before using.

After making many variations of all constituents with no marked improvement, Kober and Egerer found " on substituting hydrochloric for nitric acid, the solution not only remained practically colorless for an indefinite length of time, but was stable and gave quantitative and constant results."

2. A check on the purity of the reagents should be made by a " blank " determination and a correction made if necessary.

3. Ordinary filter paper cannot be used, since the acid solution extracts from it a substance which gives a very marked cloud on standing. The substance seems to be a phosphorus compound. Kober and Egerer[5] found satisfactory S & S No. 575, a hardened paper, and S & S No. 589, a quantitative paper. In any case, a test should be made to be certain that no error will be introduced by the filter paper.

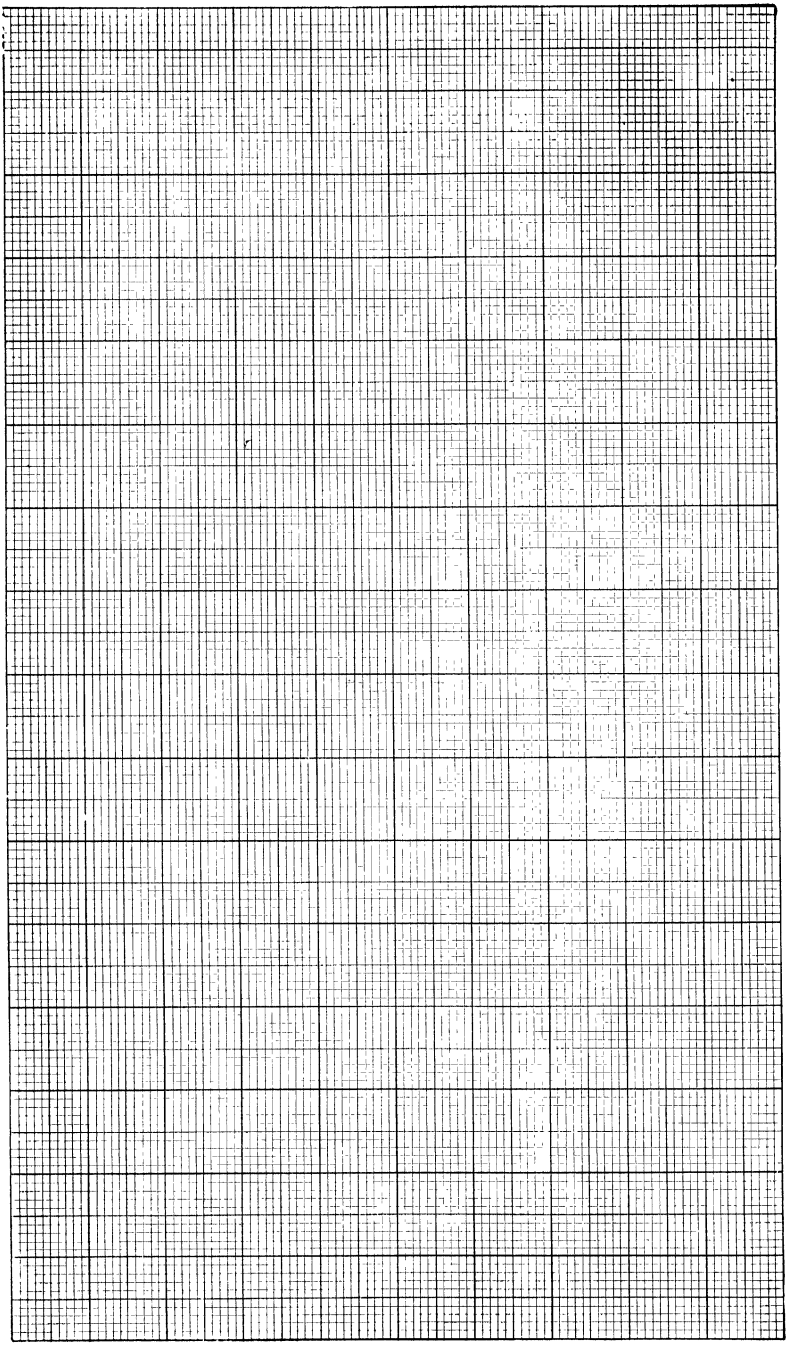
4. Usually the sample solution is so diluted that very few substances are likely to interfere with the reaction between the reagent and phosphate. In an extreme case it might be necessary to precipitate the phosphate as phosphomolybdate, filter, and dissolve the precipitate. As a rule, any solution containing phosphate, but no organic matter, may be used at once, provided the solution is clear, and either neutral or only slightly acid.

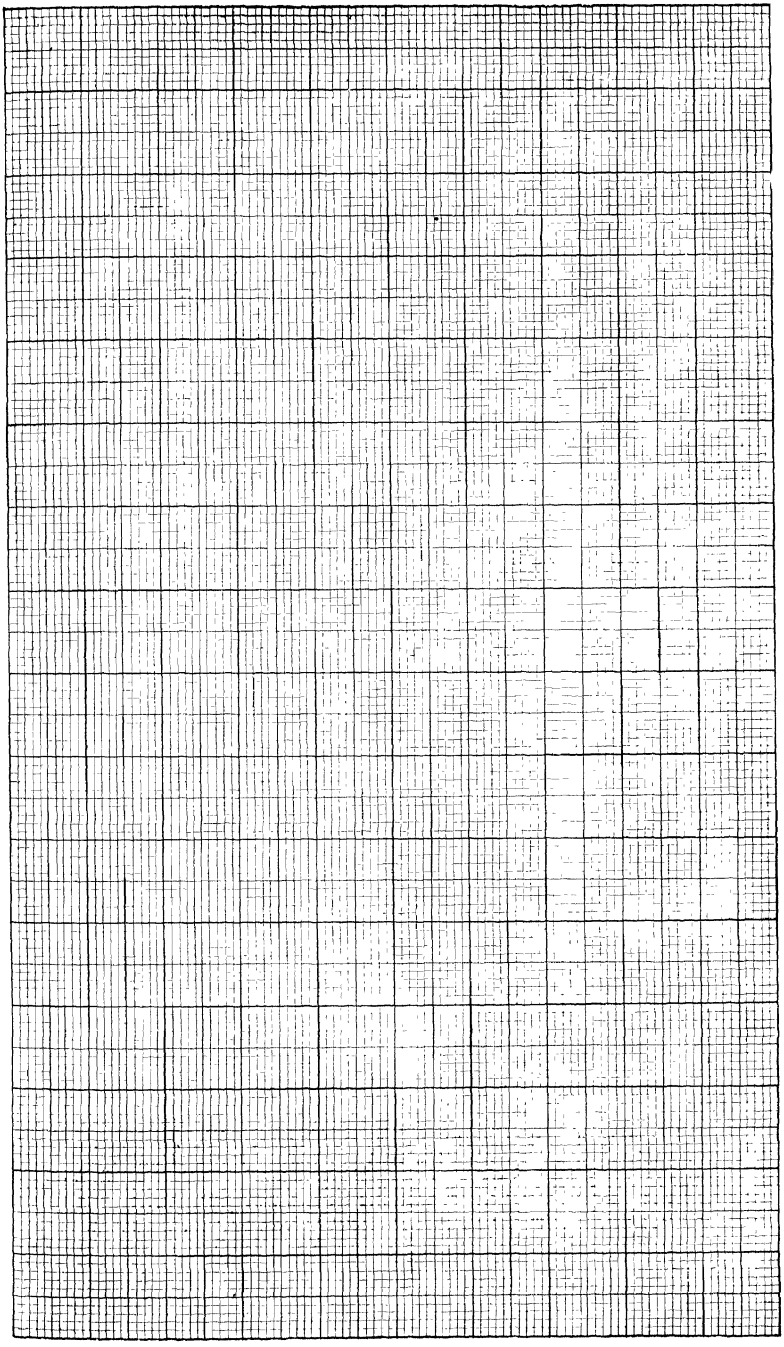
5. " Protective action in addition to any produced by the strychnine does not seem to be necessary."[6]

[4] J. Am. Chem. Soc., **37**, 2373 (1915).

[5] *Loc. cit.*

[6] P. A. Kober, J. Ind. Eng. Chem., **10**, 560 (1918).





DETERMINATION OF PHOSPHORIC ACID

By Hans Kleinmann [7]

This method depends upon the production of a nephelometric cloud in a sulfuric acid solution of phosphates by means of a strychnine-molybdenum reagent, and matching the turbidity with that of a standard similarly prepared. The turbidity is proportional to the phosphoric acid content. The directions must be clearly observed in order to obtain accurate results.

Reagents.

1. Sulfuric acid, 2 N. Use only the purest concentrated sulfuric acid. Dilute approximately 54 cc. of concentrated H_2SO_4 to 1000 cc. with distilled water and standardize against normal NaOH solution. The solution need not be exactly 2 N but its concentration must be accurately known, since an accurate control of the acidity is necessary for the determination.

2. Saturated sodium sulfate solution. Saturate a liter of solution at about 50° C., cool, and allow it to stand stoppered and in contact with the excess crystals. Filter off the solution as needed.

3. Molybdenum-strychnine reagent. This reagent is prepared in two parts. These are mixed just before using, since the addition of the acid molybdenum solution to the strychnine solution produces sediment after twenty-four hours. The sediment is probably strychnine sulfate crystals.

Solution A. Sulfuric acid-sodium molybdate solution. Thirty grams of pure molybdic acid are placed in a 500-cc. round-bottomed flask. Then 10 grams of anhydrous, freshly ignited sodium carbonate and about 200 cc. of distilled water are added. The mixture is heated slowly over a free flame and kept boiling until a perfectly clear solution has formed. This usually takes place in fifteen to thirty minutes. Traces of impurities which do not go into solution are filtered off while the solution is still hot. To the filtrate are added 200 cc. of 10 N H_2SO_4. An approxi-

[7] Biochem. Z., **174**, 43 (1926).

mately 10 N H_2SO_4 solution can be made by diluting 280 cc. of sulfuric acid, sp. gr. 1.84, to 1000 cc. A dilution of 1 to 10 of the acid is titrated with N NaOH and the factor of the solution obtained. Such amounts of sulfuric acid are used as are equivalent to 200 cc. 10 N H_2SO_4.

After cooling, the mixture is made up to 500 cc. in a graduated flask. A clear, light blue solution is thus obtained. It is advisable, since the acidity is very important, to test it by making a dilution of 1 to 100 with distilled water and determining the hydrogen-ion concentration electrometrically. The *p*H should be 1.41.

Solution B. Strychnine solution.—Dissolve 1.6 grams of strychnine sulfate (bisulfate, Kahlbaum) in about 100 cc. of distilled water with slight warming. After cooling, dilute the clear solution to 500 cc. with distilled water.

To make the reagent, solutions *A* and *B* are mixed as follows: Equal volumes of *A* and *B* are pipetted into separate flasks. Then solution *B* is quickly poured into solution *A* and the flask thoroughly shaken. A bluish-white precipitate forms a cloud but in a few minutes agglutinates and is filtered off through a quantitative filter paper. The clear colorless solution will keep about a day. It is advisable to prepare it just before using. Solutions *A* and *B* will keep separately indefinitely.

4. Standard phosphate solution. From the purest primary potassium phosphate (for enzyme-studies according to Sörensen, Kahlbaum) 1.9167 grams are weighed off, dissolved, diluted to 1000 cc. with distilled water and thoroughly mixed. This solution contains 1 mg. P_2O_5 per cubic centimeter. From this stock solution 5 cc. are measured exactly, diluted to 1000 cc. and mixed thoroughly. This solution contains 0.005 mg. P_2O_5 per cubic centimeter. One milligram of P_2O_5 is equivalent to 0.4379 mg. P. Therefore the solution contains 0.00219 mg. P per cubic centimeter. This solution serves as a standard. It should be freshly prepared.

Procedure.—*Ashing of the sample.* For the determination of P_2O_5, the substance to be analyzed is ashed. In the determination of different phosphoric acid fractions, i.e., " protein phos-

phorus," " lipoid phosphorus," etc., after isolating according to the usual methods, the fraction or an aliquot portion is ashed as described for the determination of P_2O_5. Pieces of organs (in the form of a mush) or fluids are evaporated to dryness on the water-bath in a platinum crucible, and then ashed over a Meeker burner until the residue is entirely white. In the presence of metal oxides it ought to be free from carbon. The ashing succeeds well, as a rule, if the crucible is kept covered. For example, the ashing of 1 cc. of serum takes only a few minutes.

If the ash contains heavy metals (iron up to 1 mg. per analysis of phosphorus can be neglected—see below) these must be removed.

The residue or—if no heavy metals are present—the original white ash is treated with a few cubic centimeters of 2 N H_2SO_4. Then the crucible is warmed on the water-bath five to ten minutes. This treatment hydrolyzes the meta-phosphoric acid formed by ignition and converts it into ortho-phosphoric acid. The solution is rinsed into a graduated flask and the crucible treated several times by warming with a little 2 N H_2SO_4, and rinsing into the graduated flasks.

The amount of sulfuric acid necessary for solution of the ash depends upon the amount of P_2O_5 present. If the total ash is to be used for a determination, not more than 0.5 cc. of H_2SO_4 should be used. If only a part of the dissolved ash is to be used, then make it up to a desired volume with 2 N H_2SO_4. If a number of duplicate determinations are to be made the total amount used may be 25 or 50 cc. of acid.

If alkaline earths are in the ash, the undissolved alkaline earth sulfates are filtered off, and the filtrate washed with acid to be used in making up the required volume. If the total ash is used for analysis, it is better to take a somewhat greater amount of acid than is generally used (9.5 cc.), for example, 12 cc. The solution is then centrifuged and 9.5 cc. taken from the supernatant liquor and used for analysis. This procedure of course requires allowance to be made for the aliquot portion taken, when the final calculation is made.

Treatment of the dissolved ash.—A 2 N acid solution of the ash,

or pure phosphate solution whose content of sulfuric acid is exactly 2 N, is used for the analysis. Of this solution, a volume whose content of P_2O_5 is 10 to 50 μg. (1 μg. is 0.001 mg.) is taken, but not more than 9.5 cc. for analysis. If the P_2O_5 content of the solution is not known approximately, a portion of the ash solution is taken and made up to 9.5 cc. with 2 N H_2SO_4 and then 4 cc. of saturated Na_2SO_4 are added. The volume is then made up to 23 cc. with distilled water.

Now prepare from the phosphate standard solution several solutions for comparison with varied phosphate content. For example, take 2, 4 and 8 cc. equivalent to 10, 20 and 40 μg. P_2O_5 respectively, and add 9.5 cc. of 2 N H_2SO_4 and 4 cc. of Na_2SO_4 and make up to a volume of 23 cc. with distilled water. Now add to each of the solutions 2 cc. of reagent and gauge with the eye the amount of the unknown compared to the known concentration, after allowing thirty minutes for the solutions to come to equilibrium. After a few phosphorus determinations have been made, no standard cloud will be necessary to gauge the strength of the unknown roughly, to make it suitable for the determination. A volume of the ash solution which has from 10 to 50 μg. P_2O_5 is then used for the actual determination.

A corresponding amount of the phosphate solution is diluted with 2 N H_2SO_4 to 9.5 cc. The measurement is best done with a burette. Test tubes can be used for containers, but flasks of 30 to 50 cc. capacity with ground glass stoppers are preferred because they permit shaking without contamination.

The acid does not need to be exactly 2 N but the factor must be accurately known. Then the corresponding volume is used, since it is not the acid volume but the molecular concentration that counts.

Then 4 cc. of the saturated Na_2SO_4 solution are added and the solution made up to 22 cc. with distilled water.

The solution used for comparison is made with standard phosphate by mixing the necessary number of cubic centimeters of phosphate solution with 9.5 cc. H_2SO_4 plus distilled water to make a total volume of 22 cc. It is most convenient to prepare different solutions for comparison, having say from 20 to 40 μg.

P_2O_5 (corresponding to 4 and 8 cc. of standard phosphate solution).

It is advisable to prepare duplicate or triplicate test and standard solutions. Then to each test and standard solution are added 2 cc. of reagent. The exact measurement is best done with Bang's micro-burette. The solutions are shaken and the time of the addition of the reagent noted.

If a series of tests is to be made, it is advisable after five or six analyses (including duplicates) to make ten-minute pauses in adding the reagent, in order to have ample time for the measurement.

The solutions begin to cloud gradually. After twenty-five to thirty minutes' standing the content of the test solution as compared to the standard can be gauged and decision made whether or not it contains more than 20 μg. of P_2O_5. It is therefore recommended that the operator have always a standard of 20 μg. P_2O_5 at hand. The further treatment depends upon whether it contains more or less than 20 μg. of P_2O_5.

(*a*) When measuring concentrations over 20 μg. P_2O_5 in the volume used, 1 cc. of water is added to the test solution as well as to the standard, whereupon the clouds are immediately measured nephelometrically. The measurement is made fifteen to twenty minutes after the addition of the water, i.e., during the third quarter of an hour after the addition of the reagent.

(*b*) When measuring concentrations under 20 μg. of P_2O_5, thirty minutes after the reagent is added, instead of adding 1 cc. of water, 1 cc. of 2 N H_2SO_4 is added. The clouds are then allowed to stand an additional fifteen minutes and are measured nephelometrically during the fourth quarter of an hour after the addition of the reagent.

The nephelometric measurement.—In regard to nephelometric technique, see Chapter IV. The limits of measurements are the concentrations of 10 to 30 μg. P_2O_5 in 25 cc. volume. Obviously higher concentrations can be measured by suitably diluting. Below 10 μg. per 25 cc. should not be attempted because, first, the cloud is so weak that impurities such as shreds, etc., become

noticeable and, secondly, because the proportion between cloud and concentration does not hold.

If smaller amounts than 10 μg. P_2O_5 are to be determined, use for the ordinary nephelometric cup only 12.5 cc. and then use only one-half of the above-mentioned amounts of reagent, dilution, etc. In this way, the amount of P_2O_5 that can be determined with ordinary nephelometric cups can be as small as 0.005 mg., i.e., 5μg. P_2O_5.

For still smaller amounts of P_2O_5 the micro-nephelometric cups must be used. If one takes of all solutions the fifth part, which can still be measured accurately with pipettes graduated 1/100 cc., one can still measure 0.001 mg., ie., 1 μg. P_2O_5, if one takes into consideration the somewhat larger error of the micro-nephelometric cups.

It is most suitable to work in the middle of the range of determinations from 40 to 20 μg. P_2O_5 in 25 cc. Here the error of the method is about 1 per cent. If one works near the limits of the determination, the error is ± 2 per cent.

To eliminate the occasional off result, it is recommended always to make duplicate or triplicate determinations.

Notes.

1. Influence of extraneous substances on the analysis: Admixture of salts in the solution of the ash after treatment with sulfuric acid can be present only as sulfates. Alkali sulfates do not disturb if they are not present in too great an excess. The addition of 0.5 cc. of saturated Na_2SO_4 solution to the system has little or no influence. Alkaline earth sulfates are eliminated due to their insolubility.

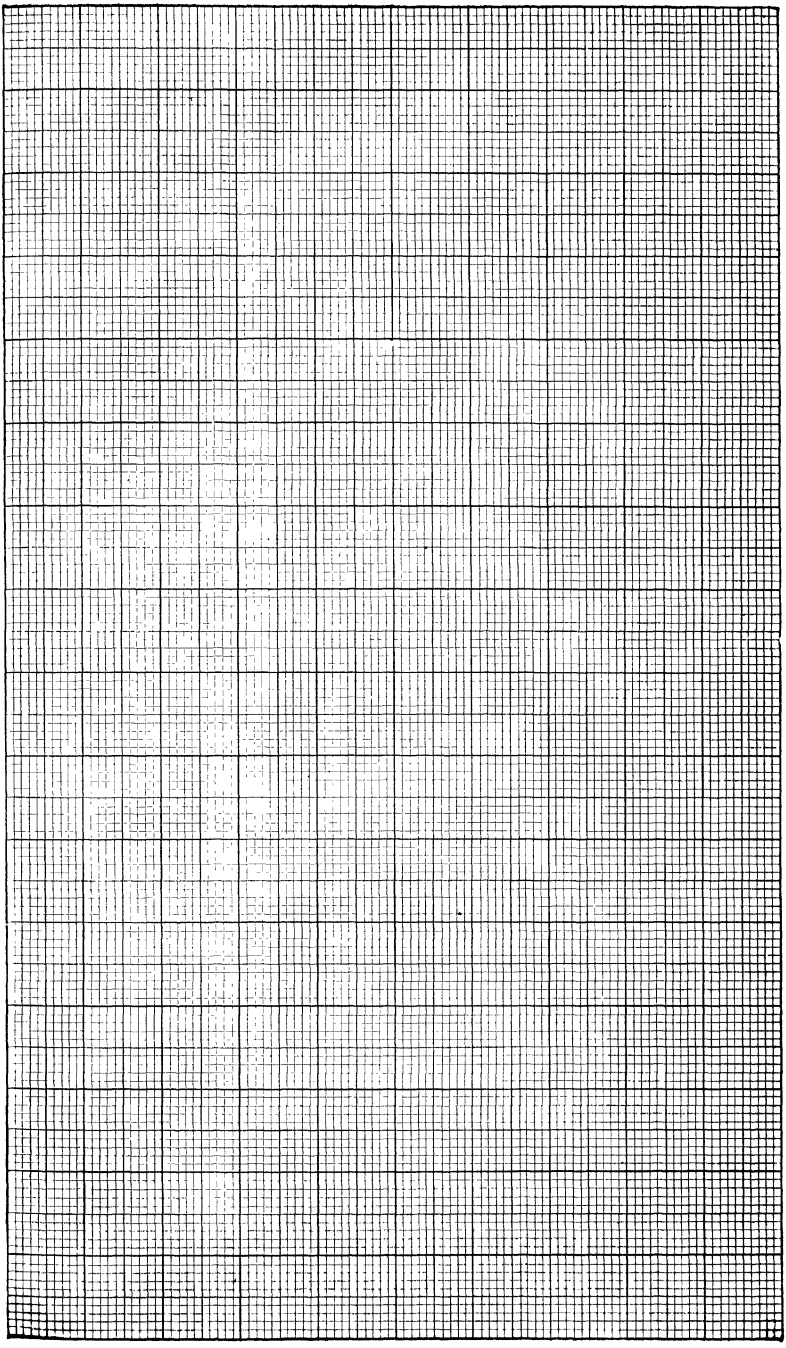
Magnesium does not disturb up to 10 mg. per 10 to 50 μg. of P_2O_5, that is, in 200- to 1000-fold amounts. Likewise, iron does not disturb in a concentration up to 7 mg. per analysis, i.e., in 20- to 100-fold amounts. Higher amounts of these admixtures (and other heavy metals) must be removed.

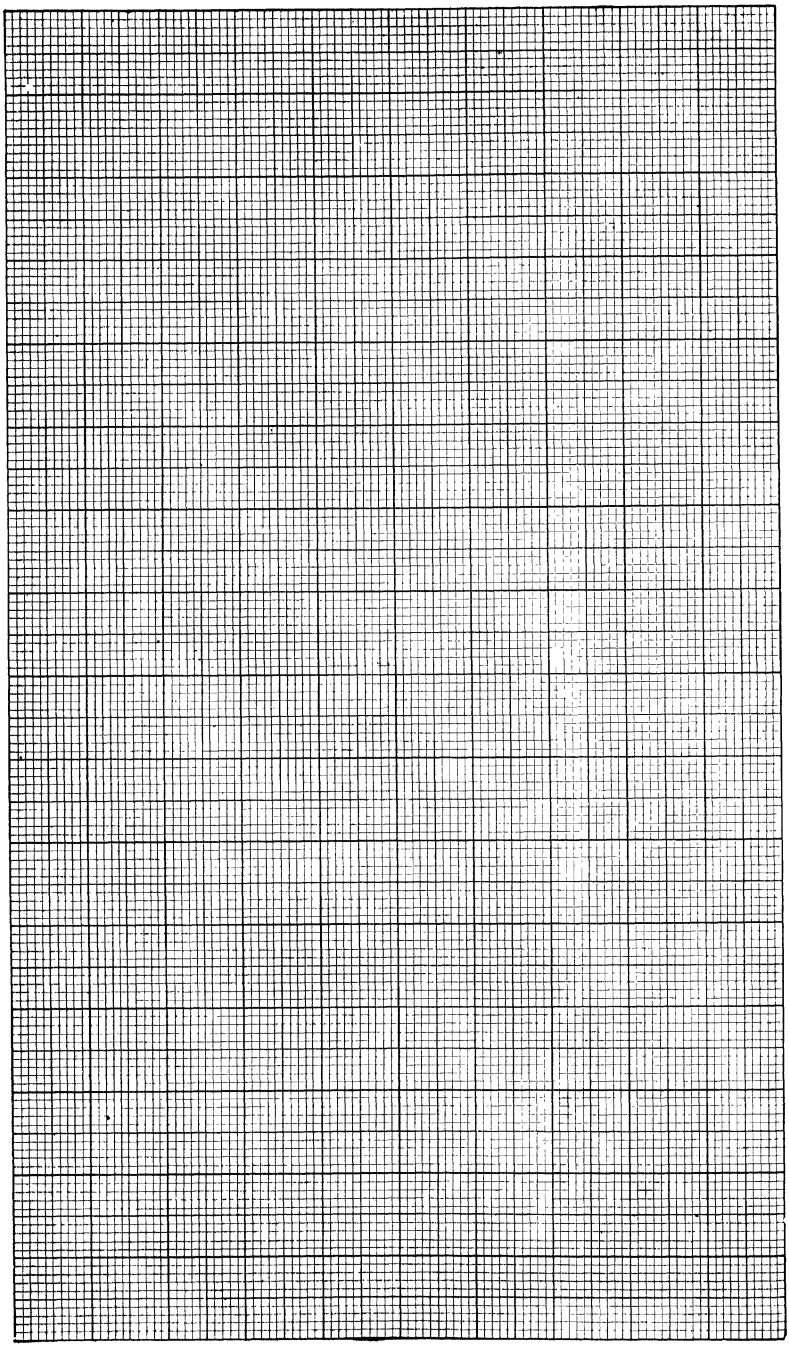
Arsenic acid does not disturb in the presence of sodium sul-

fate, since under the conditions of the determination it does not give a cloud. Therefore the reagent can be used for the determination of phosphorus in the presence of arsenic acid.

2. The method permits the determination of P_2O_5 as low as 0.005 mg. when using the macro-cups (Kleinmann's nephelometer) and 0.001 mg. P_2O_5 when using the micro-cups. The experimental error amounts to approximately ± 2 per cent. The analyses within the limits given are easily reproducible.

3. The technique of the method is much simpler than is apparent from the description. Aside from the time allowed for the clouds to form, the determination requires only a few minutes.





CHAPTER XII

SULFUR

DETERMINATION OF SULFATES AND TOTAL SULFUR IN BLOOD

METHOD OF DENIS AND REED [1]

THIS method is based upon the formation of colloidal barium sulfate in uniform suspension under certain conditions and with gelatin as a protective colloid. By means of the procedure given below it is possible to determine inorganic sulfates, total sulfates, and total sulfur in the protein-free filtrate obtained from 30 cc. of human blood or from 15 cc. of dog or beef blood, with a maximum error of 5 per cent.

Reagents.

1. Hydrochloric acid, 0.1 N and 1 N.
2. Trichloroacetic acid, 20 per cent. Use sulfate-free trichloroacetic acid prepared by redistilling the C. P. acid *in vacuo* at a pressure of 15 to 20 mm. It is usually necessary to filter this through sulfate-free filter paper, since it generally contains suspended particles.
3. Sodium hydroxide, 0.6 N. Prepare from metallic sodium, since sodium hydroxide frequently contains a trace of sulfate.
4. Barium chloride, 1 per cent.
5. Gelatin solution, 5 per cent (sulfate-free). All the specimens of high-grade commercial gelatin contain traces of sulfates and it is therefore necessary to free the gelatin from this impurity before its use as a protective colloid.

Sulfate-free solutions that will keep for long periods without the

[1] J. Biol. Chem., **71**, 191 (1926).

formation of mold, etc., may be prepared as follows: To 50 grams of any high-grade commercial gelatin [2] add 900 cc. of approximately 0.01 N hydrochloric acid and 100 cc. of a 5 per cent solution of barium chloride. Immerse the flask in a bath of boiling water and heat at this temperature, with occasional shaking, for one hour. At the end of this period of heating remove the flask from the water-bath, cool in running water, add 50 cc. of egg-white, mix thoroughly, and heat again in a boiling water-bath for about thirty minutes or until the egg-white has coagulated and carried down all traces of colloidal barium sulfate. Centrifuge while hot, using 50- or 100-cc. centrifuge tubes, pour off the clear supernatant liquid into small wide-mouth bottles of about 30 cc. capacity, stopper these bottles with cotton, sterilize the preparation by heating the bottles for one hour in boiling water, allow to cool, and store in the refrigerator. When kept in an ordinary domestic refrigerator preparations by Denis and Reed have remained in good condition for as long as eight months.

6. Zinc nitrate oxidizing mixture. This reagent is prepared as follows: 25 grams of zinc nitrate, C. P.; 25 grams of sodium chloride, C. P.; 10 grams of ammonium chloride, C. P.; 100 cc. of distilled water. When the salts are completely dissolved, the mixture is filtered through an ashless filter paper. Each lot of reagent should of course be tested for the presence of sulfates before use.

7. Standard K_2SO_4 solutions. Two standards are required. Standard 1 contains 0.5437 gram of potassium sulfate per liter. One cubic centimeter of this solution is equivalent to 0.1 mg. of S. Standard 2 is prepared by diluting 1 volume of Standard 1 with an equal volume of water. One cubic centimeter of this solution is equivalent to 0.05 mg. of S.

Procedure.—*Precipitation of protein from blood or plasma.*—Fifty cubic centimeters of citrated blood or plasma are treated with 110 cc. of distilled water and 40 cc. of a 20 per cent solution of trichloroacetic acid. The mixture is thoroughly shaken, allowed to stand fifteen minutes, the precipitate centrifuged out,

[2] The Difco brand of powdered gelatin manufactured by the Digestive Ferments Company of Detroit is satisfactory.

and the supernatant liquid filtered through acid-washed filter paper (Whatman's No. 40).

Inorganic sulfate in dog or beef blood.—Mix 15 cc. of trichloroacetic acid filtrate, 2 cc. of 0.6 N NaOH, 1 cc. of 5 per cent gelatin solution, and 5 cc. of 1 per cent barium chloride solution. Allow the mixture to stand fifteen minutes and then read in the nephelometer against the following standard which has been prepared at the same time as the unknown: One cubic centimeter of standard K_2SO_4 solution (equivalent to 0.1 mg. of S), 14 cc. of 0.1 N HCl, 2 cc. of 0.6 N NaOH, 1 cc. of 5 per cent gelatin solution, 5 cc. of 1 per cent barium chloride.

Inorganic sulfate in human blood.—On account of the very small concentration of inorganic sulfate in normal human blood it is necessary to use 25 cc. of trichloroacetic acid blood filtrate for the determination. To this add 4 cc. of 0.6 N NaOH, 1 cc. of 5 per cent gelatin, and 1 cc. of 5 per cent $BaCl_2$. Allow to stand fifteen minutes before reading in the nephelometer.

The standard is made with 1 cc. of K_2SO_4 containing 0.05 mg. of S per cc., 25 cc. of 0.1 N HCl, 3 cc. of 0.6 N NaOH, 1 cc. of 5 per cent gelatin, and 1 cc. of 5 per cent $BaCl_2$. Allow standard and unknown to stand fifteen minutes before reading in the nephelometer.

In nephritic blood where the sulfate content is high, the procedure outlined for dog or beef blood may be used.

Total sulfates in dog or beef blood.—To 10 cc. of blood filtrate add 4 cc. of normal HCl and evaporate at such a rate that solid particles begin to settle out in not less than fifteen to twenty minutes. At this point the heating should be discontinued. This evaporation is best carried out in a large (20 × 200 mm.) Pyrex test tube heated by means of a micro burner. The evaporation of the last few cubic centimeters of liquid must of course be carried out with considerable care in order to prevent undue discoloration. To the residue in the test tube add 15 cc. of water, 2 cc. of 0.6 N NaOH, 1 cc. of 5 per cent gelatin solution, and 5 cc. of 1 per cent barium chloride solution. Allow to stand fifteen minutes and then read against a standard which has been prepared at the same time as the unknown in the following

manner: 1 cc. of standard K_2SO_4 solution (equivalent to 0.1 mg. of S), 4 cc. of normal HCl. Evaporate in a manner identical with that used for the unknown; to the residue add 15 cc. of water, 2 cc. of 0.6 N NaOH, 1 cc. of 5 per cent gelatin solution, and 5 cc. of 1 per cent barium chloride solution.

Total sulfates in human blood.—The method for human blood is identical with that for dog or beef blood with one exception, namely, that it is necessary to evaporate 15 cc. of filtrate with 4 cc. of the N HCl rather than 10 cc. of filtrate. It is best to prepare two standards, the 0.05 mg. of S standard, and the 0.1 mg. of S standard.

If nephritic blood, having a high sulfur content, is used, the method for dog or beef blood may be used unaltered.

Total sulfur in dog or beef blood.—To 3 to 5 cc. of blood filtrate add 1 cc. of zinc nitrate oxidizing mixture. Evaporate to dryness in a large (20 × 200 mm.) Pyrex tube and continue to heat with a free flame until no more fumes are given off; dissolve the residue in 2 cc. of normal HCl, add 15 cc. of distilled water, 1 cc. of 5 per cent gelatin solution, and 5 cc. of 1 per cent barium chloride; allow to stand 15 minutes, then read in the nephelometer against a standard prepared as follows and as nearly as possible at the same time as the unknown: 1 cc. of K_2SO_4 solution (equivalent to 0.1 mg. of S), 1 cc. of oxidizing mixture. Evaporate to dryness and heat with a free flame by the same technique used for the unknown, then dissolve the residue in 2 cc. of normal HCl, add 15 cc. of distilled water, 1 cc. of 5 per cent gelatin, and 5 cc. 1 per cent barium chloride.

Total sulfur in human blood.—The method for total sulfur in human blood varies from that for dog or beef blood only in the amount of blood filtrate oxidized with the $Zn(NO_3)_2$ oxidation mixture. It is generally necessary to use from 5 to 10 cc. of filtrate with normal blood, though as little as 3 cc. of filtrate may be used in blood showing sulfur retention.

Ethereal sulfates are calculated by difference between the values obtained for total and inorganic sulfates; neutral sulfur by the difference from the values obtained for total sulfur and total sulfates.

Notes.

1. The quantitative precipitation of colloidal barium sulfate in uniform suspension is accomplished only under certain conditions, and in order to bring about this result, a protective colloid has been used, namely, 1 cc. of 5 per cent gelatin in 25 cc. volume. Satisfactory colloidal precipitation will not occur under these conditions if the amount of sulfur in 25 cc. volume is more than 0.16 to 0.2 mg. of S or less than 0.02 mg. of S. If more than 0.20 mg. of S is present, the precipitated particles are larger than those of a true colloidal sol and soon settle out making the suspension uneven; if smaller, the cloud is too light to read in the nephelometer. Since the amount of sulfur present as sulfate determines to some extent the size of the particles, it is best to prepare both the 0.1 mg. and the 0.05 mg. of S standard and read the unknown against the standard which appears most nearly to approach it in turbidity.

2. In precipitating barium sulfate in the presence of other salts it is necessary to take into consideration the fact that barium phosphate is also insoluble in slightly acid, neutral, or alkaline solution. The solution therefore must be acid. If, however, the solution is too acid, barium acid sulfate, a soluble salt, is formed and the precipitation of the sulfate ion is incomplete. From long experimental practice Denis and Reed have found that a *p*H of 3.0 to 3.8 is optimum for the precipitation of all the barium sulfate and none of the barium phosphate. Sometimes in work on pathological material the trichloroacetic acid filtrates are of stronger acidity than usual, due apparently to the presence of abnormally small amounts of blood proteins. In this case the directions given for the amount of sodium hydroxide to produce the correct *p*H may have to be varied. If upon precipitation of a filtrate no sulfate shows as a cloud, before deciding that none is present it is advisable to determine the *p*H. It has long been known that certain ions have a tendency to prevent quantitative sulfate precipitation and that the presence of others is beneficial to this process. Denis and Reed have confirmed this fact in that

they have found that the presence of sodium chloride is essential to obtain a correct nephelometric reading.

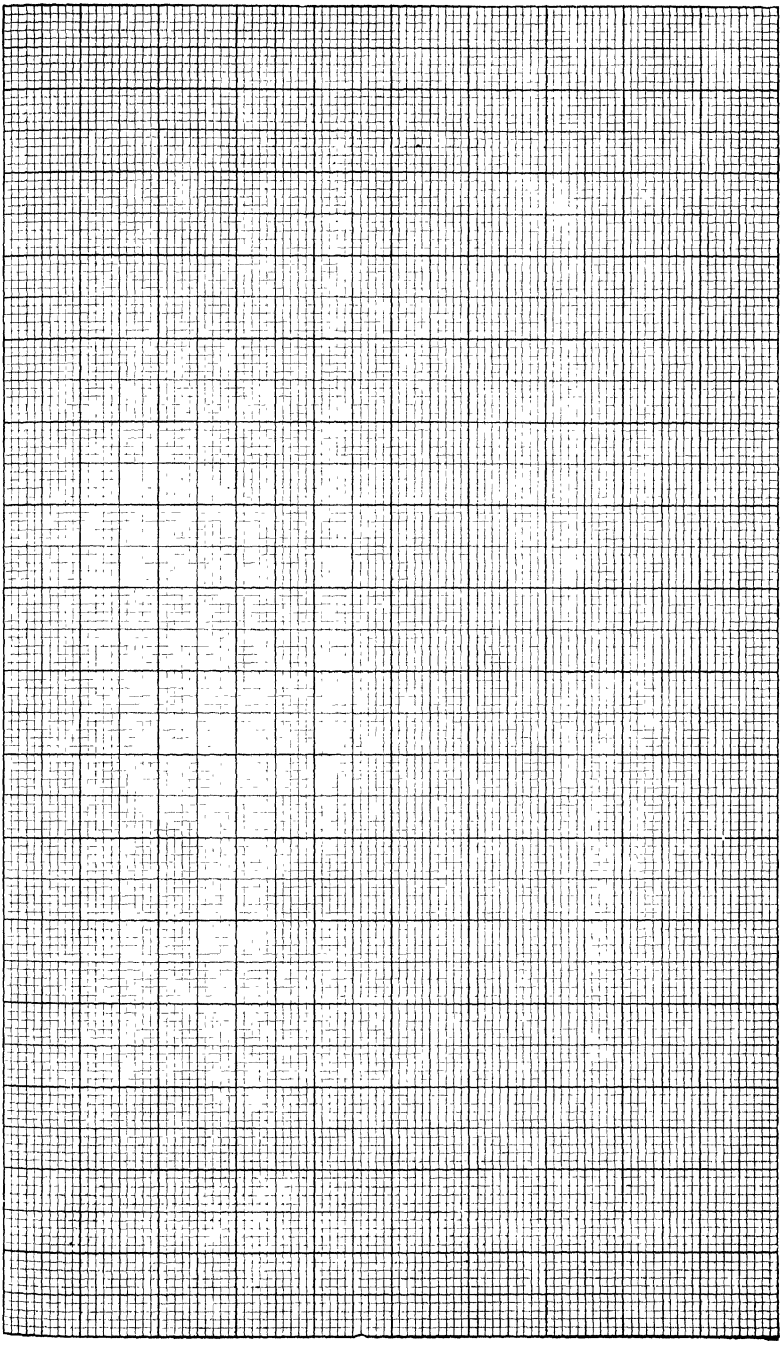
3. In all the determinations the neutralization of hydrochloric acid by sodium hydroxide is used as the source of the necessary sodium chloride with the exception of the total sulfur, in which case sodium chloride is present in the oxidation mixture.

4. If the oxidation mixture used in the determination of total sulfur is not heated until all trace of nitrate is decomposed and driven off, very low results will be obtained; indeed, in some cases the deficiency may amount to as much as 50 per cent.

5. Table X gives a comparison of results (Denis and Reed) obtained by gravimetric and nephelometric determinations of the sulfur compounds in blood.

TABLE X

Sample	Gravimetric Method, Mg. of S per 100 cc. of Blood			Nephelometric Method, Mg. of S per 100 cc. of Blood			Per Cent Error on Basis of Gravimetric Method		
	Inorganic Sulfates	Total Sulfates	Total Sulfur	Inorganic Sulfates	Total Sulfates	Total Sulfur	Inorganic Sulfates	Total Sulfates	Total Sulfur Zn Method
Dog Blood									
1	2 912	....	10 528	2 84	...	10.56	− 3	...	+4
2	4 032	....	10 592	3.86	..	10.26	− 4	..	−3
3	2 52	..	8 736	2 4		9 42	− 4		+7
4	3 25	..	8 96	3 04		9 53	− 6		+5
5	2 464	..	8 736	2 6	.	8 50	+ 4		−2
6	3 416	.	8.40	3 2	.	8 81	− 6		+5
7	3 864	...	9 74	4 00	...	9 64	+ 3		−1
8	2 408	..	9 968	2 23	.	9 81	− 7	..	−1
Human Blood									
1	1 02	2 006	4 51	1 00	2 00	4 26	− 2	0	−5
2	0 35	1 89	3 52	0 348	1 69	3 75	0	−10	+6
3	1 72	3 36	5 53	1 56	3 20	5 21	−10	− 5	−4



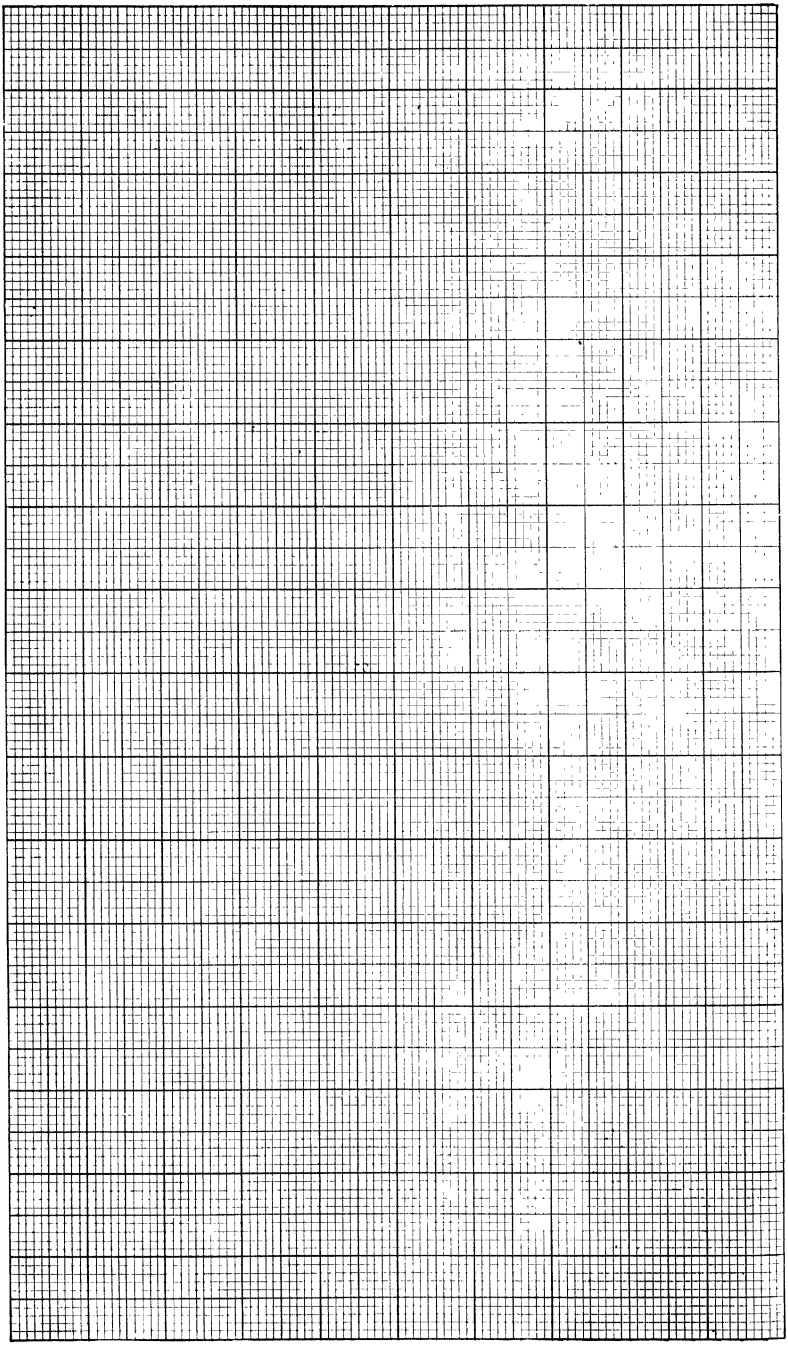


Table XI records the non-protein sulfur compounds (Denis and Reed) found in the blood of various animals.

TABLE XI

Source of Blood	Mg. of S per 100 cc. of Blood					Per Cent on Basis of Total Sulfur			
	Inorganic Sulfates	Ethereal Sulfates	Total Sulfates	Neutral Sulfur	Total Sulfur	Inorganic Sulfates	Ethereal Sulfates	Total Sulfates	Neutral Sulfur
Human	1 12	0 886	2 006	2 504	4 51	24 8	19 6	44 4	55 5
	0 35	1 54	1 89	2 21	4 10	8 5	37 6	46 1	53 9
Dog	3 64	4 65	8 29	3 58	11 87	30 6	39 1	69 8	30 1
	2 02	4 59	6 61	3 47	10 08	20 0	45 5	65 5	34 1
	3 08	3 08	6 16	2 576	8 736	35 2	35 2	70 4	29 6
Beef	2 35	3 274	5 624	1 666	7 29	32 2	44 9	77 1	22 8
	2 576	3 6	6 176	2 412	8 59	30 0	41 9	71 9	28 1
Goat	11 28	5 6	16 88	7 08	23 96	47 2	23 4	70 7	29 6
	6 48	0 672	7 156	5 696	12 852	50 7	5 25	55 9	44 5
Rabbit	0 533	2 007	2 54	4 96	7 50	7 11	26 8	33 9	66 1

DETERMINATION OF SULFATES AND TOTAL SULFUR IN URINE

Method of Denis and Reed [3]

The principle of this method is the same as the preceding one for blood. It is of special value in cases where extremely small quantities of urine are available for analysis either on account of the small size of the experimental animal, or because of the necessity of making analysis of urine collected for relatively short periods. It also possesses the advantage of requiring for its use the minimum of time and of reagents.

Reagents.

The reagents required are identical with those used in the determination of sulfates and total sulfur in blood (p. 157).

Procedure.—*Dilution of urine.*—Since the nephelometric procedure for the determination of sulfates gives the best results

[3] J. Biol. Chem., **71**, 205 (1926).

when concentration of the sulfate ion is in the neighborhood of 0.1 mg. of S, it is invariably necessary to dilute the urine. The most convenient dilutions are 1 to 2 or 1 to 5 for human urine and 1 to 10 for the urine of cats and dogs.

It is convenient in practice to dilute the urine to a proper concentration for the total sulfur determination, and if necessary to use 2 cc. of this dilution in place of 1 cc., as recommended below for the determination of inorganic and total sulfates. This additional volume must of course be balanced by the addition of 1 cc. more water to the standard.

Inorganic sulfates.—To 1 cc. of the dilute urine (which contains in the neighborhood of 0.1 mg. of S as SO_4) add 15 cc. of 0.1 N HCl, 2 cc. of 0.6 N NaOH, 1 cc. of 5 per cent sulfate-free gelatin, and 5 cc. of 1 per cent $BaCl_2$ solution. Allow to stand fifteen minutes and read in the nephelometer against a standard which has been prepared at the same time as the unknown, and in which 1 cc. of diluted urine is replaced by 1 cc. of K_2SO_4 standard equivalent to 0.1 mg. of S.

Total sulfates.—Place 1 cc. of diluted urine in a Pyrex test tube together with 4 cc. of 1 N HCl, and boil gently with a free flame for not less than fifteen to twenty minutes. When solid particles begin to settle out, heating should be discontinued, and the residue dissolved in 15 cc. of 0.1 N HCl. To this solution then add 2 cc. of 0.6 N NaOH, 1 cc. of 5 per cent gelatin, and 5 cc. of 1 per cent $BaCl_2$ solution. The standard is prepared at the same time by substituting for the diluted urine 1 cc. of K_2SO_4 solution equivalent to 0.1 mg. of S. Both standard and unknown are allowed to stand fifteen minutes and are then read in the nephelometer.

Ethereal sulfates.—This fraction is calculated in the customary manner by the difference between the values for total sulfates and inorganic sulfates.

Total sulfur.—Place 1 cc. of diluted urine in a Pyrex test tube, add 1 cc. of the $Zn(NO_3)_2$ oxidation mixture, and evaporate to dryness over a free flame. Dissolve the residue in 2 cc. of 1 N HCl, add 15 cc. of distilled water, 1 cc. of 5 per cent gelatin, and 5 cc. of 1 per cent $BaCl_2$ solution.

The standard is prepared at the same time as the unknown and is carried through the same manipulations regarding heating, etc., the only difference being that in place of the urine there is substituted 1 cc. of standard K_2SO_4 equivalent to 0.1 mg. of S.

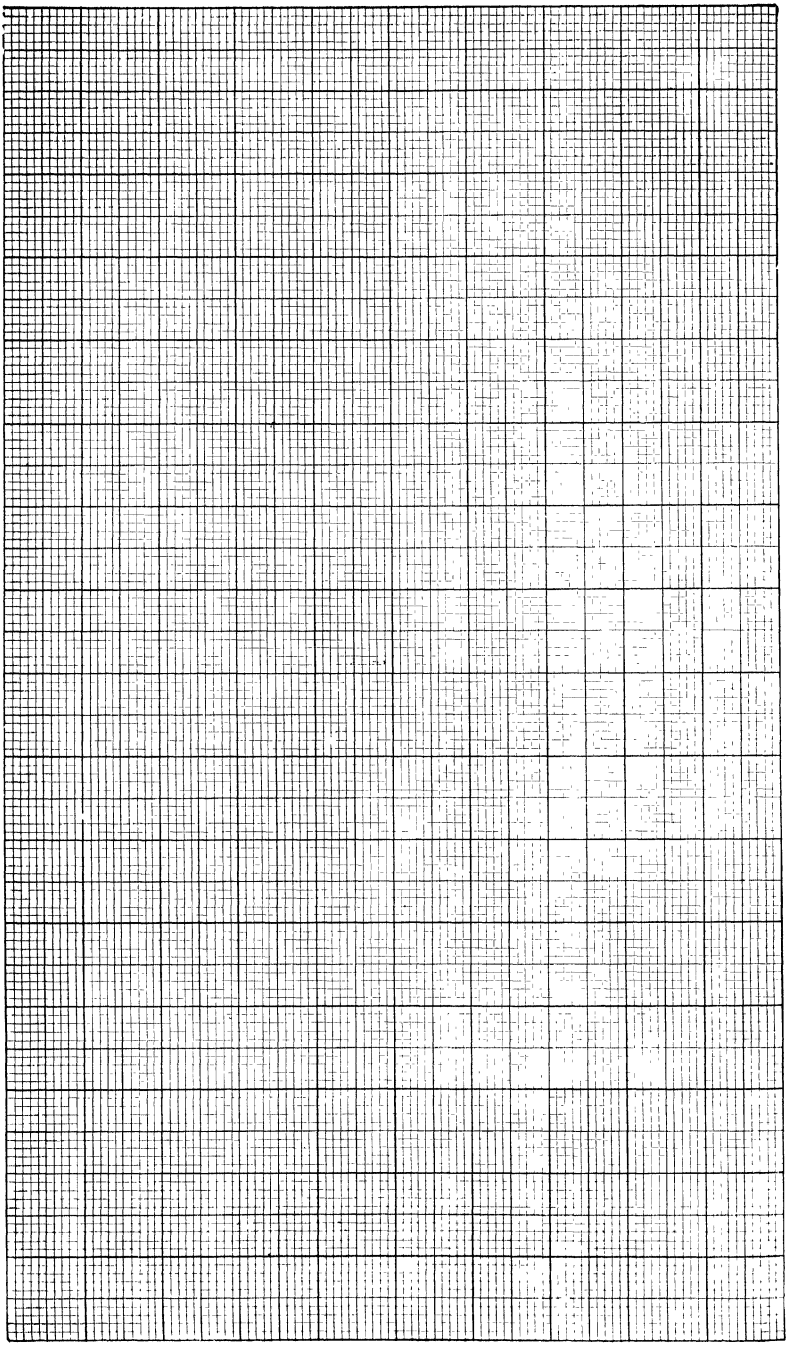
Neutral sulfur.—This fraction is calculated in the usual way by difference between total sulfur and total sulfates.

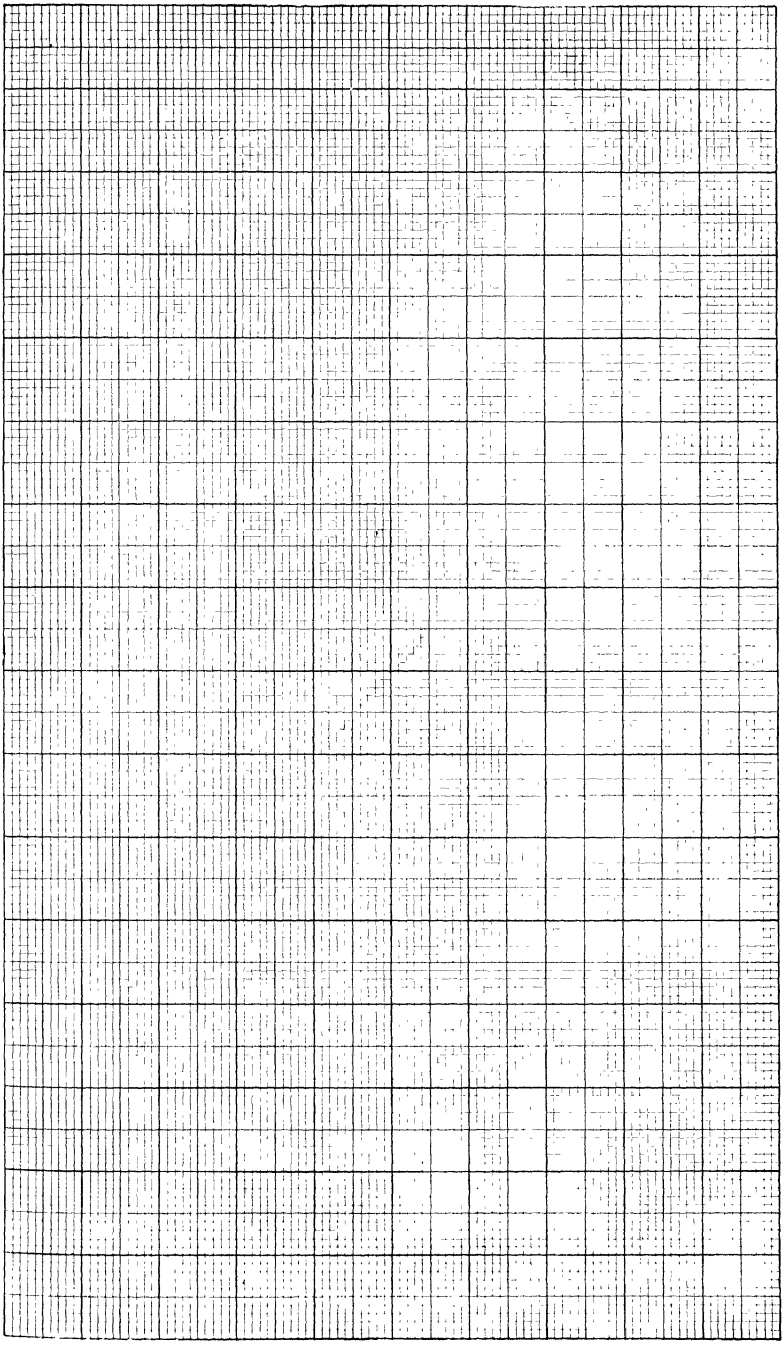
Note.—The methods outlined above have been checked by a series of comparative analyses (Denis and Reed) made by gravimetric methods. Inorganic and total sulfates were determined by the methods of Folin;[4] total sulfur by the procedure of Benedict,[5] with the use of Denis'[6] modification of the oxidation mixture employed in the original method. The results are given in Table XII.

TABLE XII

Urine	Inorganic Sulfates			Total Sulfates			Total Sulfur		
	Gravimetric	Nephelometric	Per Cent Error	Gravimetric	Nephelometric	Per Cent Error	Gravimetric	Nephelometric	Per Cent Error
Human									
I..	0 465	0.440	−5	0 504	0 504	0	0 604	0 644	+6
II...	0 190	0 186	−2	0 22	0 217	−1 3	0 250	0 251	+0.4
III...	0.325	0 319	−1 8	0 369	0 376	+2	0 408	0 426	+4
IV...	0.129	0.1294	0	0 170	0 171	+0 5	0 210	0 212	+0 9
V...	0 262	0 251	−4	0 2996	0 316	+5	0 3608	0 373	+3
VI...	0.092	0 0882	−3	0 108	0 105	−2 7	0 124	0 121	−2
VII. .	0 353	0 338	−4	0 379	0 377	−0 5	0 456	0 479	+5
VIII...	0.280	0.293	+4	0 312	0 325	+4	0 462	0 465	+0 6
Cat									
IX...	0 735	0 732	−0 4	0 866	0 856	−1	1 220	1 190	−2
X...	1 15	1 20	+4	1 39	1 452	+4	1 376	1 40	+2
Dog									
XI.. .	1 08	1 00	−8	1 132	1 162	+2	1 350	1 41	+4
XII....	0 920	0 89	−3	0 938	0 934	−0 4	1 230	1.20	−2

[4] J. Biol. Chem., **1**, 131 (1905–06).
[5] *Ibid.* **6**, 363 (1909).
[6] *Ibid.* **7**, 401 (1910).





PART III

ORGANIC

CHAPTER XIII

ACETONE

DETERMINATION OF ACETONE

METHOD OF MARRIOTT [1]

WHEN acetone is added to a silver-mercury-cyanide solution an abundant white nebulous precipitate is produced. The reaction is so delicate that a solution of freshly distilled acetone containing 0.010 mg. per liter, i.e., 1 part in 100 millions, is sufficient to cause a distinct opalescence. Moreover, the intensity of the opalescence, as measured nephelometrically, has been found, within limits, to be proportional to the amount of acetone added. The method is adapted to the determination of very small amounts of acetone such as occur, for example, in a few cubic centimeters of normal blood.

Reagents.

1. Silver-mercury-cyanide reagent. Dissolve 10 grams of mercuric cyanide and 180 grams of sodium hydroxide in 1200 cc. of water in a 2- or 3-liter flask. Stir the solution and run in slowly 400 cc. of a 0.73 per cent solution of silver nitrate. Just before using, the reagent is filtered through an asbestos mat through which has been filtered a little talcum in water suspension. Instead of filtering, the solution may be set aside for at least three or four days. At the end of this time the small amount

[1] W. M. Marriott, J. Biol. Chem., **16**, 289 (1913).

of sediment present will have settled, leaving a clear supernatant liquid which may be carefully siphoned off. The solution is then ready for use.

At least 30 cc. of the reagent is used for each milligram of acetone present or expected to be present. With a little practice, the approximate amount of acetone present can be judged by the density of the precipitate formed in the distillate obtained in the first two minutes. A dense precipitate may call for the addition of more reagent to the receiving flask.

2. Standard acetone solution. Distill a solution containing a known amount of acetone (see Note 3), collecting the distillate in an excess of the above reagent. Dilute the distillate to a definite volume and use as the standard. Both the distillation and the dilution of the distillate are carried out in the same manner as described in the following procedure for the sample.

Procedure.—Place a measured quantity of the sample solution in a distillation flask (see Note 5). Connect the flask to a condenser having the delivery tube dipping under the surface of the reagent liquid in the receiving flask and distill. The distillation is continued about fifteen minutes or until the distillate measures 75 to 100 cc. Allow the distillate to stand about thirty minutes, then transfer it to a graduated cylinder and dilute to an opalescence convenient for reading. The turbidity produced by 0.05 mg. of acetone diluted to 100 cc. is a convenient strength for reading, although much smaller or larger amounts give good results. If the opalescence is very dense, it is desirable to dilute further, say 250 cc., pipette out an aliquot portion, and dilute this appropriately. The sample and standard solutions are then placed in the nephelometer tubes and a reading made.

Since the suspensions slowly settle out, the readings should be made as quickly as possible after filling the tubes.

The nephelometer is manipulated in a dark room, a small electric flash lamp being used to read the scale.

Notes.

1. The acetone solution must be free from ammonia, aldehyde or hydrogen sulfide.

2. The standard cannot be prepared by adding a standard acetone solution directly to an excess of the reagent. Such a standard gives a lower result than when prepared by distillation.

3. A convenient stock solution contains about 0.03 mg. of acetone per cubic centimeter. The strength of such a solution is determined by titrating 200 cc. according to the method of Messinger.[2] The titration is carried out as follows: 200 cc. of the acetone solution are run into about 300 cc. of distilled water contained in a large Florence flask. In delivering the solution the pipette is always kept under the surface of the water in the receiving vessel. A measured amount (an excess) of standard iodine solution is then run in, 10 cc. of 60 per cent sodium hydroxide added, the flask stoppered, shaken a little, and allowed to stand for five or ten minutes, after which 15 cc. of concentrated hydrochloric acid are added and the excess iodine titrated with standard sodium thiosulfate solution in the usual manner. One cubic centimeter of 0.1000 N iodine solution is used up by 0.968 mg. of acetone.

Kober [3] suggests using a standard solution made with 0.25 N sulfuric acid so that it contains 0.5 mg. of acetone per 10 cc. Such a standard will keep a few weeks at least, while without the sulfuric acid the acetone solution quickly polymerizes. It may be noted that no additional protective colloid is added, since the organic nature of the complex is sufficient protection, or, better stated, makes the rate of agglutination low.

4. As originally pointed out by Richards,[4] the amounts of precipitate are not exactly inversely proportional to the scale readings. Kober [5] has constructed a curve of correction for use with his modification of the nephelometer. When the two solutions for comparison are of nearly the same concentration, the correction is within the limits of observational error and may be disregarded. Further, by using Kober's equation for a correction curve it is seen that the difference between observed and

[2] Cf. Marriott, J. Biol. Chem., **16**, 282 (1913).

[3] J. Ind. Eng. Chem., **10**, 561 (1918).

[4] Richards, Z. anorg. Chem., **8**, 269 (1895); Richards and Wells, Am. Chem. J., **31**, 235 (1904).

[5] J. Biol. Chem., **13**, 485 (1913).

corrected values becomes proportionately less with readings taken with greater depths of solution. If the unknown suspension is so diluted as to be not more than 20 per cent different from the standard and if comparisons are made with scale readings in the neighborhood of 50 mm. or 60 mm., no corrections are necessary.

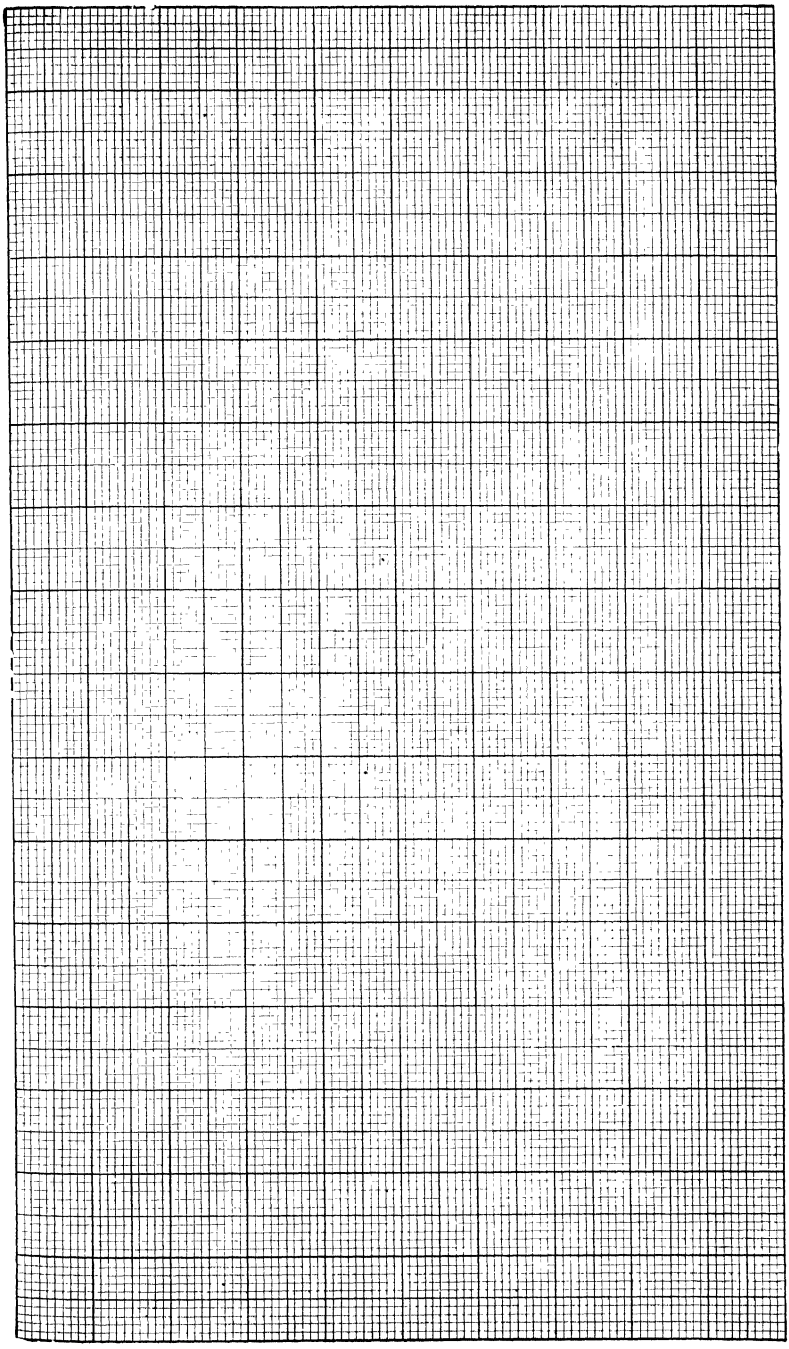
In making a series of determinations a single standard suspension is used and the various unknown suspensions are diluted in graduated cylinders to approximately the same opalescence. Little difficulty is experienced in thus obtaining suspensions differing from the standard by not more than 10 per cent.

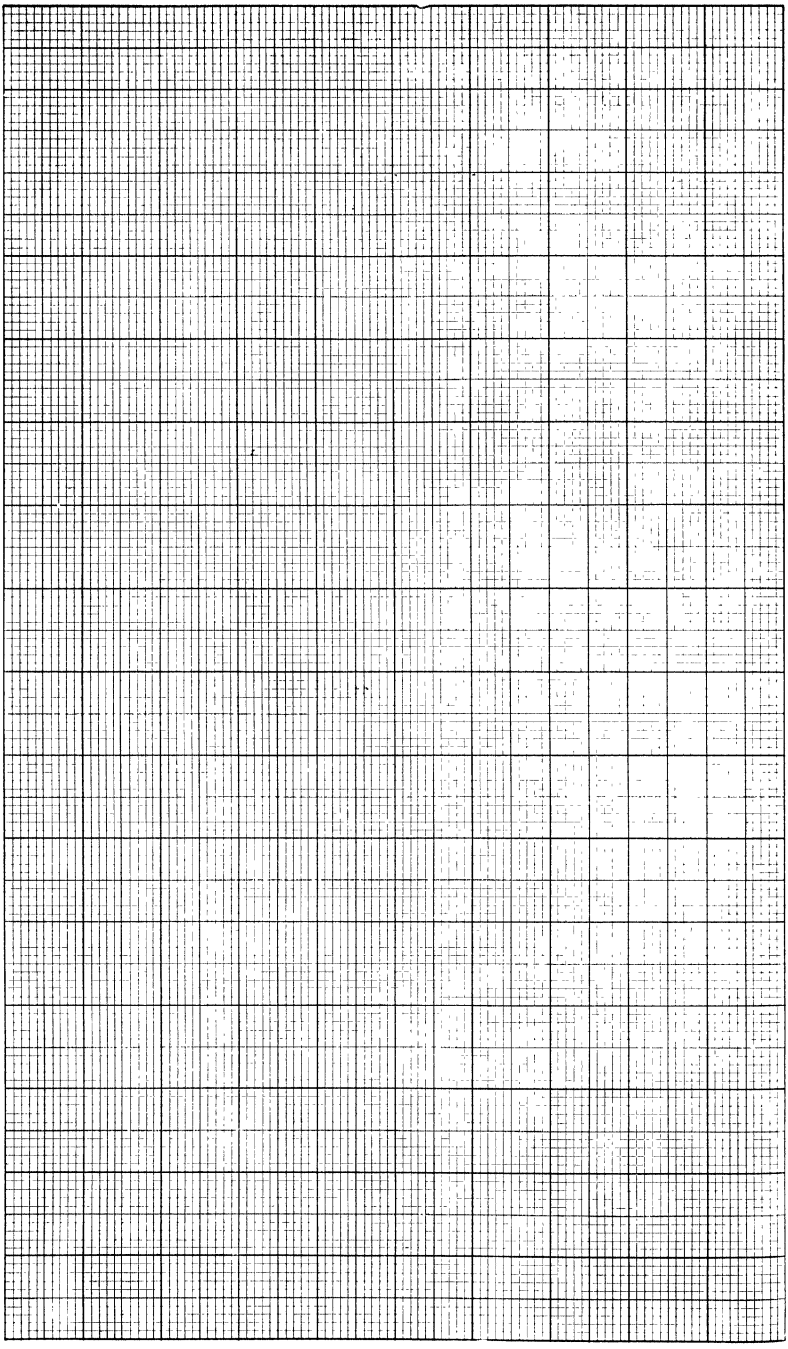
5. Instead of distilling the acetone from the original solution into the reagent it may be aerated [6] at room temperature (or 35–40° C.) into sodium bisulfite solution, the solution diluted to about 50 cc., the reagent added, the mixture diluted with water to 100 cc., mixed, and the acetone determined nephelometrically. Ten cubic centimeters of a 2 per cent aqueous solution of sodium bisulfite are used. The exact concentration is unimportant, so that a fresh solution may be made in a few minutes as needed.

The sodium bisulfite solution has two disadvantages: (1) it does not keep very long (about a week); and (2) it diminishes the amount of turbidity obtained, so that the same amount of it must be added to the standard acetone solution in order to secure strictly comparable turbidities. These disadvantages are, however, not serious.

The time required to remove all the acetone depends on the rapidity of the air current, and the temperature and volume of the liquid. In general, ten minutes is sufficient to remove 2 mg. of acetone from 5 cc. of liquid at 35–40° C. when a moderately rapid air current is used.

[6] O. Folin and W. Denis, J. Biol. Chem. **18**, 263 (1914).





CHAPTER XIV

AMYLASE

NEPHELOMETRIC METHOD FOR THE STUDY OF AMYLASE

By Hans Kleinmann [1]

The principle of the nephelometric amylase determination depends upon a homogeneous and stable colloidal glycogen solution acted upon by amylase and the undigested glycogen measured against a standard solution of glycogen. Under the conditions of the method the nephelometric cloud is an accurate measure of the concentration of the glycogen. The turbidities of different concentrations of glycogen are exactly proportional to the content of glycogen when compared in a Kleinmann nephelometer.

Comments on the Method.—The nephelometric determination of glycogen has the advantage that with it very dilute substrate solutions (0.15 to 0.5 per cent glycogen solutions are used) can be determined and therefore very dilute ferment solutions can be used. The method permits rapid completion of numerous solutions and makes possible the running of a series. Finally, in distinction from the methods which determine the digestion products (reduction methods) it measures directly the decrease in substrate.

It ought to be particularly emphasized that not all kinds of glycogen are suitable for nephelometry. Many glycogens change their colloidal condition upon dilution, i.e., permit impurities to precipitate, so that under some conditions dilute solutions have as much turbidity as, or more than, concentrated solutions. While previously Kahlbaum's preparations produced clouds

[1] Cf. P. Rona and C. van Eweyk, Biochem. Z. **149**, 179 (1924).

which were strictly proportional to the concentration, at the present time both Kahlbaum's and Merck's preparation are unsuitable for the purpose. Therefore, it is recommended that glycogen be prepared as described below.

Reagents.

1. Preparation of the glycogen.—In connection with the method of Pflüger, glycogen is prepared from rabbit's liver (dog's liver is not suitable) as follows:

By means of a stomach tube 50 cc. of glucose solution of approximately 50 per cent are given a rabbit. After two to three hours the animal is bled to death as rapidly as possible and the liver taken out and after the removal of the gall bladder is quickly rinsed off with conductivity water. Then the liver is cut into pieces of the size of a walnut and placed in an ice-salt mixture until the pieces are frozen hard. They are then ground to a fine condition in a mortar with infusorial earth. The ground mixture is treated with 3 per cent trichloroacetic acid and thoroughly stirred, after which it is thoroughly treated with distilled water and filtered. The filtrate, a white cloudy liquid, contains most of the glycogen. The residue is again treated with distilled water and filtered for a second time. The second filtrate, which is still decidedly cloudy, is united with the first filtrate. The combined filtrates are now precipitated with an equal volume of alcohol, and allowed to stand over night, when the glycogen will have precipitated as a fine flocculent precipitate which does not cling to the sides of the vessel. After decanting the mother liquid the precipitate is dissolved again with distilled water, placed in centrifuge tubes, again precipitated by the addition of sufficient alcohol and then centrifuged. After pouring off the supernatant liquid the glycogen is again dissolved, reprecipitated with alcohol and centrifuged. After repeating this operation five times, the residue is finally stirred with absolute alcohol and centrifuged, after which the alcohol is poured off and the residue stirred in ether and filtered. The result is a fine white powder which easily makes white stable suspensions in water. The viscosity of these suspensions is not appreciably greater than that

of water. Their turbidity in the Tyndall beam is strictly proportional to the concentration when a Kleinmann nephelometer is employed.

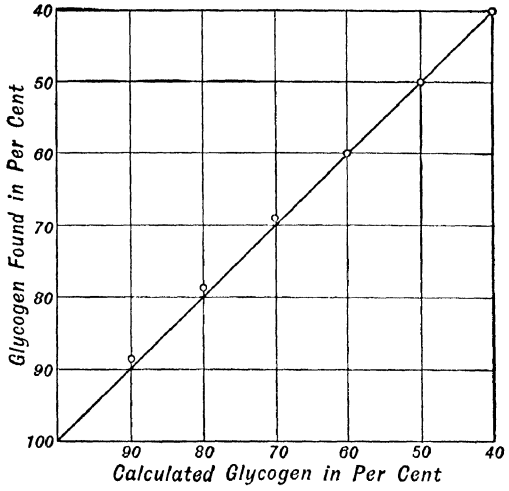


FIG. 35.

In Fig. 35 is a standard curve of a glycogen preparation in which the abscissas represent the calculated glycogen content and the ordinates the nephelometrically found glycogen content (Table XIII).

TABLE XIII

The concentration of the starting solution was 0.3 per cent. Therefore a 50 per cent digestion or concentration was 0.15 per cent.

Amount of Glycogen, Per Cent	Nephelometer Readings		Glycogen Found, Per Cent
	Left Tube	Right Tube	
90	20	22 1	88 5
80	20	25 0	78 5
70	20	28 2	69 6
60	20	32 8	59 8
50	20	39 1	50 0
40	20	47 6	40 05

The concentration of the 0.3 per cent solution is put at 100 so that 50 per cent represents a solution of 0.15 per cent. A new glycogen preparation should be used only after this proportionality between turbidity and concentration is determined.

2. Phosphate buffer, M/3, having the *p*H which is to be maintained during the digestion. The most suitable acidity is a *p*H of 6.5.

3. Sodium Chloride, 0.1 N.

Procedure.—Into a series of 100-cc. graduate cylinders are measured 91 cc. of a glycogen solution such as is suitable for nephelometry. The solution is obtained by dissolving the glycogen powder obtained under (1) in distilled water; usually a 0.3 per cent solution is suitable. One to three cubic centimeters of M/3 phosphate buffer of such acidity as is necessary for the proper hydrogen-ion concentration of the digestion, and 1 cc. of 0.1 N sodium chloride solution are added and the volume made up to 95 cc. with distilled water. The graduate is then put in a thermostat and the temperature maintained at 37° C. (±0.05°). As soon as the temperature has reached an equilibrium, 5 cc. of amylase are added. For example, 5 cc. of diluted human saliva could be added. The solutions are thoroughly mixed and, after thirty seconds, 5 cc. from each cylinder are taken out and put into a dry flask which has been cooled in ice water. Additional samples are taken out after intervals of ten to twenty minutes. The ice-cold suspensions, the values of which do not change appreciably with time, are compared as quickly as possible, using the first sample as a standard.

Calculation of Results.—The calculation of results is in accordance with the rule that the concentrations of the solutions compared in a Kleinmann nephelometer are inversely proportional to the readings, after the two fields in the eyepiece show equality of light. If C_1 is the concentration of the solution digested, C the concentration of the standard solution, which is placed at 100 per cent, H_1 the nephelometric reading of the digested solution, H the nephelometric reading of the standard solution, then

$$C_1 = \frac{100\,H}{H_1}.$$

This gives the concentration of the undigested glycogen in per cent, and by subtraction from 100 the digested glycogen is found. As an example of the method, a test in duplicate is given, from which within two hours six samples of each were taken.

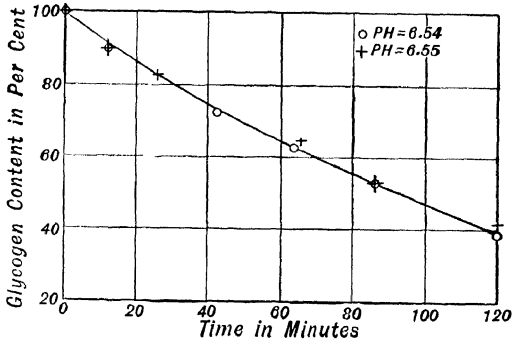


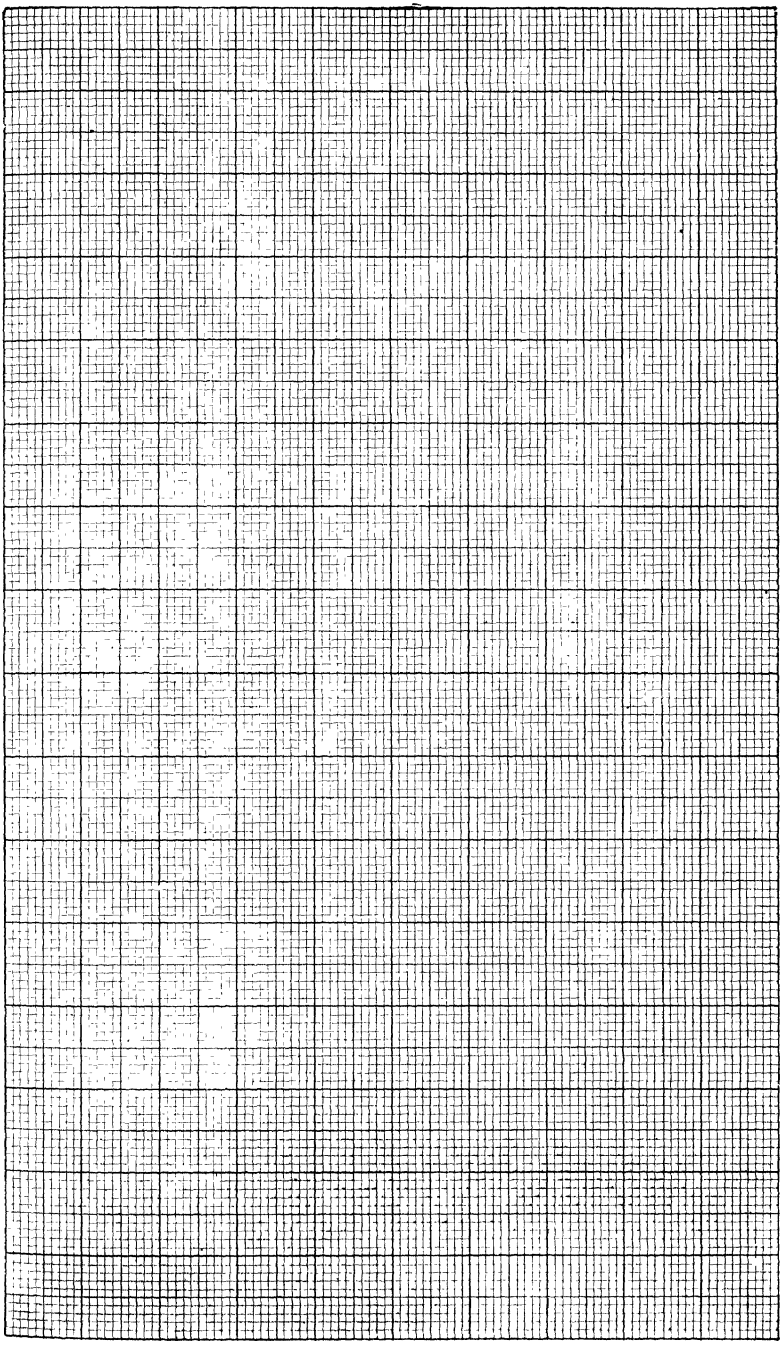
FIG. 36.

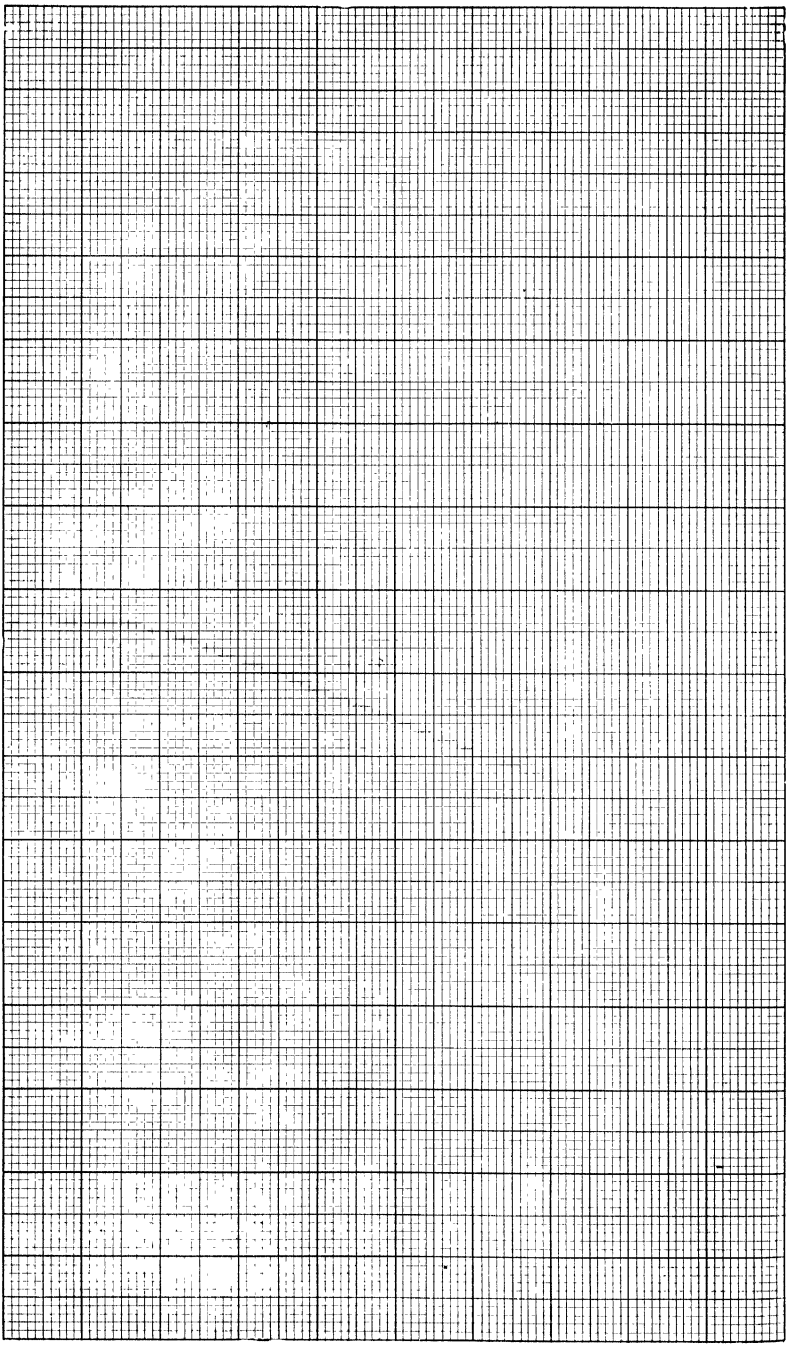
The experiment is given in Table XIV, and in Fig. 36 is shown graphically the course of digestion.

TABLE XIV

Solutions Used	Time in Minutes	Nephelometer Readings		Calculated in Per Cent	x	$\frac{x}{t} \cdot 10^3$	$K \cdot 10^5$	
		Left	Right					
0 4 per cent glycogen soln . . . . . 91 cc.	½	20	23 4	100 0				
	11	20	25 4	92 0	8 0	725	327	
Ferment soln. (1:100). 5 cc.	26	20	28 4	83 0	17 0	655	308	
Buffer N/3. . . . . 3 cc	63	20	36 1	65 0	35 0	555	296	316
NaCl N/10. . . . . 1 cc.	86	15	33 4	52 5	47 5	552	326	
pH=6.54	119	12	34 5	40 8	59 2	498	326	
Duplicate solutions as above, pH=6.55	½	20	22 9	100 0				
	11	20	25 0	91 8	8 2	743	336	
	42	20	31 7	72 0	28 0	670	338	
	63	20	38 0	60 3	39.7	633	347	337
	86	15	32 8	52 3	47 7	555	327	
	119	12	34 5	39 9	60.1	505	336	

The example shows that the value $\frac{x}{t}$ runs a distinct course, while the constant K of a monomolecular reation is in satisfactory agreement.





CHAPTER XV

DICHLORO-ETHYLSULFIDE

DETERMINATION OF DICHLORO-ETHYLSULFIDE (MUSTARD GAS)

METHOD OF YABLICK, PERROTT AND FURMAN [1]

THIS method was developed during the World War. It consists essentially in reducing a 1 per cent solution of selenious acid in 1 : 1 sulfuric acid, by means of dichloro-ethylsulfide vapor, to an orange-red suspension of selenium. The solution is heated to about 85° C. to facilitate the reaction. As little as 0.005 mg. of the substance can thus be detected.

The amount of the suspension produced is nearly directly proportional to the amount of dichloro-ethylsulfide present. Amounts of the compound from 0.10 to 0.01 mg. can be determined nephelometrically with a maximum error of about 0.005 mg.

The method was developed to meet the need of a qualitative test for mustard gas, sufficiently delicate to indicate the presence of dangerous amounts of the substance in the air. An idea of the sensitivity required of the test is obtained when it is known that concentrations of mustard gas as low as 0.0005 mg. per liter (0.08 part per million) will cause discomfort on exposure for twenty-five minutes and concentrations between 0.001 mg. per liter and 0.005 mg. per liter (0.2 p.p.m. and 0.8 p.p.m.) will cause skin burns if exposure is as long as thirty minutes.

The method has had a variety of uses, among which may be mentioned the detection of the presence of mustard gas in field and factory, securing of quantitative data as to the persistence

[1] J. Am. Chem. Soc., **42**, 266 (1920).

of the gas over contaminated soil, and determining the permeability of protective fabrics to the vapor and liquid.

Reagents.

1. Selenious acid reagent. Dissolve 1.0 gram of selenium dioxide in 100 cc. of cold 1 : 1 (by volume) sulfuric acid.

2. Standard dichloro-ethylsulfide solution. For a stock solution, dissolve 0.01 gram of dichloro-ethylsulfide in 100 cc. of cold 1 : 1 sulfuric acid and mix thoroughly. For use, dilute 10 cc. of the stock solution to 100 cc. with cold 1 : 1 sulfuric acid. One cubic centimeter of this solution contains 1.0 mg. of dichloro-ethylsulfide. The solution will remain unchanged for at least several weeks.

Procedure for Solutions of Dichloro-ethylsulfide.—First prepare a standard nephelometric curve as follows: Prepare a standard selenium suspension (say one corresponding to 0.05 mg. of dichloro-ethylsulfide per 20 cc.) by diluting the proper amount of the standard solution with sufficient 1 : 1 sulfuric acid to make a total volume of 10 cc., and then add 10 cc. of the selenious acid reagent. Thoroughly mix the resulting suspension, keep it at 85° (±5°) for ten minutes, cool to room temperature, and put it in both cups of the nephelometer. Set the left-hand cup at a definite height and then adjust the right-hand cup until equal intensity of illumination is obtained in both fields of the eyepiece. Record the reading of the right-hand cup. Next place in the right-hand cup suspensions containing 2.0, 1.9, 1.5, etc., 0.9, 0.8, 0.7, etc., times as much dichloro-ethysulfide per 20 cc. as the standard contained and note the respective readings. Take the precaution always to rinse the cup with three portions of the solution to be tested and to wash the outside of the cup with distilled water and dry before making a comparison. From the readings construct a curve, plotting milligrams of dichloro-ethylsulfide per 20 cc. against millimeter readings on the nephelometer scale. The hypothetical curve may also be drawn by assuming that the scale readings vary inversely as the amount of suspended substance present. It will be noted that the actual curve is in close proximity to the hypothetical curve, thus indi-

cating that the reaction between dichloro-ethylsulfide and selenious acid is almost complete under the conditions outlined. See Fig. 37.

Having obtained the nephelometric curve, the strength of an unknown solution may be determined by treating a measured volume of it in the same manner as directed above and then matching against the standard suspension. From the reading and the curve, the concentration of the sample is easily calculated.

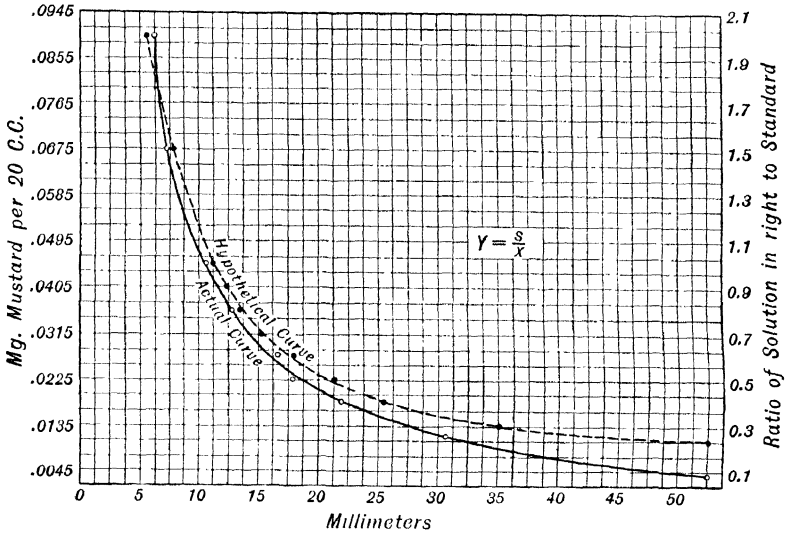


FIG. 37.—Nephelometric Standard Curve for Mustard Gas.

Procedure for Air-Dichloro-ethylsulfide Mixtures. - The apparatus illustrated in Fig. 38 was developed by Yablick, Perrott, and Furman [2] for producing low concentrations of dichloro-ethylsulfide vapor. It was arranged to deliver 1 liter per minute of an air mixture containing about one part per million of dichloro-ethylsulfide.

The bulb *D* contains dichloro-ethylsulfide redistilled *in vacuo* and is kept in a water bath at 20°. Air purified by passing through sulfuric acid and a charcoal, soda-lime mixture is passed

[2] *Loc. cit.*

through the liquid in bulb D at a rate of 11 cc. per minute as measured by the flowmeter C_1. The saturated air passes into the mixing chamber F where it is mixed with pure air sufficient to bring the entire flow up to 1000 cc. per minute as measured by the flowmeter C_2. The air mixture, containing 0.0062 ($\pm$0.0004) mg. per liter,[3] now passes through the absorbing solution in the bubblers E.

Ten cubic centimeters of the absorbing agent used are placed in the bubbler and the mixture passed through it for ten minutes. Then 10 cc. of a solution of selenious acid in sulfuric acid are

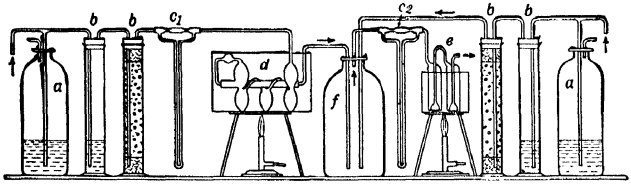


FIG. 38.—Apparatus for Producing Low Concentration of Mustard Gas Vapor.

(*a*) constant pressure bottle.
(*b*) air-purifying bottle.
(*c*) flow meter.
(*d*) mustard bubbler in bath at 20°.
(*e*) selenious acid bubbler in bath at 85°.
(*f*) mixing jar.

added, the sulfuric acid being of such strength as to make the resulting mixture 1 : 1 sulfuric acid and the selenious acid present in the same per cent as in the original 10 cc. of absorbing agent; for example, if 10 cc. of a 1 per cent solution of selenium dioxide in water were used as the absorbing medium, then 10 cc. of a 1 per cent solution of selenium dioxide in concentrated sulfuric acid are added, making 20 cc. of a 1 per cent solution of selenium dioxide in 1 : 1 sulfuric acid.

The solution is then heated for ten minutes in a bath at 85°, cooled, and the strength determined by comparison with a standard solution prepared as previously described.

The following notes are based upon experimental data obtained by Yablick, Perrott, and Furman.

[3] Calculated from British chemical warfare data on vapor pressure of dichloroethylsulfide.

Notes.

1. There is no apparent difference between results when hot and cold selenious sulfuric acid solutions are used as absorbents for the mustard gas vapor.

2. The results are in closer agreement and more closely approach the theoretical, when sulfuric acid is present in the original absorbing solution.

3. A very large increase in sensitivity is obtained by using a concentrated hot solution of selenium in water.

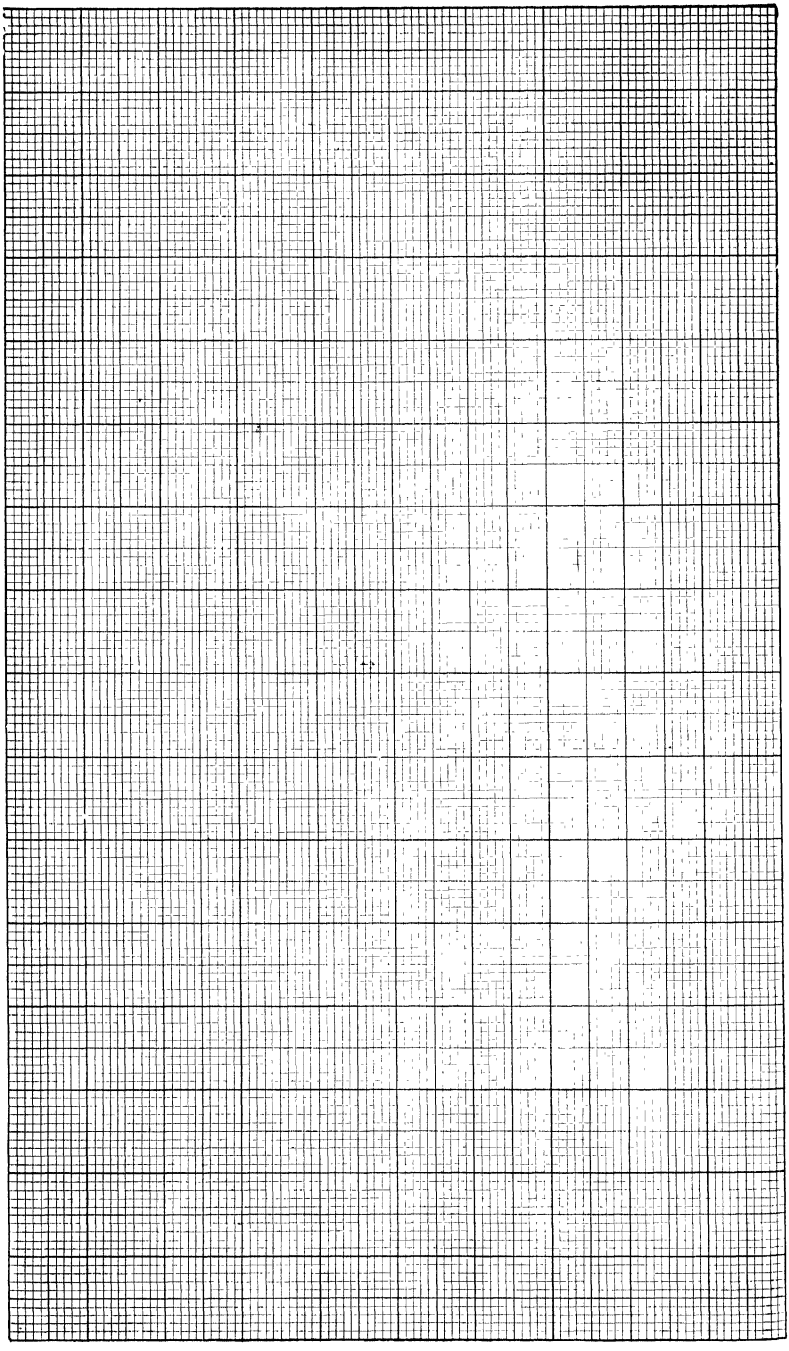
4. The selenious acid solution should not be used for twenty-four hours after it is made. After this time the solution is stable for at least two weeks.

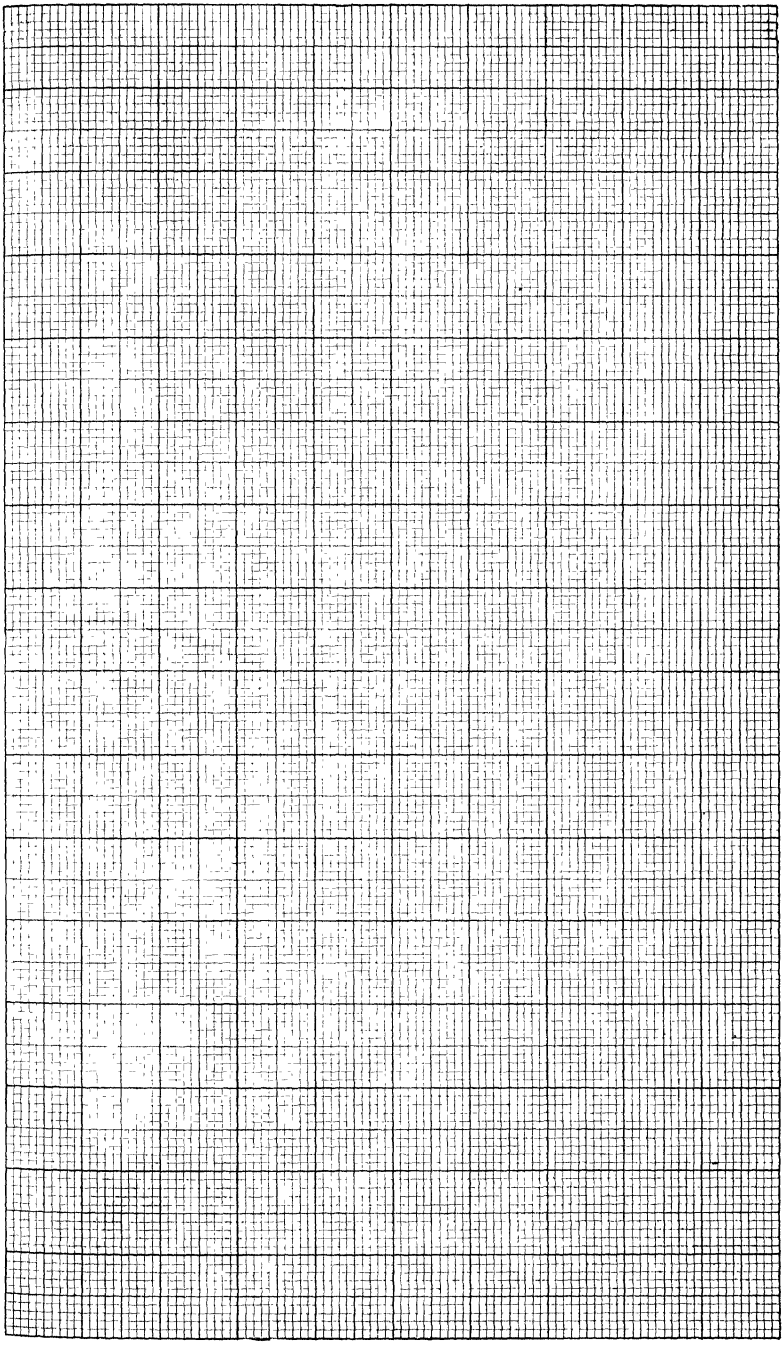
5. The standard solution of dichloro-ethylsulfide shows no apparent deterioration over a period of at least four weeks.

6. The temperature at which the solution is heated in process of " developing " can vary 5° in either direction without affecting the character of the suspension. The method is remarkably free from the necessity for extreme purity of original solutions, cleanliness of glassware, etc., which obtains in silver halide nephelometry.

7. It has not been found possible to use good solvents for dichloro-ethylsulfide as absorbing solutions and then determine the strength nephelometrically, for the reason that the only suitable solvents (alcohol and acetic acid) reduce the selenious acid to some extent.

8. The selenious acid reagent is not specific to dichloro-ethylsulfide. Arsine, the substituted arsines, and several other toxic gases react with it vigorously.





CHAPTER XVI

FATS, OILS AND FATTY ACIDS

DETERMINATION OF FATS AND OILS

Method of Bloor

In quantitative work where a precipitate is easily thrown down, easily washed, dried and weighed, gravimetric analysis imposes no difficulty, although it may fail to estimate small quantities. Fats and oils are difficult to filter and to free from solvent and, hence, Bloor [1] devised a nephelometric method which avoids these difficulties. The fat or oil is extracted with an alcohol-ether solution and the extract poured into water. The fats or the oil separate out in fine globules forming a cloudy suspension. Bloor's method will easily estimate quantitatively 0.05 mg. of fat and will show the presence of 1.0 part of fat in a million of water. The method is useful in the nephelometric determination of fat in blood and in milk, and has been applied by Woodman, Gookin and Heath [2] to the nephelometric estimation of essential oils.

A. DETERMINATION OF FAT IN BLOOD.

Reagents.

1. Hydrochloric acid, 2.5 N.

2. Alcohol-ether solution. Mix 750 cc. of freshly redistilled absolute alcohol and 250 cc. of pure dry ether.

3. Sodium ethylate, 1 N. Prepare from carefully cleaned sodium and freshly distilled absolute alcohol.

[1] J. Biol. Chem., **17**, 377 (1914); J. Am. Chem. Soc., **36**, 1300 (1914). See also Kober, J. Ind. Eng. Chem., **10**, 561 (1918).

[2] J. Ind. Eng. Chem., **8**, 128 (1916).

4. Standard fat solution. Use an alcohol-ether solution (prepared as in (2) above) containing 2.0 mg. of fat or oleic acid per 5 cc.

Procedure.—About 2 cc. of blood are drawn from the vein with a graduated syringe and run at once, with stirring, into a weighed graduated flask containing about 40 volumes of the alcohol-ether solution. After again weighing to find the weight of blood added, the solution is raised to boiling in a water-bath, cooled under the water-tap, diluted to the mark with the alcohol-ether solution, mixed and filtered. The filtrate is water-clear and almost colorless.

From 5 to 20 cc. of the extract (containing about 2 mg. of fat) are measured with a pipette into a small beaker and saponified by evaporating just to dryness with 2 cc. of 1 N sodium ethylate. The dry residue is gently warmed with 5 cc. of the alcohol-ether until all the flakes of alkali are dissolved, then poured slowly with stirring into 100 cc. of distilled water in a beaker and the saponification beaker well washed out with the water.

A similar solution of the standard is prepared by adding 5 cc. of the standard fat solution from a pipette with stirring to 100 cc. of distilled water. To the standard and to the test solutions are added simultaneously 10-cc. portions of dilute 2.5 N hydrochloric acid and the solutions allowed to stand for five minutes, after which they are transferred to the comparison tubes of the nephelometer.

For the comparison, the two tubes, filled to the same height with the solutions, are placed in the nephelometer with the standard tube always on the same side. If bubbles appear on the walls of the tubes they should be removed by inverting the tubes two or three times. The movable jacket on the standard tube is set at a convenient point, generally 50 mm. (Richards' nephelometer) and comparisons made by adjusting the jacket on the test solution until the images of the two solutions show equal illumination. Not less than five readings are taken, alternately from above and below, and the average taken as the correct reading.

Notes.[3]

1. Running the freshly drawn blood slowly into the solvent which is kept in motion causes the precipitation of the blood proteins in a finely divided flocculent form—in excellent condition for extraction. The solvent combines the penetrating power of alcohol with the greater solvent power of the ether. Under these conditions the presence of the great excess of solvent and the short heating are believed to be adequate to extract all but the most difficultly extractable fatty material. No claim is made that the extraction is complete, for it can readily be shown that such is not the case, but it is believed that the above treatment extracts all the fat from the blood except what is contained in the interior of the corpuscles.

2. Sodium ethylate is used to ensure saponification of the cholesterin esters. Heating the saponification mixture after it has reached dryness generally does no harm, but in some cases, particularly where a small aliquot of the fat solution is used, too high results are sometimes obtained. The reason for the difference is not clear, but since the addition of a drop or two of water before the evaporation prevents the increase in value it is believed to be due to the action of the sodium on some constituent of the mixture.

3. The choice of triolein as the standard is empirical, triolein being readily obtainable of a sufficient degree of purity. A mixture of fats approximating the composition of blood fat offers no advantage as a standard; besides, the composition of blood fat varies.

The use of a suspension of unsaponified triolein as standard for the comparison of a suspension of saponified blood fat is quite satisfactory and simplifies the procedure.

4. It was pointed out by R. C. Wells [4] with regard to solutions of this nature that since very small particles do not reflect (ordinary) light, the reflection must be due to aggregates of particles, and since also when aggregates pass a certain size the light

[3] Notes 1 to 6 are from the published work of Bloor, J. Biol. Chem., **17**, 378 (1917).

[4] Am. Chem. J., **35**, 99 (1906).

reflected diminishes because of the decreased surface, the reflecting power must increase to a maximum and then diminish. In agreement with the above it has been found that both standard and test fat solutions change continuously in reflecting power and it is necessary to determine approximately the rates of change in the solutions so as to find out under what conditions accurate comparisons may be made. This has been done by Bloor, who found (*a*) that the maximum, if any, is reached very quickly—before the first readings could be made; (*b*) that the suspensions decrease in value during the observations (a ground-glass plate was used for a standard), rapidly during the first five minutes and then more slowly until in the course of some days all the fat separates out; (*c*) that the rate of decrease in the value of the triolein suspension is greater than that of either a saponified triolein suspension or a saponified blood fat suspension; and (*d*) that differences in the readings of the triolein suspension and the saponified blood fat (or saponified triolein) suspension are within the error of the instrument (2–3 mm. for a single reading) up to ten minutes. The standard and sample suspensions are allowed to stand about five minutes to equalize differences in the time of addition of the precipitating acid and the readings are then made during the succeeding five minutes.

5. Calibration of the nephelometer. Care must be taken to see that both tubes when filled with the same solution give the same readings on both sides of the instrument. If the tubes do not match, then each is marked and always used on the same side of the nephelometer. Always using the tube for the standard solution on the left, the average of a number of readings of the right-hand tube gives the potential height of the standard.

Since the amount of light reflected is not exactly proportional to the depths of solution (because of different distances of the reflecting surfaces from the eye, absorption of light by the upper layers of liquid, etc.), it is necessary to calibrate the nephelometer for different strengths of test solution, and for different standards, if more than one is used. The values obtained are plotted on a curve and corrections are made in the readings made from it. (See p. 46).

Again, since the total light reflected from a given depth of solution depends as well on the size of the particles as on their number, it is obvious that success with a nephelometric method depends on securing as nearly as possible the same aggregation of the particles in the solutions at different times. Care must be taken therefore to secure the same chemical and physical conditions—regarding concentration of alcohol-ether, acid, etc.; also that the solutions to be compared should not be too different in value. Readings above 70 and below 30—with the standard at 50—are discarded.

6. Correction for reagents. Using the purest reagents prepared as directed above reduces the correction very much but does not eliminate it entirely. The most convenient way to determine the correction is to superpose an equal volume of the alcohol-ether and of the sodium ethylate on the amount of blood fat solution ordinarily used for a determination, and then make the determination in the usual way. From the differences in value thus obtained the correction is easily calculated.

7. Murlin and Riche[5] have modified the method of Bloor by pouring the fat solution into 0.05 per cent gelatin solution (prepared by dissolving 1 gram of gelatin in 2 liters of water containing 5 cc. of glacial acetic acid). The addition of this small amount of protein as protective colloid reduces the rate of coalescence of the fat globules and thereby keeps the suspension nephelometrically constant for hours.

8. The nephelometric method has the advantage that only a small volume of blood (0.5 to 5 cc., ordinarily about 2 cc.) is required. It has been found to be accurate to within 5 per cent of the total fat. A determination can be made in about three-quarters of an hour.

[5] Private communication to P. A. Kober. See J. Ind. Eng. Chem. **10**, 561 (1918).

B. DETERMINATION OF FAT IN MILK

Reagents.

The reagents are the same as those used in the determination of fat in blood, p. 192, omitting the sodium ethylate.

Procedure.[6]—Measure out with an accurate pipette 1 cc. of the milk (the measurement may be checked by weighing the milk withdrawn) and run it slowly, with stirring, into about 80 cc. of the alcohol-ether solution contained in a 100-cc. volumetric flask. Raise the mixture to boiling by placing the flask in a boiling water-bath, cool to room temperature, make up to 100 cc. with the alcohol-ether solution, shake and filter. The filtrate is clear and almost colorless.

Five cubic centimeters of the filtrate are run from a pipette, slowly and with stirring, into 100 cc. of distilled water in a beaker, the tip of the pipette being kept below the surface of the liquid during the outflow. A slightly opalescent colloidal solution is formed. Prepare a similar solution with 5 cc. of the standard fat solution. Now add, simultaneously, to the sample and standard solutions 10-cc. portions of 2.5 N hydrochloric acid. Stir the solutions, let them stand for five minutes, then transfer them to the comparison tubes and make the readings during the succeeding five minutes.

For the comparison, the two tubes, filled to the same height with the solutions, are placed in the nephelometer with the standard tube always on the same side. If bubbles appear on the walls of the tubes they are removed by inverting two or three times. The movable jacket on the standard side is set at a convenient point, generally 50 mm. (Richards' nephelometer), and comparisons made by adjusting the jacket on the test solution until the images of the two tubes show equal illumination. At least five readings are taken, alternately from above and below, and the average taken as the reading. This reading is corrected from the calibration curve of the instrument and the fat value of the milk calculated from the corrected reading.

[6] W. R. Bloor, J. Am. Chem. Soc., **36**, 1302 (1914).

Notes.

1. Read Notes 1, 3, 4 and 5 under the Determination of Fat in Blood, p. 194.

2. The calibration of the nephelometer for a given standard is carried out as follows: Suspensions of triolein of various known values are prepared as in the method, and compared with the similarly prepared standard triolein suspension in the nephelometer, with the movable jacket on the standard set at its usual place. The readings obtained are compared with the theoretical readings for the different values and the correction for each strength of solution determined. From these values a curve is plotted, from which intermediate values are obtained. In general, it is found that suspensions stronger than the standard give readings higher (i.e., lower fat values) than the theoretical, and solutions weaker than the standard give lower readings, indicating that probably the greater speed of flocking in the stronger solutions is the main factor in the abnormal readings.

3. Ordinarily a correction for reagents is unnecessary when freshly distilled alcohol and ether are used.

4. Duplicate determinations made on the same milk agree with one another within the limits of error of the instrument (about 3 per cent). Table XV taken from Bloor's[7] pages shows a comparison of results obtained by the nephelometric method, the Babcock method and also in some cases the Adams method.

The cows' milk used for the analyses on p. 199 was mixed dairy milk collected from various localities and the human milk was from patients in the obstetric division of the Washington University Hospital. The samples of human milk were from cases in various early stages of lactation and were selected so as to obtain as wide range of fat content as possible.

5. The nephelometric method has the advantage that only a very small volume (ordinarily 1 cc.) of milk is required and is therefore of special value in working with human milk or with that of small animals where it is inconvenient or impossible to obtain the large samples required for other methods. Moreover,

[7] *Loc. cit.*

the time required is no longer than that for the " rapid " methods, while the accuracy is limited only by the accuracy of the instruments.

TABLE XV

COMPARISON OF RESULTS OBTAINED BY THE NEPHELOMETRIC AND OTHER METHODS FOR FAT IN MILK

Milk Sample	Babcock	Nephelometric	Adams (Soxhlet)
R. Kingston, Can. . .	2 9	2 85	. . . .
B. Kingston, Can	3 1	3 00	. . . .
Hospital supply I., St Louis, Mo.	3 5	3 6	. . . .
U. D. I., St. Louis, Mo	3 0	3 1	3 01
Hospital supply II, St. Louis, Mo.	3 0	3 1	. . . .
M. Kirkwood, Mo	3 6	3 8	3 80
J. A., St. Louis, Mo. . .	3 5	3 6	3 77
			. . . .
Human Milk			. . . .
I. L. 22 days post partum	4 7	4 8	4 70
II. V. 10 days p.p. (child still-born) . .	8 2	8 1	. . . .
III. W. 11 days p.p. . .	3 9	3 83	. . . .
IV. G. 4 days p.p	3 75	3 8	. . . .
V. N. 3 days p.p. (colostrum)	3 3	3 4	. . . .
VI. M. 16 days p.p.	2 0	2 2	2 15
VII. B. 8 days p.p. (breast caked a little)	5 6	5 60	5 71

C. DETERMINATION OF ESSENTIAL OILS

This method is based on the fact that water, or dilute hydrochloric acid, added to an alcoholic solution of many essential oils, precipitates the oil as minutely divided globules and thus forms a fairly stable emulsion. The amount of oil present may then be estimated by measuring the amount of light reflected from a column of the emulsion. The reflected light is matched in a nephelometer with the light reflected from an emulsion containing a known amount of the same oil (see Notes 1 and 2) and prepared under the same conditions as used in the case of the unknown. By this method essential oils can be determined with great

accuracy in concentrations up to 1 per cent and, by suitable dilution with alcohol, in higher concentrations. Obviously the process is not applicable to those essential oils which do not form an emulsion on adding water or dilute hydrochloric acid to their alcoholic solutions.

The procedures given below were worked out by Woodman, Gookin and Heath[8] and were used to estimate small amounts of the oils of peppermint, rose, anise and nutmeg.

Reagents.

1. Hydrochloric acid, 2.5 N.
2. Alcohol, 95 per cent (redistilled).

Peppermint Oil

Procedure.—In general, dilute 5 cc. of the extract with 25 cc. of water or better 2.5 N hydrochloric acid (see Note 4) and compare in a nephelometer with a standard similarly prepared.

The following table is a typical series of readings obtained by Woodman, Gookin and Heath in the case of peppermint oil precipitated by adding water to alcoholic extracts:

Standard	0 5 per cent solution						
Column length	15 mm.						
Test solution:							
Concentration, per cent.	0.75	0 625	0 56	0.5	0.44	0 375	0.31	0.25
Column length, mm.	7 4	10 7	13 0	15.0	17.1	20.9	25 0	36.5

Note.—The column lengths are in each case the average of nine readings.

These values are expressed graphically in Fig. 39, the ordinates representing the length of column of the test solution and the abscissae representing the ratio of the concentrations of the two solutions.

In the figure is also shown the theoretical curve, in which the lengths of column of two solutions are inversely proportional to their concentrations. A consideration of the figure will show that peppermint oil follows the theoretical curve fairly closely when the ratio of concentrations lies between the limits of 0.7 and 1.2.

[8] J. Ind. Eng. Chem., 8, 128 (1916).

On both sides of these limits the actual plot deviates distinctly from the theoretical curve and corrections must be applied to get the true percentage.

There are thus two methods of procedure in determining the percentage of oil in an unknown sample which lies outside these limits of concentration ratios:

1. Compare the unknown with the standard in the usual way and read off the percentage on the plot.

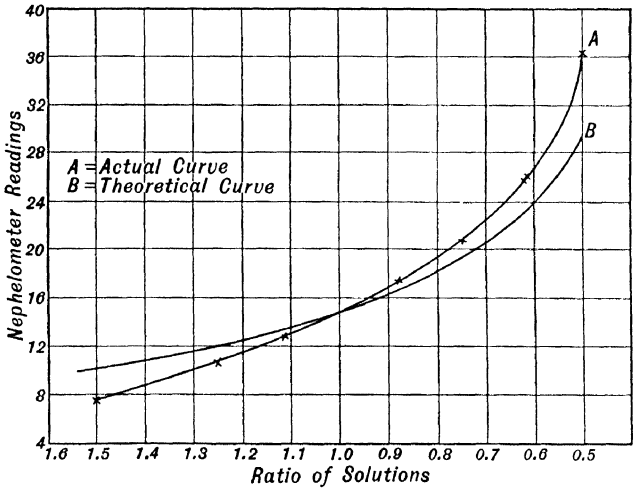


FIG. 39.—Nephelometric Curve for Oil of Peppermint.

2. Compare the unknown with the standard approximately and from the indicated result prepare a standard more nearly approaching the concentration of the unknown. Using this as a standard, the percentage of oil in the unknown can be computed.

The second method seems to be much safer and serves also as a check on the approximate determination, thus reducing the chance of error. It is in general easier because the correction curves are not the same for different oils.

Nutmeg and Anise Oils

Woodman, Gookin and Heath obtained in the cases of nutmeg and anise oils results similar to those for oil of peppermint. These

oils when diluted follow the theoretical curve of the nephelometer even more closely than peppermint oil and give results in very close agreement with the theoretical values.

Oil of anise gives a heavy precipitate with water and it is best to precipitate 5 cc. of the extract with 5 cc. of water or dilute hydrochloric acid instead of the larger volume used to precipitate the other oils.

Oil of Rose

Take 5 cc. of the extract and precipitate the oil by adding 15 cc. of water or better 2.5 N hydrochloric acid. Use 0.4 per

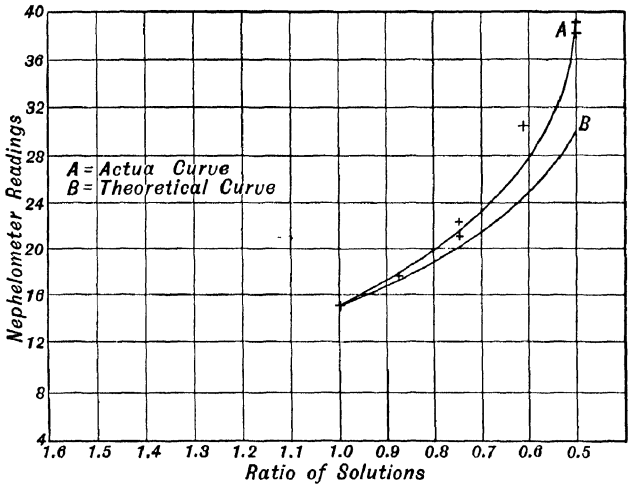


FIG. 40.—Nephelometric Curve for Oil of Roses.

cent or 0.2 per cent alcoholic solutions as standards (see Note 4). For accurate results, the unknown and standard should have nearly the same strength. Figure 40 shows the actual and theoretical curves as given by Woodman, Gookin and Heath.[9] It may be noted that the actual and theoretical values agree only for concentration ratios near unity.

Essential Oil in Cordials

As a rule the cordials contain less alcohol than the extracts. They also usually contain much sugar and are frequently highly

[9] *Loc cit.*

colored. The low alcohol content has very little effect on the determinations. The sugar, on the contrary, either prevents the precipitation of a part of the oil, or alters the size of the oil globules in the precipitate so that the amount of light reflected is less than that reflected from a solution of the same strength but containing no sugar.

By adding to the standard approximately the same proportion of alcohol and sugar present in the sample a correction for the error due to the alcohol and sugar is easily made.

If the cordial is deeply colored, the oil should be removed by distillation. Take 100 cc. of the cordial, dilute with an equal volume of water and distill 100 cc. This procedure gives a quantitative separation of the oil, no special precautions being required except to distill at a fairly slow rate. The distillate is colorless and has the same volume and approximately the same alcoholic content as the original sample.

If the cordial is only slightly colored, distillation is unnecessary.

Notes.

1. There is no single oil that can be used as a standard for the determination of all oils, since emulsions formed by adding the same amount of water to alcoholic solutions of different oils of equal strength differ widely in the amount of light reflected.

2. The nephelometric reading is not affected by slight variations in the chemical composition of the oil, hence it is not necessary that the standard employed should be from exactly the same source as the oil being determined.[10]

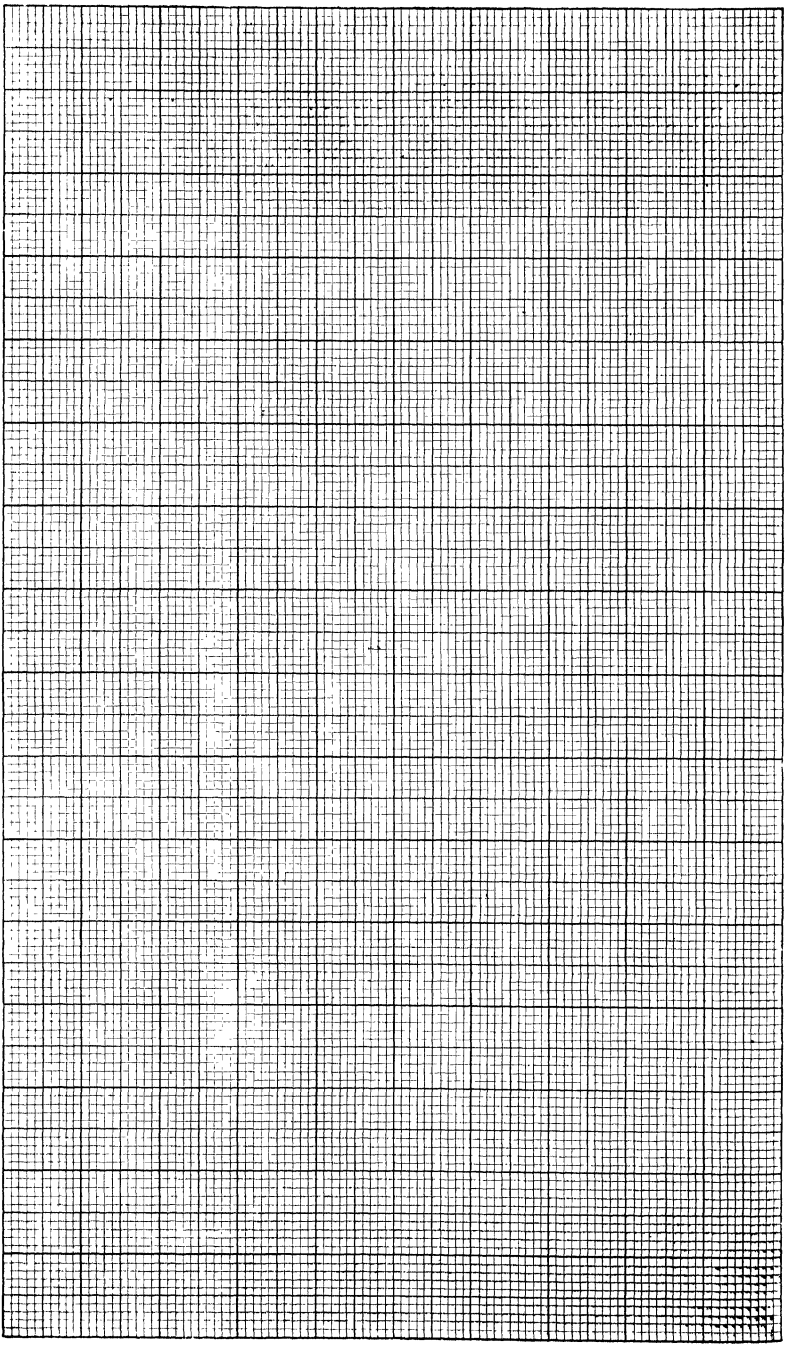
3. Woodman, Gookin and Heath give the following table of typical results in the analysis of alcoholic extracts, containing amounts of oil unknown to the analyst:

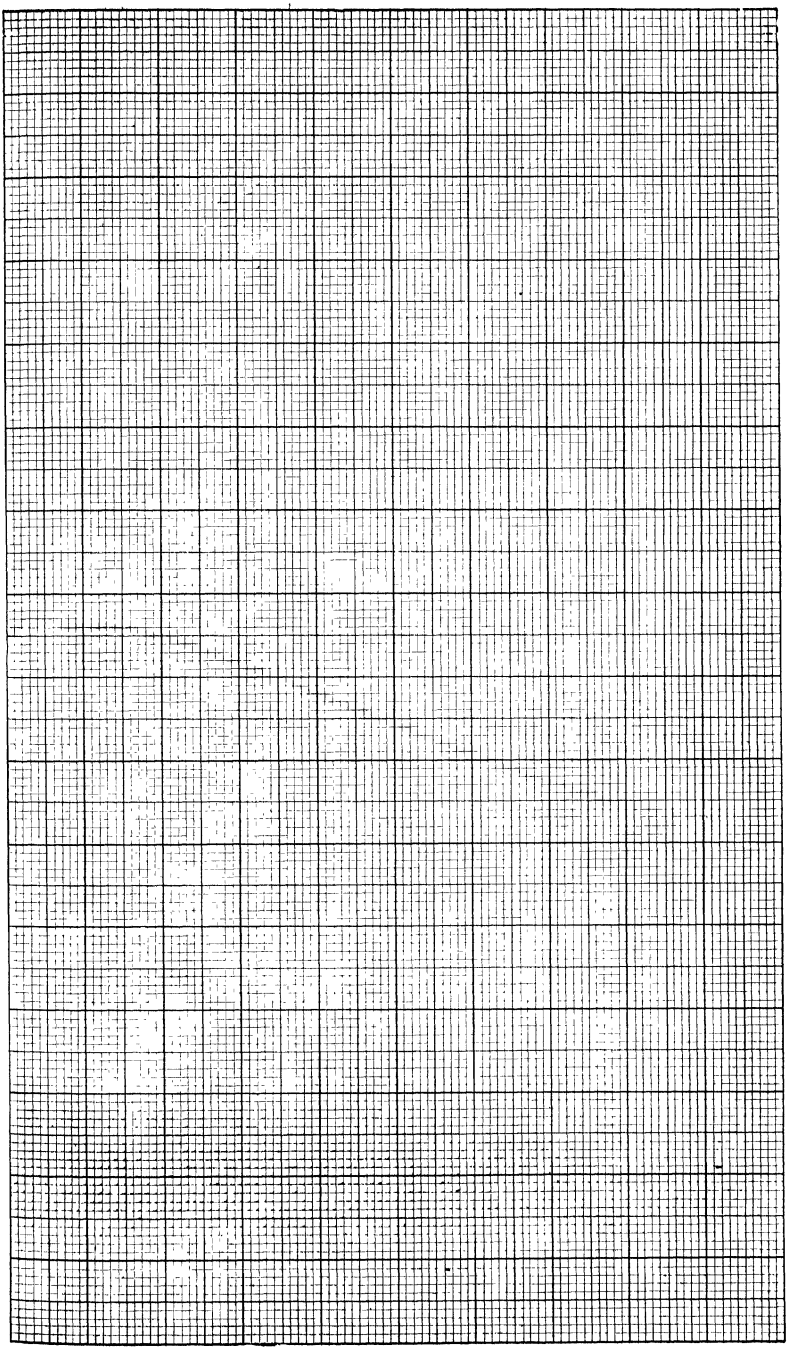
Per Cent	Peppermint			Anise		Nutmeg		Rose	
Oil added..	0.88	0.40	0 198	0 625	0 33	0 375	0 31	0 325	0 317
Oil found..	0 89	0 40	0 200	0.62	0.33	0 375	0 302	0.323	0.310

4. Kober [11] found that by adding 2.5 N hydrochloric acid as used in the Bloor method a much weaker standard than that

[10] Woodman, Gookin and Heath, *loc. cit.*

[11] J. Ind. Chem., **10**, 562 (1918).





recommended by Woodman, Gookin and Heath can be used and still suitable nephelometric clouds will be secured. He recommends precipitating with dilute hydrochloric acid instead of with water.

DETERMINATION OF FATTY ACIDS IN BLOOD PLASMA

Method of Bloor, Pelkan and Allen [12]

Hot alcohol-ether solution is used to extract the lipoids from the blood plasma. The extract is then saponified, the cholesterol extracted with chloroform and determined colorimetrically, and the soaps extracted with hot alcohol. The fatty acids (in the forms of soaps) are determined nephelometrically by the turbid solution obtained upon acidifying the soap solution. For the colorimetric method for determining the cholesterol see Volume I, p. 474.

Reagents.

1. Hydrochloric acid, 1 : 3.
2. Sulfuric acid, 1 : 3.
3. Alcohol (redistilled).
4. Ether (redistilled).
5. Chloroform. The chloroform used must be neutral in reaction and free from moisture and alcohol.
6. Sodium hydroxide. Made from metallic sodium by exposing the metal, in a closed vessel containing distilled water at room temperature, over a receiver to catch the hydroxide which drips off the metal. The action is slow but the apparatus requires little attention and a strong pure hydroxide is obtained.
7. Standard fat solution. This is a 95 per cent alcoholic solution of palmitic and oleic acids of which 5 cc. contain 2 mg. of a mixture consisting of 60 per cent oleic acid and 40 per cent palmitic acid. The standard is conveniently made by first preparing solutions of oleic and palmitic acids, each containing 200 mg. of

[12] J. Biol. Chem., **52**, 191 (1922).

fatty acid in 500 cc. of alcohol. For use 60 cc. of the oleic acid solution are mixed with 40 cc. of the palmitic acid solution.

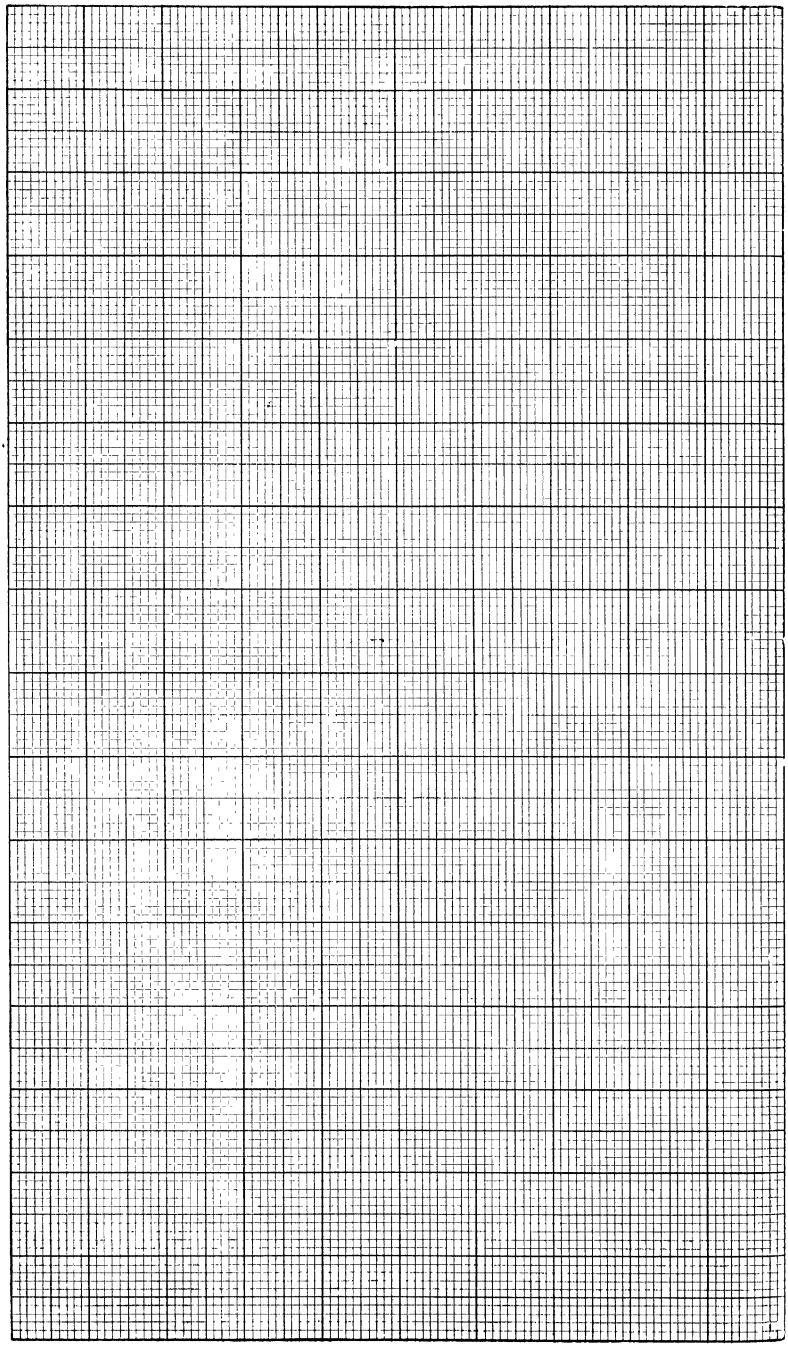
Procedure.—*Extraction and Saponification.*—Five cubic centimeters of blood plasma are measured into a 100-cc. flask containing about 75 cc. of a mixture of three parts alcohol and one part ether (both redistilled). The plasma is made to enter in a slow stream of drops and the liquid in the flask is kept rotating rapidly to prevent the formation of large aggregates of precipitate. At once, or after standing till a convenient time, the flask is immersed in boiling water with frequent and strong rotation (to prevent superheating) until the liquid begins to boil, then cooled to room temperature, made up to volume, mixed, and filtered. For the determination, a volume (10 to 20 cc.) containing about 2 mg. of fatty acid is measured into a small Erlenmeyer flask (50 to 100 cc.) of Non-sol glass (Pyrex is less suitable because it is readily attacked by the strong alkali), 0.1 cc. of concentrated NaOH made from sodium is added and the mixture evaporated on the water-bath. When the volume of liquid has been reduced to a few drops the flask should be rotated or shaken occasionally so as to distribute the liquid evenly over the bottom (but not over the sides). The drying is then continued until only two or three drops of liquid remain and the odor of alcohol is entirely gone. The alkali is then *partially* neutralized by the addition of 0.1 cc. of dilute sulfuric acid (1 volume concentrated acid, 3 volumes water), and the liquid well mixed and distributed over the bottom of the flask as before. The drying is then continued on the water-bath until the residue is dry and all the moisture has disappeared from the sides of the flask. The process of drying is a very important step in the method, since the separation is not quantitative if the drying is either carried too far (in which case some of the cholesterol cannot be recovered by the cold treatment), or not far enough (when a part of the soap or fatty acids is extracted with the cholesterol). The amount of acid added should be somewhat less than enough to neutralize the alkali since otherwise fatty acids would be set free and dissolve in the chloroform. For the same reason the added acid should be well mixed with the residue in the flask so as to insure its complete

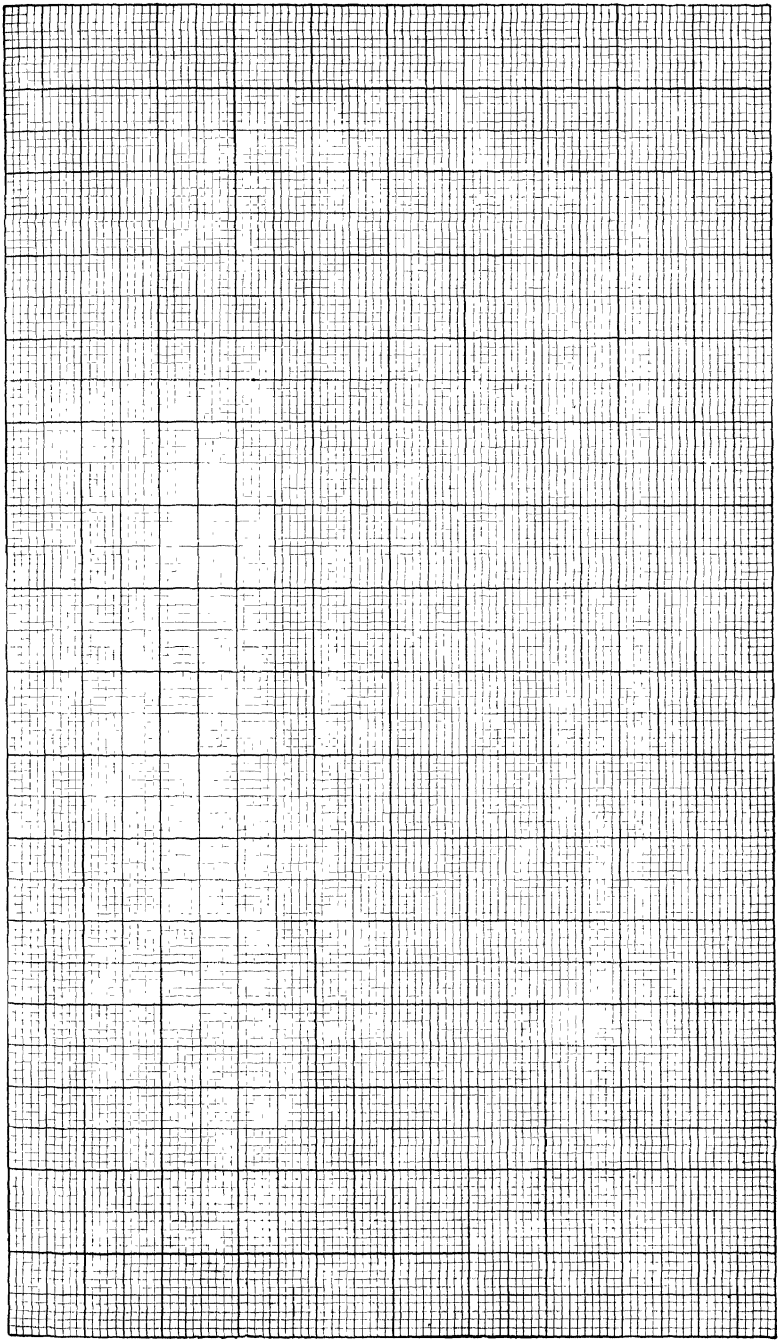
neutralization. If there is not enough liquid in the flask to allow complete mixing, a drop or two of distilled water should be added. The reason for the addition of acid is twofold, first to prevent destruction of cholesterol by the strong alkali (for, contrary to the statements in the literature, cholesterol is altered—at least as far as its color-producing properties are concerned—by heating with strong alkali), and second by the formation of the crystalline sodium sulfate the residue is made porous so that the solvents penetrate readily. The heating should be carried through all its stages on a water-bath and not on an electric hot-plate since it has been found impossible to prevent overheating on the latter.

Separation and Determination of Fatty Acids.—After the cooling, 10 cc. of chloroform are added and the flask is allowed to stand ten minutes; it may be shaken occasionally so that the solvent may reach any material adhering to the sides. The chloroform extract is poured through a 5½-cm. hardened paper filter into another small flask and the extraction twice repeated with 5 cc. of chloroform. If the drying and distribution of the salt have been carefully carried out, very little of the salt will break loose from the bottom of the flask during the chloroform extraction and the fatty acids are quantitatively retained. The combined chloroform extract is then evaporated down to 2 or 3 cc., poured into a 10-cc. glass-stoppered, graduated cylinder, made up to 5 cc. with chloroform washings from the flask, and the cholesterol then determined colorimetrically as directed on page 476 in Volume I.

The residue in the small flask after extraction with chloroform is treated with boiling alcohol for the extraction of the fatty acids (in the form of soaps) in the following way: 10 cc. of redistilled alcohol are added to the flask, the mixture is raised to boiling on an electric stove or water-bath and kept boiling very gently for a period of ten minutes. The hot alcohol is then poured through the small hardened paper filter which was used in filtering the chloroform into a 100-cc. Erlenmeyer flask. The extraction with alcohol is repeated once, using 5 cc. of alcohol, the hot extracting fluid being poured through the filter into the flask. The combined filtrates are evaporated to small volume, about 2 to

3 cc., then transferred quantitatively to a small graduated, glass-stoppered cylinder, and the flask is rinsed out with just enough alcohol to bring the volume in the cylinder up to 5 cc. One hundred centimeters of distilled water are next measured into a 200-cc. beaker and the alcoholic extract of the fatty acid is added, with stirring through a small funnel with the stem drawn out to form an opening about 1 mm. in diameter and extending nearly to the bottom of the beaker. The cylinder is rinsed once with the solution in the beaker and the rinsings are poured back into the beaker through the funnel. To another beaker containing 100 cc. of water are added through a pipette, with stirring, 5 cc. of the alcoholic standard containing 2 mg. of a mixture of oleic and palmitic acids containing 60 per cent oleic and 40 per cent palmitic acids in 95 per cent alcohol (redistilled). Ten cubic centimeters of dilute hydrochloric acid (1 part concentrated acid, 3 parts water) are added to each beaker, with stirring, and after standing not less than three nor more than ten minutes the solutions are compared in a nephelometer.





CHAPTER XVII

LIPASE

NEPHELOMETRIC METHOD FOR THE STUDY OF LIPASE

By Hans Kleinmann [1]

The principle of the nephelometric lipase determination consists in splitting a homogeneous and stable emulsion of triolein by means of lipase. The course of the digestion is determined by the comparison of the digested and undigested triolein solutions by means of the nephelometer. The measurement of the turbidity of the triolein suspension gives, under the conditions of the method, the exact amount of the triolein content. The method involves the neutralization of oleic acid which is set free in the digestion and which would produce a turbidity, with a certain amount of ammonia converting the acid into soluble ammonium oleate. As the ammonium oleate emulsifies the undigested triolein depending upon the concentration of the ammonium soap, a certain excess of ammonium oleate is added at the beginning, so that the oleic acid split off measured as ammonium oleate is 100 to 150 per cent of the concentration theoretically possible. Within this range of concentration the emulsification of the triolein by the soap is at a maximum and perfectly uniform; therefore the undigested triolein has, in spite of the varying quantities of soap formation, a uniform and maximum emulsification. The turbidities of the emulsions are proportional to the content of triolein.

The nephelometric method for the determination of lipolytic activity makes it possible to work with emulsions of very low concentrations. The emulsion described below is 0.05 per cent,

[1] P. Rona and H. Kleinmann, Biochem. Z., **174**, 18 (1926).

that is, N/590. Such emulsions are not determinable by means of titration methods nor by Rona and Michaelis' drop (falling) method, as this method requires solutions of tributyrin, i.e., it cannot use a dispersed system. With the nephelometric method one can study theoretical problems, such as the relationship between substrate dispersion and ferment action; for these the method has already been applied. The method can be used wherever special problems are to be solved, or where the unsuitability of other methods makes its application desired. One limitation the nephelometric method has, namely, buffers and electrolytes cannot be added stronger than N/500. Electrolytes affect strongly the emulsification of the triolein by means of soap. The method has therefore been worked out so that electrolytes other than those required by the method are not introduced into the system.

Reagents.

For the purpose of comparing emulsions of different substrate dispersion, for which purpose the method was developed, different fat emulsions, both fine and crude, had to be prepared. It must, however, be mentioned that for the nephelometric determination of lipase, the described method of preparing, through condensation, highly dispersed emulsions (Ef) is most suitable. This emulsion is relatively uniform and stable, and merely comes into consideration when one wants to determine lipolytic action. As example the description of an emulsion (Eg) of crude fat suspension is given; it is not stable, and much less suitable for nephelometry and is only described for special purposes.

1. *Substrate Solution.*—For nephelometry 10 cc. of 0.05 per cent triolein emulsion is used. Triolein shall have 0.5 per cent of oleic acid. As starting material triolein (Kahlbaum) will do. The age, i.e., the fatty acid content, of the triolein is important. For the purpose of reproducibility it is best to add the fatty acids in the amount needed artificially (see below), and to start with a perfectly neutral triolein, as it may be obtained from Kahlbaum. If perfectly neutral triolein is not obtainable (1 gram dissolved in

alcohol-ether solution is titrated using phenolphthalein as indicator, and using the same volume of alcohol-ether mixture as a control) it must be made acid-free.

For this purpose fats can be made acid-free using experience gained in other work. The fats are dissolved in low boiling benzin and then treated with the calculated amount of NaOH solution required to neutralize the fatty acids. By this procedure the soaps settle out in flakes and, on warming, gum together and usually contain all of the water added with the alkali, and floating on the surface of the liquid. The concentration of the NaOH depends upon the amount of fatty acids. Usually a 1 N NaOH is suitable. The benzin solution is filtered from the soap and separated from traces of water in a separatory funnel, and the fat freed from benzin by vacuum distillation.

The acid- and soap-free triolein is dissolved in a mixture of three parts of absolute alcohol and one part of ether (by volume) so that 1 gram of triolein is contained in 100 cc. of solution. The ether should be of the purest ether for anesthesia.

To the alcoholic-ether solution of triolein before it is made up to volume, 0.5 cc. of 1 per cent alcoholic oleic acid solution (oleic acid I, Kahlbaum) is added for every 100 cc. of volume, and alcohol-ether added to the mark. The solution is therefore 1 per cent triolein and 0.005 per cent oleic acid; in other words, the triolein used is 0.5 per cent oleic acid.

(*A*) For the preparation of a fine, highly dispersed emulsion, designated in the following as " Ef," 5 cc. of triolein solution are used for every 100 cc. of emulsion.

About 250 to 270 cc. of a N/1000 K_2CO_3 solution are placed in a 500-cc. suction flask. With constant shaking, 15 cc. of the triolein solution are allowed to flow in. There is formed a complete blue-white opalescent fat emulsion. From this the alcohol-ether can be removed by either of the following methods:

(1) *Through boiling.*—For this purpose the flask is placed in a boiling water-bath, and allowed to remain there for one-half to three-quarters of an hour until the slightest odor of alcohol has disappeared. The emulsion does not change through this treatment; its turbidity remains the same. Also digestion experi-

ments with boiled emulsions gave the same results as those obtained with the following method.

(2) *Through aëration.*—Carbon-dioxide-free air is bubbled (by means of a soda lime tube inserted in the line) through the flask (suction), for two to three hours. This removes the alcohol so that it is no longer detected by smell. To remove the last traces the suction flask is heated to 50° C. while being aerated.

As this method avoids with certainty even the smallest saponification of the emulsion, it is preferred to method (1).

After driving off the alcohol-ether the emulsion is put into a 300-cc. graduated flask. Usually the suction flask has a ring of fat deposited which represents the meniscus of the emulsion. This ridge is dissolved with a few cubic centimeters of alcohol-ether mixture and precipitated with 20 cc. of N/1000 K_2CO_3 solution, whereby an emulsion is produced which is freed from alcohol-ether as described above. Shaking the suction flask often during the aeration prevents the formation of a new meniscus ring. After the alcohol is completely removed, the remainder of emulsion is added to the main portion, and the flask rinsed with a few cubic centimeters of K_2CO_3 solution, using a rubber " policeman " to clean the sides of the suction flask, and the rinsing liquid to bring the emulsion up to 300 cc.

The emulsion will keep many hours without change. After twenty-four hours one can see a light deposit on the walls of the flask. Therefore the emulsion should be prepared on the day it is to be used in ferment work.

(*B*) For the production of cruder dispersed emulsions (designated in the following always by " Eg ") of the same concentration, 15 cc. of triolein solution are put into a 500-cc. wide-mouth flask having a ground-glass stopper that fits perfectly. The flask is then put into a boiling water-bath, whereby in a few minutes the alcohol and ether are evaporated. The flask is then quickly removed from the water-bath so that oil residues clinging to the sides of the flask are not heated unnecessarily. These residues cannot be emulsified perfectly as they cling to the sides of the flask too tightly. Therefore the drops of oil are treated with a few cubic centimeters of ether (anesthesia); from 5 to 10 cc. are

sufficient. Then 150 cc. of K_2CO_3 solution are added and the floating oil is emulsified roughly by shaking the flask with the hand. The ether is then removed with carbon-dioxide-free air. Finally the flask is placed in a water-bath at a temperature of 50° C. Within a half-hour the ether has totally disappeared.

The emulsion resulting from the aeration is placed in a 300-cc. graduated flask. The wide-mouth flask usually shows residues of oil sticking to the sides. These are dissolved in alcohol-ether mixture and the alcohol-ether removed by placing the flask in a boiling water-bath. The remaining oil is again dissolved in a little ether, precipitated by the addition of 100 cc. of K_2CO_3 solution, and the ether evaporated as above. The emulsion is added to the rest in the graduated flask. The graduated flask must be shaken often in order to prevent the formation of a ring of fat at the line of the meniscus.

Usually it suffices to redissolve the fat residues only once, but if necessary the redissolving must be repeated a second time. All the emulsions are placed in the graduated flask and made up to the mark with N/1000 K_2CO_3 solution. The whole emulson is now returned to the wide-mouth flask and shaken fifteen minutes by hand rapidly and vigorously. For the purpose of testing, the resulting emulsion has a stability of about one-half hour. Immediately before using it is given another shaking for ten minutes. Naturally it is much less stable than emulsion " Ef," from which it differs in not having a blue-white color and in being decidedly weaker nephelometrically.

The emulsions have a *p*H of 7.5.

2. Ammonium hydroxide, 1/25 N.

3. Oleic acid-glycerin emulsion. One gram of oleic acid (Kahlbaum) is dissolved in 100 cc. of 95 per cent alcohol. An emulsion made with 4.75 cc. of this solution in 100 cc. of water corresponds, as far as the oleic acid content is concerned, to the 5 in 100 cc. of the alcoholic triolein solution. In order to save volume the oleic acid emulsion is made of double strength and of half this volume. Therefore for the production of an oleic acid emulsion, 19.14 cc. of the 1 per cent alcoholic oleic acid solution are put into a 200-cc. graduated flask or cylinder and emulsified

by quickly pouring in distilled water and stirring. Before bringing the solution up to the mark, the amount of glycerin produced in fat digestion is added (see below) and the volume made up to 200 cc. with distilled water. This produces a perfectly homogeneous, strong, bluish opalescent emulsion which keeps several days without settling. However, as there is a certain amount of aggregation of particles noticeable from its increasing turbidity, it is best to prepare this oleic acid emulsion on the day it is to be used. Five cubic centimeters of the emulsion correspond to 10 cc. of the triolein emulsion so far as oleic acid and glycerin are concerned; that is, with these volumes it is 100 per cent.

4. Glycerin solution. An aqueous 1 per cent solution of glycerin is prepared. Of this solution, 2.1 cc., which correspond to 19.14 cc. of the oleic acid solution, are added to the 200 cc. of oleic acid emulsion before it is finally made up to volume. The glycerin is added to the oleic acid—although in the small concentration it can hardly have any influence—in order to have not 100 per cent oleic acid but 100 per cent " digestion products " in the receivers.

Procedure.—As an example of the nephelometric study of a lipolytic digestion, the digestion of two emulsions of fat of equal concentration but of different dispersions will be described.

For the measurement of a triolein cloud, which may vary up to 50 per cent of oleic acid and glycerin split off, a receiver is prepared consisting of a 25-cc. test tube containing 5 cc. of 1/25 N ammonia, and 5 cc. of the oleic acid-glycerin emulsion, for every 10 cc. of triolein emulsion.

The addition of the ammonia to the oleic acid-glycerin emulsion produces a clear solution of ammonium oleate. A skin-forming cloud is without significance. After several hours the receivers begin to cloud up. The test should, therefore, be made within two to three hours after setting up the receiver.

Into these receivers are put 10 cc. of triolein emulsion. There is produced thereby, particularly if emulsion " Ef " is used, a highly dispersed fine suspension of fat, which is completely homogeneous and stable for hours. It is, however, advisable to measure the cloud with the nephelometer within the first hour.

The receiver contains enough ammonium hydroxide to neutralize and dissolve amounts of oleic acid within 50 per cent equivalent of the triolein. This can be proved by the addition of 2.5 cc. of oleic acid-glycerin emulsion to the clear soap solutions in the receivers.

Therefore the fatty acids produced through lipolytic activity are dissolved completely by the contents of the receivers. After mixing, the cloud that remains is a pure fat turbidity, which, through the ammonium soap content of the receivers, is brought to a definite dispersion. The fatty acid-glycerin produced through digestion is without influence upon the proportionality relationship, as they vary between 100 and 150 per cent (instead of from 0 to 50 per cent) and within these limits an optimum and uniform emulsion is effected.

The proportionality relationship holds accurately to about 40 per cent digestion (error of measurement 1.0 per cent) for emulsion " Ef."

The emulsion " Eg " does not give such a homogeneous fat suspension, but shows under a magnifying glass some individual drops. Therefore the accuracy of the method is not so good as with the emulsion " Ef." The law of proportionality, however, holds up to 30 per cent digestion.

The making of a digestion experiment is given in an example involving two emulsions " Ef " and " Eg " in which their digestions are compared.

The emulsions " Ef " and " Eg " are prepared on the day of the tests. Also the oleic acid-glycerin emulsion is freshly prepared. The emulsion " Eg " is shaken again vigorously by hand ten minutes just prior to the digestion. Two digestions are started for each emulsion. In order to prevent disintegration of the emulsion " Eg," the digestion is carried out in the shortest possible time. Portions are taken every four minutes for a total of seven, making the time of digestion twenty-four minutes.

Put 73 cc. of emulsion into each of the 75-cc. gr duated cylinders. Since each test is made in duplicate, four graduates will be necessary. Place a 10-cc. graduated pipette in each cylinder. Then place the graduated cylinders in a thermostat whose tem-

perature is accurately regulated to 40° C. (±0.05°). Warm the emulsions for about fifteen minutes until they have reached the temperature of the thermostat.

Thirty cubic centimeters of ferment solution are heated in the thermostat along with the emulsions. The ferment solution is obtained by treating pancreatin powder (e.g., "Pankrean," "Pankreatin Rhenania") with water and filtering. Use such portions of a solution containing 0.25 g. of pancreatin in 100 cc. of distilled water as will, within the specified time, produce a digestion of 30 per cent. The ferment concentration should be so chosen that the 30 per cent digestion is not exceeded on account of the working limits of the methods, but on the other hand this limit should be closely approximated in order to avoid too small readings or results. The pancreatin extraction mixture is allowed to stand fifteen to thirty minutes, at room temperature, with frequent stirring, after which it is filtered. If the filtrate is not perfectly clear, it is filtered again through the same filter, after which it is usually clear. Five to ten cubic centimeters of the clear filtrate of the ferment solution are taken and boiled for a few minutes, whereby it first clouds and then precipitates some protein, which is filtered off. The unboiled filtrate is then put into the thermostat.

In the meanwhile the receivers for the ferment portions are prepared. Since seven portions are to be taken from each, twenty-eight receivers are carefully cleaned and placed in a drying oven. After drying, the cleaned test tubes each receive 5 cc. of 1 25 N ammonia and 5 cc. of oleic acid-glycerin emulsion.

Into the first test tube of each of the four rows, of seven tubes each, is put 1 cc. of the boiled ferment solution. This serves the purpose of equalizing the 9.0-cc. portion taken for the first sample, in distinction from the regular portion of 10 cc. As the total amount of fat substance must be the same in all portions, the first portion is only 9 cc. on account of the increase of dilution of the fat emulsion of 10 per cent due to the ferment solution.

The reason for using boiled ferment solution instead of distilled water is as follows: The salts present in the ferment extract produce an emulsifying action upon the fat emulsion, especially

those of higher concentration. This effect, unless equalized in the original sample, would make it appear as a slight digestion. That this phenomenon is due to the salts and not the protein content of the ferment solution can be seen from experiments using, side by side, a boiled and an unboiled ferment solution. The nephelometric reading immediately after the addition shows the same intensity of clouds, and therefore the slight protein content of the ferment solution (which may cause a slight opalescence when the ferment solution is measured alone with the contents of the receivers) has no influence and that the skin-forming cloud due to the protein disappears completely in the massive fat suspensions.

From the triolein emulsions in the thermostat 10 cc. are now withdrawn and 1 cc. discarded and 9 cc. put into the first receiver, whose total volume is now 20 cc. It is recommended that these first portions which represent the total undigested fat, i.e., 100 per cent, and which are used as standards for the measurements of the other portions, be made ready for the nephelometer. For this purpose two pairs of nephelometric tubes are set up, and in one of each pair the first portion of emulsions " Ef " and " Eg," respectively, is added. Into the second tube the duplicate of each emulsion is put and measured in the nephelometer to see if they check. Then the duplicate solution of each is emptied and the other of each emulsion left is filled and used as standard for the following.

Now while the observer stays at the nephelometer, an assistant takes 7 cc. of the prewarmed ferment solution and adds it to the 63 cc. of the triolein emulsion, at intervals of one minute each. The time of adding the ferment solution is accurately marked with a stop-watch. One minute after the last addition of ferment, that is, four minutes after the first addition, the assistant takes the first portion from cylinder or graduate No. 1. This first portion is 10 cc. after first stirring the solution by aeration by blowing through the pipette. After the interval of another minute the next portion is taken out of cylinder 2, etc., so that from every cylinder 10 cc. is taken every four minutes. The receivers are very carefully and very slowly rotated so as to mix the con-

tents but not to form any foam, which disturbs the nephelometric measurement very much. They are immediately measured in the nephelometer, each sample against the first portion of its kind of emulsion as a standard. That is the reason why two pairs of nephelometric cups are prepared. With a little practice it is possible to take the nephelometric readings just about as fast as the samples are prepared. If it should go somewhat slower it does no harm because at room temperature the digestion proceeds only very slowly.

It is not possible to check the ferment action otherwise since any additions for that purpose change the emulsions. Also the preservation with ice is not suitable, as the subsequent warming has a tendency to produce air bubbles which make nephelometric measurements difficult. The immediate measurement of the digested solutions and the collaboration of two workers, one taking samples every minute while the other operates the nephelometer, were found to be advantageous.

While operating the nephelometer, air bubbles, which are easily formed from the tendency of the emulsions to foam, must be avoided, and particularly when using emulsion " Eg," is it necessary to make control tests of the standard by interchanging the nephelometric tubes. The plungers must also be carefully wiped with a linen cloth between every measurement on account of the fatty deposits that may accumulate upon them.

After the seventh portion has been taken, there remain 10 cc. of solution in the substrate-ferment container. This serves for a *p*H determination.

That the *p*H hardly changes is seen from the following example: The *p*H of the fat emulsion was 7.5–7.6; after diluting with the protein-containing ferment solution it sank to 7.26; after digestion to about 30 per cent, the *p*H of the fat solution was 7.1 to 7.2.

The results using emulsion " Ef " are perfectly sure and reliable but those with emulsion " Eg " show variations on account of its sensitivity to stirring, etc.

Calculation.—If C_1 is the concentration of the digested solution, C is the concentration of the starting solution of the diges-

tion, which is equal to 100 per cent, and H_1 the nephelometric reading of the digested solution, and H the nephelometric setting of the standard solution, i.e., starting solution, then

$$C_1 = \frac{100 \times H}{H_1}.$$

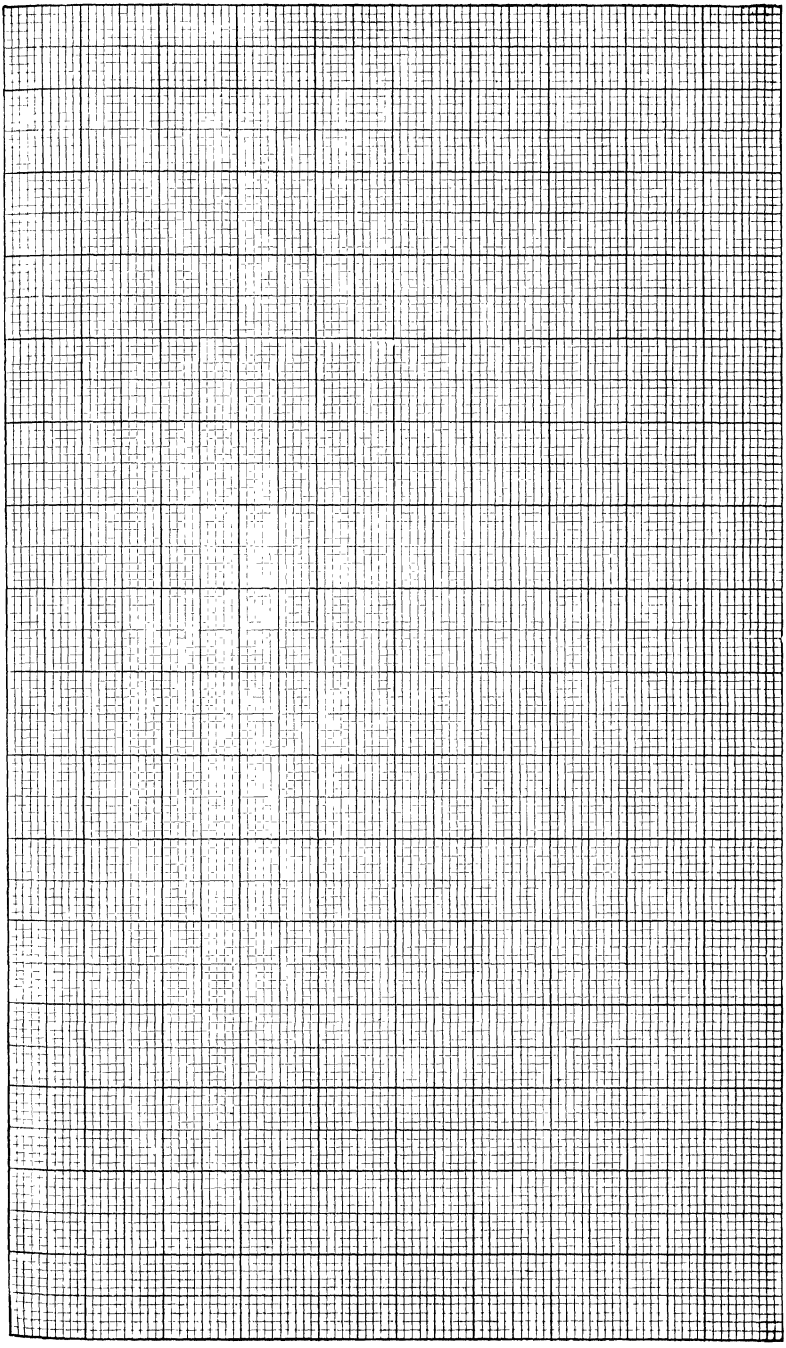
The fat concentration in the digested solution C_1 is obtained in per cent. By subtraction from 100 the amount of digested fat, in per cent, is obtained. Unless a Kleinmann nephelometer is employed, a calibration curve may be required.

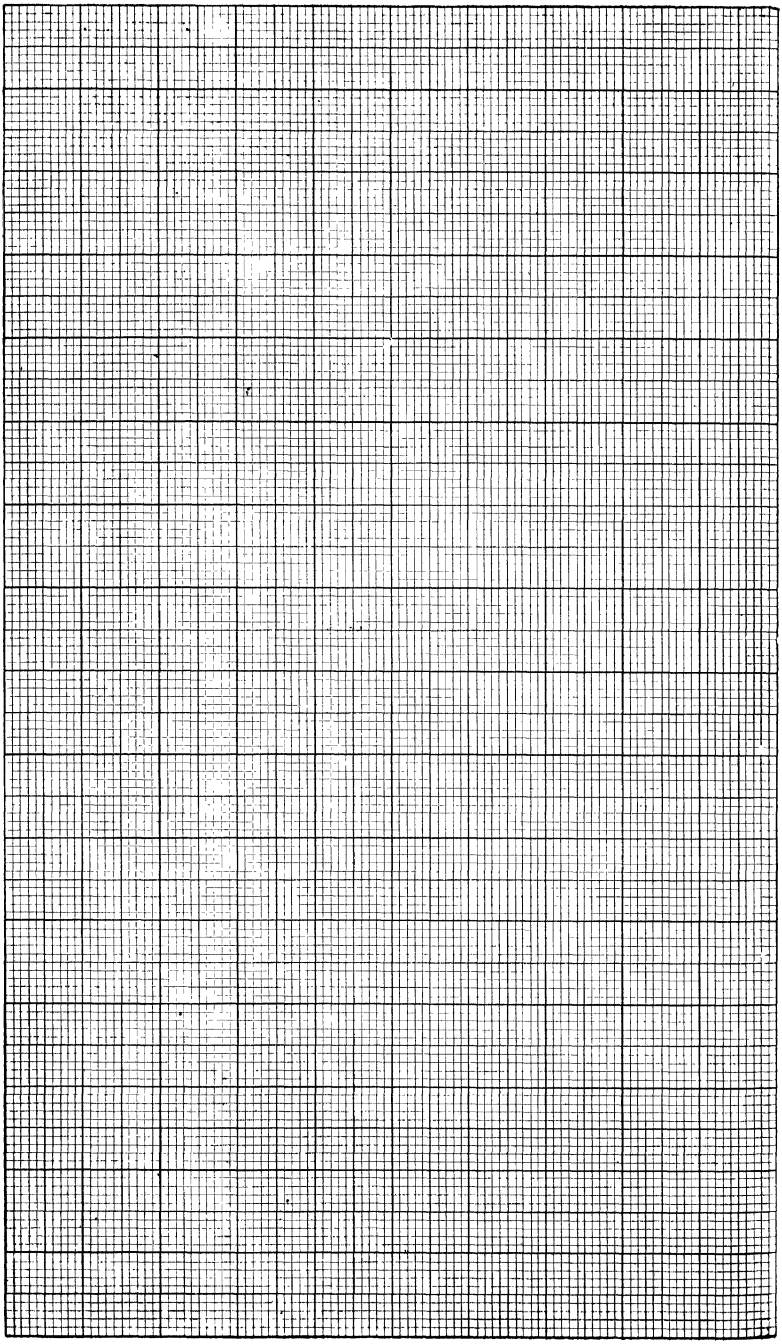
As example of the method, the digestion of the emulsion "Ef" is given using two different ferment concentrations (1 : 3). The error of the method amounts to about 2 per cent.

TABLE XVI

THE DIGESTION OF THE SAME EMULSIONS EF WITH DIFFERENT CONCENTRATIONS OF PANCREATIN RHENANIA. ALSO ITS PROTEOLYTIC POWER

Time of digestion, in minutes . .	0	4	8	12	16	20	24
1. Ferment solution nephel. readings .	20	23 4	24 2	26 0	27 2	29 0	30 2
(1 to 100) nephel. readings .	20	23 4	24 2	26 0	27.2	29 0	30 2
2. Ferment solution nephel. readings	20	20 2	21 0	21 9	22 1	22 5	22 7
(1 to 300) nephel. readings	20	20 2	21 0	21 9	22 1	22 5	22 7
1. Fat digestion, per cent. .	0	14 5	17.4	23.1	26 5	31 0	33 8
2. Fat digestion, per cent	0	1 0	4 6	8 5	9 5	11 2	12 0





CHAPTER XVIII

NUCLEIC ACIDS

DETERMINATION OF NUCLEIC ACIDS

METHOD OF KOBER AND GRAVES [1]

THIS method was developed for the purpose of estimating undigested nucleic acids. It consists in adding a 0.2 per cent solution of acid egg albumin to dilute solutions of nucleic acids and estimating nephelometrically the resulting suspensoids. This reagent will easily detect one part of nucleic acid in a million parts of solution and is not appreciably affected in dilute solutions by most substances met with in physiological work.

Reagents.

1. Albumin solution, 0.2 per cent. Prepare an approximately 1 per cent solution of egg albumin (assuming egg-white contains 10 per cent albumin by weight) from the whites of four to six fresh eggs by shaking thoroughly with 0.1 N acetic acid (0.5 cc. per gram of egg-white) and then diluting with ten volumes of water, allowance being made for the volume of acid used. Filter the solution twice. Place 50 cc. of the clear 1 per cent solution in a 250-cc. volumetric flask, add 3 cc. of 0.1 N acetic acid and dilute to the mark with distilled water. This gives a 0.2 per cent solution of albumin.

2. Standard nucleic acid solution. Prepare a 0.1 per cent stock solution by dissolving 0.1000 gram of nucleic acid in distilled water, diluting to 100 cc. and thoroughly mixing. This stock solution is further diluted before using.

[1] J. Am. Chem. Soc., **36**, 1304 (1914).

Procedure.—To one volume (10 cc.) of nucleic acid (not stronger than 0.01 per cent) add two volumes (20 cc.) of 0.2 per cent albumin solution by means of a pipette, taking care that the tip of the pipette is below the surface of the liquid and thus avoiding the entrance of air bubbles which sometimes cause agglutination. The solution is gently shaken in a rotary fashion and then compared in a nephelometer with a standard suspension of nucleic acid similarly prepared. From the readings and the nephelometric curve calculate the percentage of nucleic acid in the sample.

Notes.

1. Strictly fresh eggs (preferably not over forty-eight hours old) should be used in the preparation of the albumin solution.

2. Egg albumin solutions, although clear, have a tendency to form shreds on the surface. These shreds increase considerably upon shaking, hence precaution must be taken to mix the albumin and nucleic acid solutions by shaking gently in a rotary manner.

3. The formation of "nuclein" precipitates is very much affected by hydrogen ions. For example, 0.2 per cent egg albumin solution without acetic acid gives very little "nucleins," but the addition of only 1 cc. of 0.1 N acetic acid to 50 cc. of the albumin solution is sufficient to give complete precipitation.

4. The formation of "nucleins," being easily affected by hydrogen ions, ought to be influenced by almost any substance, since most substances change the hydrogen ion concentration of a solution. However, when diluted as is necessary for nephelometric work, an equal weight of substance ought to have no appreable effect. This was actually found by Kober and Graves to be the case.

One part by weight of the substances given below was dissolved with one part of nucleic acid and the latter estimated nephelometrically. Within experimental error, the controls gave the same readings as these mixtures:

Inorganic: NaCl, $NaNO_3$, Na_2SO_4, $(NH_4)_2SO_4$, NH_4Cl, $BaCl_2$, $Ba(NO_3)_2$, NaH_2PO_4, $CaCl_2$, $NaC_2H_3O_2$, $KC_2H_3O_2$, $Ca(NO_3)_2$.

Organic: Glycero-phosphoric acid, alanine, tyrosine, histidine, asparagine, phenylalanine, urea, peptone, ereptone, dextrose, levulose, urine (assumed 5 per cent solids), stomach contents (assumed 5 per cent solids), extract of pancreas (dried).

5. To show that the above method is applicable in physiological work, Kober and Graves added normal specimens of urine, stomach contents, and pancreatin to nucleic acid solutions and then formed suspensions in the usual way. The figures in Table XVII were obtained by these workers.

TABLE XVII

Mixtures	*K*	Ratio of Solutions					
		1 0 mm.	0 9 mm.	0 8 mm.	0 7 mm.	0 6 mm.	0 5 mm.
I. Urine and nucleic acid	0 23	11 7	12 6	13 7	15 1	16 7	19.4
II. Pancreatin and nucleic acid . .	0 11	11 2	12 1	13 5	15 2	17 1	19 3
III. Stomach contents and nucleic acid	0 19	12 0	12 9	14 2	15 7	18 5	21 0

These figures are the average of five or six readings. In addition to 0.05 gram of nucleic acid, solution I contained 2 cc. of urine; solution II 0.02 gram of pancreatin, and solution III 2 cc. of filtered stomach contents. As may be seen, the mixtures run practically the same as the nucleic acid alone.

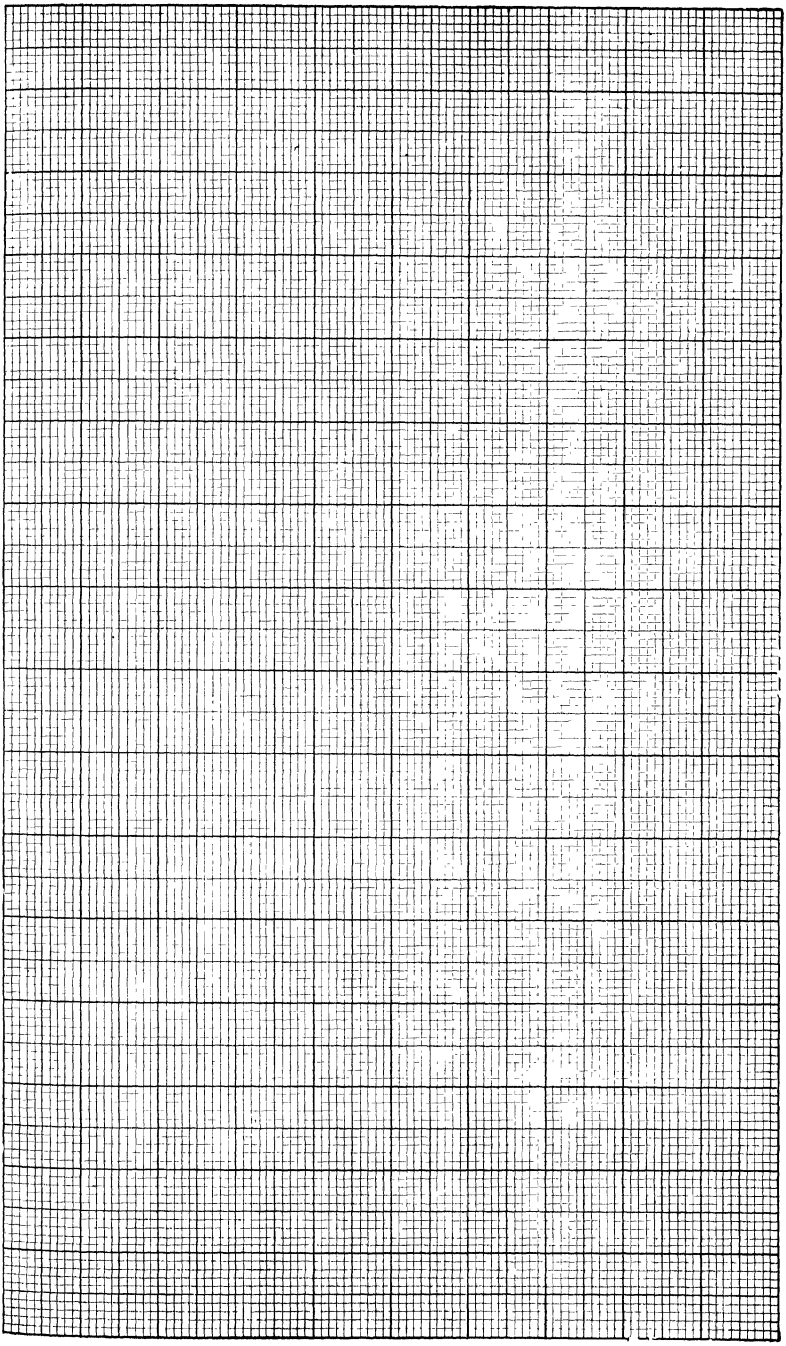
To determine the presence of nucleases and their relative activity, solutions of nucleic acid were incubated with pancreatin, and the digestion determined by comparing at intervals with known amounts of nucleic acids and precipitating in both cases with 0.2 per cent solution of egg albumin.

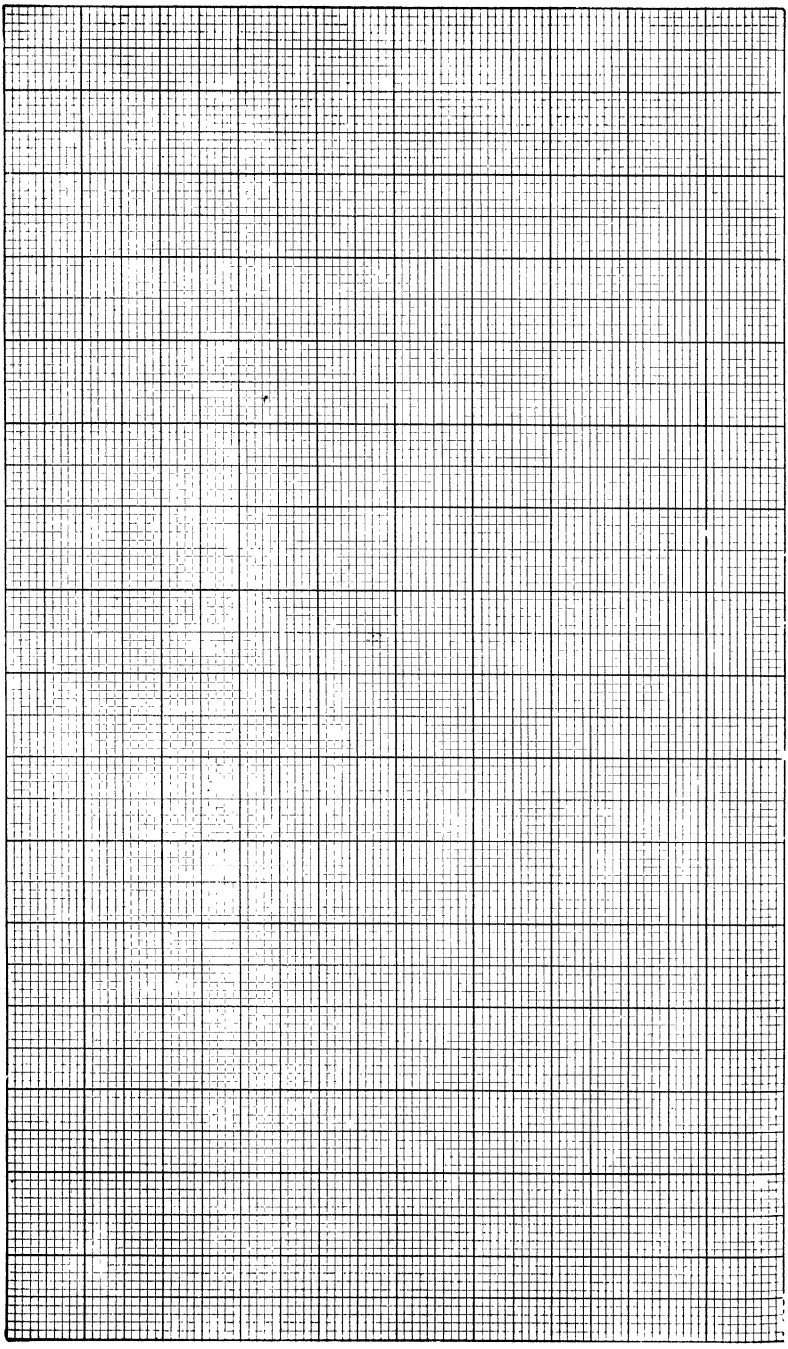
Solution I.—Contained 0.05 gram of nucleic acid, 2.5 cc. of 2 per cent pancreatin solution (Merck's), 2.5 cc. of 2 per cent tricresol, made up to 50 cc. with distilled water. *Solution II* was made in the same manner, except that it contained 5 cc. of 2 per cent pancreatin solution (Eimer & Amend).

TABLE XVIII

NUCLEIC ACID DIGESTED

Pancreatin	0 Times, Per Cent	1 Hr., Per Cent	2 Hrs., Per Cent	3 Hrs., Per Cent	4 Hrs., Per Cent	5 Hrs., Per Cent
I. Merck. . .	0 0	38 0	65 0	75 3	76 8	78 3
II. Eimer & Amend. .	0 0	21 5	28 5	33 0	(33 0)	41 5





CHAPTER XIX

β-OXYBUTYRIC ACID

DETERMINATION OF β-OXYBUTYRIC ACID IN BLOOD

METHOD OF MARRIOTT [1]

THE β-oxybutyric acid is oxidized to acetone and the latter determined nephelometrically by means of Marriott's silver-mercury-cyanide solution.

The method is useful in estimating β-oxybutyric acid in blood and tissues. Since oxidation of β-oxybutyric acid by chromic acid gives only 90 per cent of the theoretical yield of acetone, 10 per cent should be added to the results thus obtained.

Reagents.

1. Sulphuric acid, 6 N and 1 : 1.
2. Glacial acetic acid.
3. Ammonium hydroxide, sp. gr. 0.90.
4. Sodium hydroxide, 10 per cent.
5. Sodium carbonate, 10 per cent.
6. Potassium bichromate.
7. Potassium oxalate, 0.5 per cent.
8. Basic lead acetate, U. S. P.
9. Hydrogen peroxide, 3 per cent.
10. Silver-mercury-cyanide solution (acetone reagent). See p. 171.
11. Standard acetone solution. See p. 172.

Procedure.—The blood (2 to 5 cc.) is drawn from a superficial vein by means of a sterile syringe and run into about 50 cc.

[1] W. M. Marriott, J. Biol. Chem., **16**, 295 (1913).

of 0.5 per cent potassium oxalate solution, contained in a small weighed flask. The increase in weight of the flask gives the weight of sample taken.

One hundred cubic centimeters of water and 1 cc. of glacial acetic acid are placed in an 800-cc. Kjeldahl flask, provided with a dropping funnel and connected with a condenser, the delivery tube of which dips beneath the surface of the water in a receiving flask. The liquid in the Kjeldahl flask is heated to boiling and the diluted blood run in slowly through the dropping funnel. The liquid is kept boiling for about thirty minutes after the last of the blood has been added. The distillate is then redistilled with a little dilute sulfuric acid (see Note 1) and again with 20 cc. of hydrogen peroxide and a slight excess of sodium hydroxide (see Note 2). The final distillate is caught in a small Erlenmeyer flask containing an excess of the acetone reagent, care being taken that the delivery tube dips under the surface of the liquid. Ten minutes suffice to distill off all the acetone, which represents acetone preformed plus acetone from diacetic acid. The acetone is determined by matching the suspension in a nephelometer with that of a standard suspension as directed on p. 172.

Precipitate the residue in the Kjeldahl flask while still hot by adding 8 cc. of 10 per cent sodium carbonate solution. Boil a few seconds, filter on a Büchner funnel and wash the precipitate with hot water. To the clear filtrate add 15 cc. of basic lead acetate (U. S. P.) solution, 10 cc. of ammonium hydroxide, sp. gr. 0.90, allow the precipitate to settle and then filter on a dry folded filter. Use the filtrate for the β-oxybutyric acid determination in the following paragraph.

Boil the filtrate till most of the ammonia has been expelled, cool, add an excess of sulfuric acid to precipitate the lead, filter off the lead sulfate, add 30 cc. of 1 : 1 sulfuric acid to the filtrate and transfer to a 1-liter Kjeldahl flask fitted with a dropping funnel. The contents of the flask are then distilled while a solution of potassium bichromate is run in at a rate such as to keep the liquid a pale yellow. Usually not over 0.5 gram of bichromate is required and an excess is to be avoided. Slow distillation

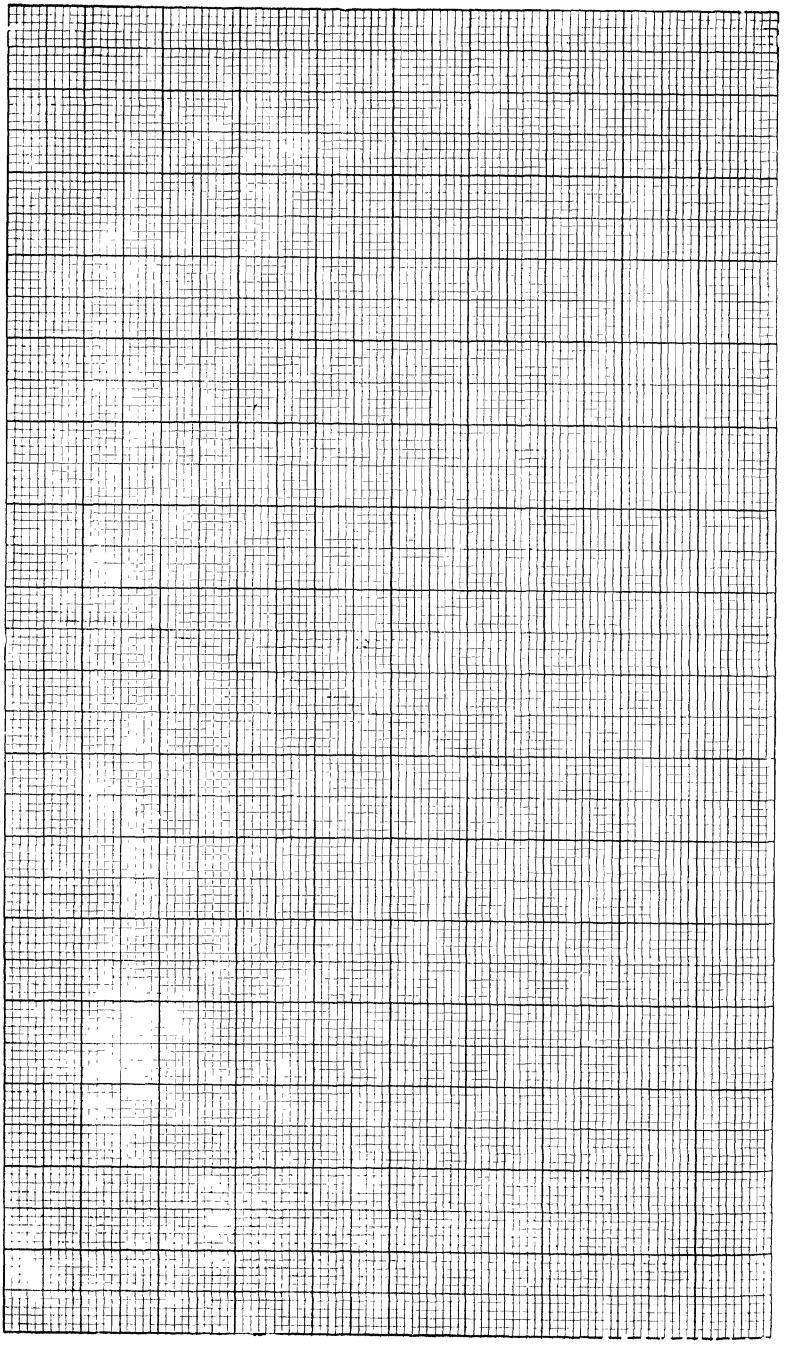
is continued for about two hours, care being taken always to have the delivery tube under the water in the receiving flask. The distillate is then redistilled with 20 cc. of 3 per cent hydrogen peroxide and 5 cc. of 10 per cent sodium hydroxide, the final distillate being caught in an excess of the acetone reagent. The resulting suspension is then matched in a nephelometer as directed on p. 172. The acetone thus obtained may then be calculated to its β-oxybutyric acid equivalent, but it must be remembered that only 90 per cent of the theoretical yield of acetone is obtained and hence 10 per cent must be added to the above nephelometer estimation.

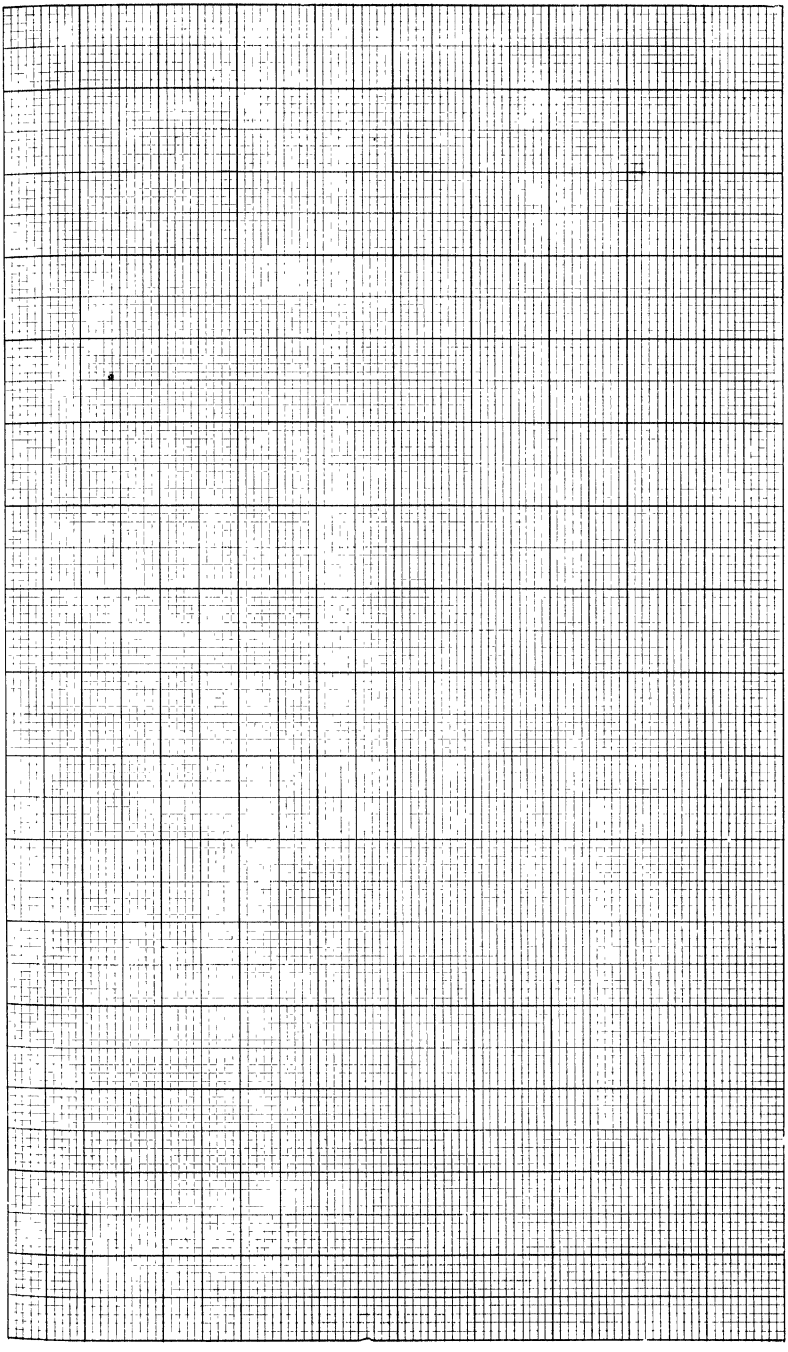
Notes.

1. The distillate is redistilled with a little dilute sulfuric acid, since some ammonia may be present.

2. The distillation with hydrogen peroxide and a slight excess of alkali serves to destroy or hold back hydrogen sulfide, aldehydes, if any, and volatile acids.

3. The above procedure is for small amounts of blood; for large amounts of defibrinated blood or tissues, a 2- or 3-liter flask containing 500 cc. of water, 3.5 cc. of glacial acetic acid, and a little powdered talc is used. Distillation is continued till about 300 cc. have distilled. Complete the analysis as directed in the procedure above, redistilling with a little dilute sulfuric acid and again with peroxide and a slight excess of alkali, etc., etc. Use 15 cc. of the 20 per cent sodium carbonate solution, 30 cc. of basic lead acetate solution and 15 cc. of concentrated ammonium hydroxide, instead of 8 cc., 15 cc., and 10 cc., respectively, as given in the procedure.





CHAPTER XX

PEPSIN

NEPHELOMETRIC METHOD FOR THE STUDY OF PEPSIN

By Hans Kleinmann

The nephelometric method for the determination of pepsin is based upon the splitting of highly diluted solutions of serum protein or sodium caseinate (adjusted to the proper hydrogen-ion concentration through the addition of acids or buffers) by means of ferment solutions and the analysis of the undigested and digested substrate solutions by means of the nephelometric principle. The latter depends upon the transformation of the clear test solution into homogeneous, and for the time of the test, stable clouds, by means of a suitable reagent, and the comparison of the turbidity of the digested with the undigested solutions by means of the nephelometer. So far as the principle and technique of nephelometry are concerned, reference must be had to the detailed presentation given in Chapters IV and VI.

Comments on the Method.—The nephelometric determination of pepsin shows the following characteristics:

1. It makes possible the use of protein solutions of extremely small concentrations. This permits the use of very highly diluted ferment solutions, so that relatively very small amounts of foreign substances get into the digestion mixture through this source.

2. It makes possible the carrying through of a series of studies, since a single analysis can be completed in a few minutes. Moreover, the accuracy of a determination is always satisfactory; an error of 1 to 2 per cent is not exceeded. The sensitivity of the method permits one to follow the beginning of a digestion which is of particular interest in kinetic investigations.

3. It permits the measurement of ferment action by the decrease of the substrate. In this respect it differs from most methods for the determination of proteases, which measure the action of the ferment by the appearance of certain digestion products. Since the formation of certain digestion products is not necessarily continuous or proportional to the digestion process, it appears desirable to have a method which measures directly the decrease in substrate. In this method the standard, against which the digestion is measured, is arbitrarily chosen. Everything that gives a precipitate with the reagent of the method (sulfosalicylic acid-alkaloid reaction) is considered protein. Practically, the method works in such a way that the substrate system is exposed to the action of the ferment at a temperature of say 37° C. At definite time intervals samples or test portions are taken from the system. Each portion removed has the ferment action of 37° C. stopped suddenly either by heat or by the addition of a substance which affects unfavorably the acidity of the ferment system. A portion is also taken from the system before the addition of the ferment. After a number of test portions have been taken in the course of a certain time, the clear solutions are precipitated. The turbidities are strictly proportional to the protein content of the test portions; and therefore with progressive digestion the turbidities become weaker. The clouds are measured against the portion taken before the addition of the ferment, which therefore contains 100 per cent of the original protein. The change in concentration produced by the addition of the ferment solution is taken into account.

The ratio of the clouds gives the ratio of the concentrations of the protein. The decrease in concentration during the course of the digestion gives the ferment action. The method not only measures the disappearance of the clouds of a system through ferment action—" is not a method with limits "—but also gives, through the production of clouds, the amount of protein in the clear solution after digestion. By means of this method it is possible to study the digestion of proteins under varied conditions (change of acidity, addition of foreign substances, etc.) as the conditions for the actual measurement (such as correct acidity,

concentrations, etc.) need only be made after the digestion, just before the determination. Therefore the method of study is independent of the method of determination.

The study of pepsin can be made with (*a*) serum protein and (*b*) sodium caseinate.

DETERMINATION OF PEPSIN MIXTURES BY THE DIGESTION OF SERUM PROTEIN

Reagents.

1. Substrate solution. The protein substrate is prepared by diluting animal sera with physiological salt solution. It is practicable to use human sera having a final dilution for digestion of about 1 in 60 to 1 in 80. A digestion of 50 per cent producing a concentration of protein of 1 in 160 is still easily measured. In general, sera in dilutions of 1 in 50 to 1 in 500 can be employed. The serum must be carefully centrifuged. It is kept in an ice box for future use in a dilution of 1 in 20, using toluol as a preservative. For scientific purposes the use of an isolated protein of the serum, as for example, serum albumin, is more suitable than the whole serum. To prepare albumin substrate, animal serum, e.g., horse serum, is treated with an equal volume of saturated ammonium sulfate solution, the precipitated globulin is filtered off and the filtrate dialyzed several weeks against running water, and finally against distilled water until the dialysate shows no appreciable reaction for ammonium sulfate. This long dialysis may be avoided by the use of electrodialysis. The final solution is covered with toluol and kept in a refrigerator but, owing to the slight cloud usually present, it must be filtered just before using. The necessary dilution must be determined empirically. It must be such that 5 cc. of the diluted solution, under the conditions of the method, produce a nephelometric cloud.

2. Ferment solutions. Extracts made with physiological salt solution of pepsin preparations may be used for ferment solutions. These must be clear and colorless. This requirement is not difficult to fulfill when using the high dilutions. The solu-

tions should, however, always be filtered free from any residues. From a commercial pepsin powder (Gr. Pharmacopœia 5), one can prepare a dilution in the final system of 1 to 17,000 which gives a suitable digestion. With stronger preparations, as for example "Grübler," greater dilutions are necessary. Thus, a pepsin "Finzelberg" gave a good digestion in a dilution of 1 in 500,000.

3. Hydrochloric acid, 1 N.
4. Hydrochloric acid, 25 per cent (by volume).
5. Sodium hydroxide, 0.025 N.
6. Sodium sulfosalicylate, 20 per cent.

Apparatus.—1. Water thermostat.

2. Kleinmann's Nephelometer (Schmidt & Haensch). Any other make of nephelometer may of course be employed but these may require the construction of a correction or standardization curve. (See Chapter IV.) Once this curve is correctly drawn, the nephelometer is capable of yielding accurate determinations.

Procedure.—The technique of the determination varies somewhat according to the kind of substrate, the time of digestion desired, or the special conditions of the research.

For the digestion of serum protein the following example of the study of two concentrations of ferment is given. Twenty cubic centimeters of serum (diluted 1 to 20) are filtered into an Erlenmeyer flask (150 cc.) To this are added 3 cc. of 1 N hydrochloric acid, and finally the volume is brought to 75 cc. with physiological salt solution. In a similar manner a second flask is prepared. Both flasks are now placed in a thermostat equipped with a mechanical stirrer, thermoregulator, and thermometer. The thermostat should be adjusted to a temperature of 37° C. ($\pm 0.05°$). In addition to the Erlenmeyer flasks the thermostat should have two test tubes containing diluted ferment solution, of concentrations a and b, whose action is to be studied in this particular example. While the ferment solutions and substrates are being brought to the temperature of the thermostat, prepare as many beakers as there are points to be established during the digestion. A half-hour digestion with seven points of measurement are assumed in the present case. Since duplicate measurements are always made, there will be needed for the comparison

of these two concentrations of ferment, twenty-eight beakers each of 25-cc. capacity. These beakers must be most scrupulously cleaned, rinsed with distilled water, and then thoroughly dried. After use they must always be cleaned with sodium hydroxide solution to remove the last trace of protein.

Into each beaker are put 5 cc. of sodium hydroxide solution. This serves the purpose of neutralizing the acid of the test portion and thus stopping the digestion. The sodium hydroxide should be equivalent to the acidity of the digestion mixture (or somewhat stronger). With a maximum digestion acidity of pH 2, a 0.025 N sodium hydroxide solution is satisfactory. If the neutralization causes the production of a cloud in the test portion no harm is done, because, as will be shown later, an addition of strong acid is necessary for the analysis, in which the proteins are perfectly clear. When the beakers are ready and the solutions in the water-bath have reached the temperature of the bath, 4.85 cc. of each Erlenmeyer flask solution are run into a beaker from a 5-cc. pipette graduated in 0.01 cc., and the remaining 0.15 cc. of the solution is discarded. The reason for taking this amount (4.85 cc.) of solution is that the 65-cc. residue in each of the flasks is diluted to 67 cc. with 2 cc. of ferment solution and hence the subsequent 5-cc. portions taken are equivalent to 4.85 cc. of the original solution. The 2 cc. of ferment solution are added to the Erlenmeyer flasks with an accurately graduated pipette. The flasks are now shaken and the time of addition of the ferment solution marked with a stop-watch. Now in the course of time (every five minutes is recommended) a 5-cc. portion of the digestion mixture is withdrawn by means of an accurate pipette and placed in a beaker containing sodium hydroxide. A separate pipette is recommended for each flask on account of the different ferment concentrations. The time interval can be changed as desired, so long as the time of flowing into the alkali, that is, the stopping of the digestion, is accurately marked with a stop-watch; but it is more practical to use the same time interval of five minutes and to choose the time in such a manner that the second portion always follows a few minutes after the first. If the sampling is made every two or three min-

utes, that is sufficient time for leisurely making the necessary adjustments. Before each portion is taken, the solution in the Erlenmeyer flask is thoroughly stirred with the pipette. In order to get uniform working conditions, the pipettes are always blown out before taking a new portion of the digestion mixture.

After the seventh portion has been taken, the digestion is ended after thirty minutes. The residue of 7 cc. left in the Erlenmeyer flask serves for the determination of the hydrogen-ion concentration. The solutions in the beakers can stand for some time but it is better to make the nephelometric determination at once. For this purpose 5 cc. of 25 per cent hydrochloric acid and 8 cc. of 20 per cent sodium sulfosalicylate are added, the solution gently rotated, allowed to stand three minutes, and then measured in the nephelometer.

In regard to the general technique of nephelometry, see Chapters IV and VI. It is understood of course that the conditions of the digestion experiment can be varied, additions made, the volume of the substrate or the number of portions changed, all depending upon the purpose of the investigation.

Thus, in determining the influence of ions upon the digestion of serum albumin with pepsin, Rona and Kleinmann employed the following procedure: Measure into test tubes 2.5 cc. of serum albumin solution (2 to 4 cc. tested out according to the albumin concentration of the solution) and 5 cc. of hydrochloric acid five times as strong as is desired in the actual digestion. To this are added in the control test 12.5 cc. of distilled water, in the salt tests 12.5 cc. of salt solution twice as strong as desired in the digestion, and the tubes made up to 23 cc. with distilled water. For every experiment two duplicate tests or tubes are used, and for every tube, four beakers are prepared. If, as is usual, one control and three experiments with different ions or concentrations of ions are used, then eight test tubes and thirty-two beakers are prepared. In each beaker place 5 cc. of sodium hydroxide of such strength as to neutralize the acid employed in the test. For example, if in the test tubes 5 cc. of 0.02 N hydrochloric acid were added and the final volume was 25 cc., then the concentration of the hydrochloric acid was 0.004 N. Hence the beakers

should receive 5 cc. of 0.004 N sodium hydroxide. This serves to neutralize the acid of the portion withdrawn and thus sharply stop the digestion. The 23 cc. of solution in each test tube are carefully stirred with a pipette, 5 cc. taken out, 4.5 cc. put into one of the beakers and the remaining 0.5 cc. discarded. To the beaker is added 0.5 cc. of water. The reason for not taking a 5-cc. portion as is done later, but only using 4.5 cc. for the first sample, is that the 18 cc. of solution remaining in the test tubes are now diluted with 2 cc. of ferment solution. For this purpose the test tubes and the ferment solution are separately brought to a temperature equilibrium in the thermostat by allowing them to warm in the latter for fifteen minutes. Then at a definitely marked period of time 2 cc. of ferment solution are added to each test tube and the contents well mixed. The test tubes are allowed to remain in the thermostat at 40° C. In exactly ten, twenty, and thirty minutes, respectively, 5 cc. are taken from each test tube and allowed to flow into the 5 cc. of sodium hydroxide solution already in each of the beakers. After thirty minutes the experiment is discontinued and the 5 cc. of solution remaining in the test tubes are used for the determination of hydrogen-ion concentration. This is done potentiometrically. From every experiment there are samples on hand, taken before digestion, and after ten, twenty, and thirty minutes, respectively.

The samples taken during digestion are measured against the sample taken before digestion. The latter is used as a standard whose protein content is taken as 100 per cent. For this purpose 5 cc. of 25 per cent hydrochloric acid and 8 cc. of 20 per cent sodium sulfosalicylate solution are added to each beaker. After standing three to five minutes, the solutions are measured nephelometrically against the standard and their protein content determin d.

Calculation of Results.—The calculation of the results is done as follows: With equal illumination of the field in the eyepiece, the concentrations of protein vary inversely with the readings of the nephelometer (Kleinmann's).

If the standard solution C is set on 20 mm. (H), and if the

unknown concentration is C_1 and the height of the unknown solution 25 mm. (H_1), the ratio of the concentration of the unknown to that of the standard, or the digested to the undigested respectively, is 20 to 25, i.e., $C_1 = \frac{C \cdot H}{H_1}$ and as C is 100 per cent, then

$$C_1 = \frac{100 \cdot 20}{25}$$

or 80 per cent.

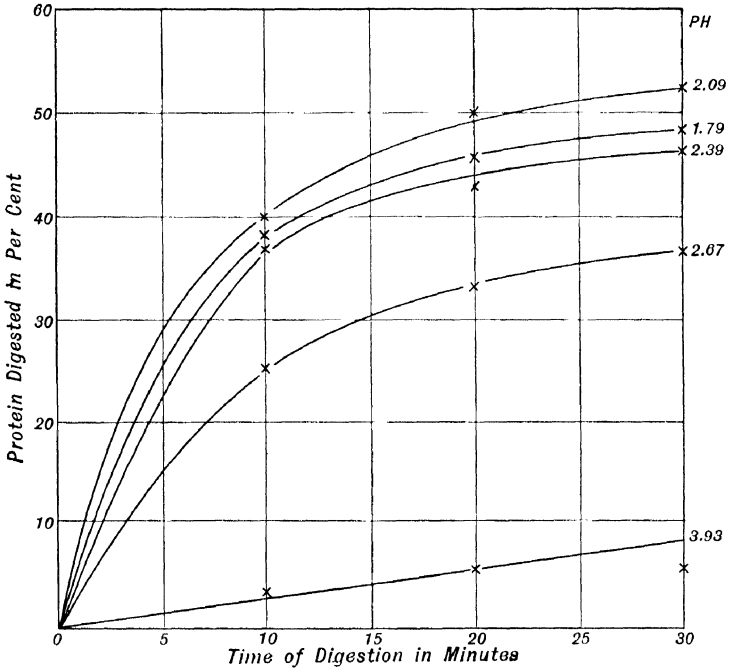


FIG. 41.—Influence of Acidity upon the Digestion of Serum Albumin.

The digested solution contains, therefore, 80 per cent of the protein of the undigested solution. Hence the amount of protein digested by the ferment is 100 − 80, or 20 per cent.

Notes.

1. An example of how the method works is given in a digestion experiment requiring thirty minutes and three portions taken, and shows the influence of hydrogen ions on the digestion.

TABLE XIX

INFLUENCE OF ACIDITY ON THE PEPTIC DIGESTION OF SERUM ALBUMIN

Number of sample	I*b*	I*c*	I*d*	II*b*	II*c*	II*d*	III*b*	III*c*	III*d*	IV*b*	IV*c*	IV*d*	V*b*	V*c*	V*d*
Time of digestion, minutes.	10	20	30	10	20	30	10	20	30	10	20	30	10	20	30
HCl	aprox. n/63			aprox. n/100			aprox. n/160			aprox. n/250			aprox. n/630		
*p*H.	1 79			2 09			2 39			2 67			3.93		
Nephelometer readings, mm.	32 3	37 2	38 7	33 8	40 0	42 5	31 9	34 7	43 4	27 2	30 5	31 8	20 9	21.0	21.3
	32 5	37.0	38 6	33 6	40 0	42 3	31 8	34 9	43.4	27 3	30 5	31 8	20 6	21 4	21 2
	32 5	37.0	38 9	33 5	40 4	42 3	31 7	34 8	43.4	27 2	30 5	32 3	20 8	21 1	21.3
Average	32 4	37.1	38 7	33 3	40 1	42 4	31 8	34 8	43 4	27 2	30 5	32 0	20.8	21 2	21.3
Standard solution.	I*a* = 20			II*a* = 20			III*a* = 20			IV*a* = 20			V*a* = 20		
Amount of protein digested, per cent	38 3	46 1	48 3	39 9	50 2	52 9	37 1	42 6	53 9	26 3	34 4	37 5	3 7	5 7	6 2

TABLE XX

Substrate, 10 cc. of serum albumin solution, 10 cc. 0.1N HCl. Distilled water to make 46 cc., and 4 cc. of ferment solution (1 to 29,000). *p*H 2.38

Time, minutes . .	0		30		60		90		120		150		180	
Number. . . .	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
Nephelometric readings, mm.	20 0	20 0	24 1	24 0	28 6	28 4	32 1	31 5	36 4	36 4	41 6	39 9	32 2	32 2
	20 0	19 9	23 9	24 2	28 4	28 6	32 0	31 2	36 1	36 1	41 8	41 2	32 4	32 1
	20 0	20 1	23 9	24 2	28 7	28 6	31 9	31 2	36 3	36 3	41 8	41 1	32 5	32 2
Average . .	20 0		24 1		28 5		31 6		36 3		41 2		32 3	
Height of standard. .	I=20		20		20		20		20		20		15	
Digestion, per cent. .	0		16 9		29 8		36 7		44 9		51 5		53 6	

In working over a period of 150 minutes, the method shows parabolic curves for peptic digestion, which in close approximation are represented by a bimolecular reaction equation.

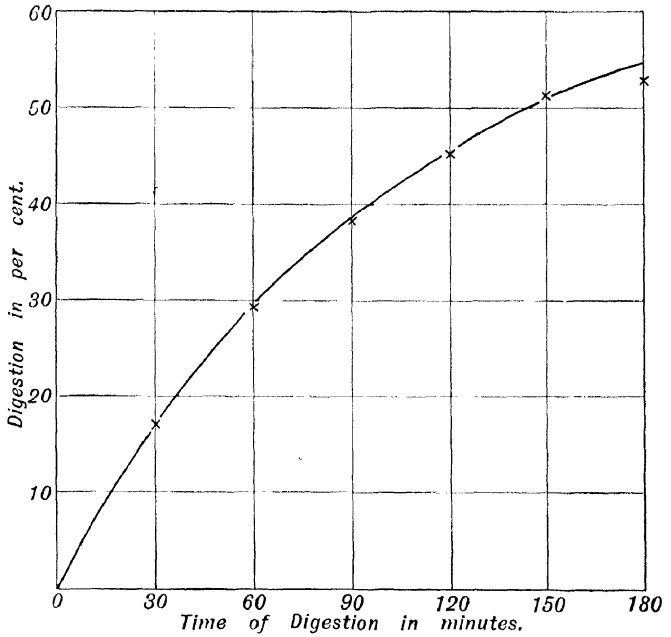
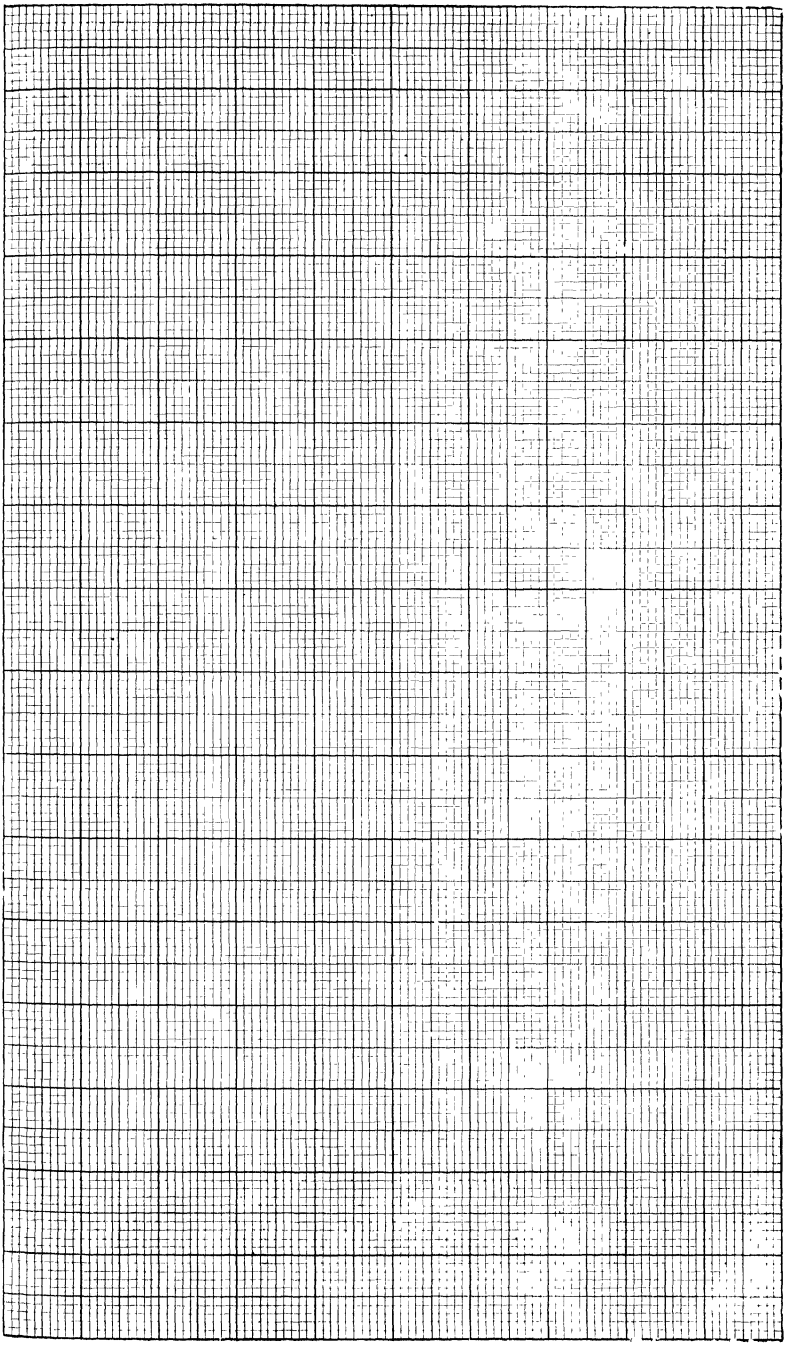


FIG. 42.—Peptic Digestion of Serum Albumin.

TABLE XXI

DATA FOR A BIMOLECULAR REACTION

Time of Digestion, Minutes	x	$a-x$	$\frac{x}{a\ (a-x)\ t}\ \frac{l}{} = k \cdot 10^5$
30	17 0	83 0	6.84
60	29 0	71 0	6 81
90	37 5	62 5	6 70
120	45 0	55 0	6 82
150	51 0	49 0	6 94
180	55.5	44 5	6.92
			Average 6 84



2. The results obtained with the nephelometric method for pepsin did not show any verification of " Schulz's rule." The percentage of error is about 2 per cent.

REFERENCES

P. RONA AND H. KLEINMANN, Biochem. Z., **140**, 478 (1923); ibid., **140**, 461 (1923); ibid., **150**, 444 (1924); ibid., **155**, 34 (1925).

DETERMINATION OF PEPSIN IN GASTRIC JUICE

BY HANS KLEINMANN

The determination of pepsin in gastric juice is based upon the same principle as the nephelometric determination of peptic activity, as shown on p. 236. It depends upon digesting highly diluted serum protein solution by means of gastric juice of a certain definite acidity. The digested protein solutions are transformed into nephelometric clouds in strongly acid solution by means of sulfosalicylic acid.

The application of the method for clinical purposes demands certain treatment of the gastric juice as well as the simplification of the usual technique for determining pepsin.

Reagents.

1. Substrate solution. For clinical purposes it suffices to use the simplest substrate solution, namely, serum diluted with physiological salt solution. With human serum the dilution amounts to 1 in 80. The serum is most suitably preserved in a dilution of 1 to 20.

2. Hydrochloric acid, 1 N.

3. Hydrochloric acid, 25 per cent (by volume).

4. Sodium hydroxide, 0.025 N.

5. Sodium sulfosalicylate, 20 per cent.

6. Gastric juice, pumped out after a white bread and tea breakfast. For the determination of the pepsin bound to the bread of the test meal, in the diminished acidity, it is necessary to have:

7. Phosphate mixture. Dissolve 2.723 grams of KH_2PO_4 and make up to 300 cc. Dissolve 3.558 grams of Na_2HPO_4 and make up to 300 cc. Mix these two solutions in equal proportions.

PREPARATIONS FOR PEPSIN DETERMINATION

1. **Production of Substrate System.**—First the proper dilution for the substrate system is determined. Usually the dilution of serum from 1 to 75–80 is found suitable. For testing, 5 cc. of diluted serum (1 to 80) are treated with 5 cc. of 25 per cent hydrochloric acid, 3.0 cc. of water and 7.0 cc. of sodium sulfosalicylate. After three minutes a uniformly strong cloud should have developed, which is not so strong that it flocculates within thirty minutes, and not so weak but that a one-half dilution is still easily measured.

2. **Preparation of the Receivers.**—For a digestion taking thirty minutes it is best to have three samples taken at ten-minute intervals, together with the preliminary sample taken before digestion starts. These require four receivers. As it is advisable to work in duplicate, it is necessary to prepare eight receivers. For this purpose eight test tubes of 25 cc. capacity are given each 5 cc. of 0.025 N sodium hydroxide. The alkali serves to neutralize the samples and thereby stop the digestion (ferment action). For the pair of test tubes which are used to receive the sample before the addition of the ferment, 0.5 cc. of water is added also.

All solutions, especially alkalis, are filtered through S & S paper (or a paper of similar quality) until they are free from shreds and fibers.

DIRECTIONS FOR PEPSIN DETERMINATION

In gastric juice after its removal following a test meal the pepsin is not always present in the free condition. This is particularly noticeable in gastric juice of hypoacidity. According to Ege,[1] pepsin in an acidity of *p*H 2 is absorbed slightly, at *p*H 3–4 pepsin is strongly absorbed, and around *p*H 6 it is not absorbed at all. It was found that a *p*H 3–4 pepsin was appreciably

[1] R. Ege, Biochem. Z., **145**, 66 (1924).

absorbed, while at a *p*H 1.5–2.0 it was found perfectly free (unabsorbed). In normal gastric juice of *p*H 1.5–2.0 the pepsin determination gives practically the entire pepsin content. With hypoacid stomach contents it is necessary either to bring the acidity up to *p*H 1.5–2.0 with 0.1 N hydrochloric acid, or, according to Ege, to mix the contents with phosphate buffer of *p*H 6.8, and then only when the pepsin is freed is the mixture to be filtered. By determining the total and free pepsin the amount of absorbed pepsin can be calculated in stomach contents of hypoacidity. Therefore the filtration varies depending upon the acidity and the purpose of the determination.

1. **Determination of Free Pepsin.**—For the determination of the free pepsin the stomach contents are filtered directly. A portion of the filtrate is diluted to 1 part in 80 with distilled water. This dilution will digest the substrate under normal conditions in thirty minutes to the extent of 20 to 70 per cent. With subacid stomach contents in which a portion of the pepsin is bound, and therefore lacking in the filtrate, stronger concentrations are used, i.e., the gastric juice is diluted to a less extent, as for example 1 to 20.

2. **Determination of Total Pepsin.**—With normal stomach contents the free pepsin is the total pepsin. With subacid contents the total pepsin may be determined according to two methods.

(*a*) In the first method, one takes a measured portion of the content including solid and bread particles well mixed and brings it with a measured portion of 0.1 N hydrochloric acid up to normal acidity for stomach contents. For example, if a stomach contents having a titration value of 7 (which means that it has 7 cc. of 0.1 N hydrochloric acid for 100 cc. of gastric juice) is used, it is brought up to 40 by adding 3.3 cc. of 0.1 N hydrochloric acid to 10 cc. of the mixture. In most cases it is only necessary to make the contents decidedly acid to Congo red paper. The amount of 0.1 N acid used is noted, the solution filtered and diluted with distilled water so that the total dilution is 1 to 60.

(*b*) In the second method, one dilutes 1 cc. of stomach contents with 19 cc. of phosphate mixture (see above), filters, and

dilutes 10 cc. of filtrate to 30 cc. with distilled water (total dilution of 1 to 60). With this method the filtrate must be used within five minutes, otherwise the pepsin loses its activity by keeping it too long at a *p*H of 6.8.

3. **Determination of Bound Pepsin.**—The bound pepsin is obtained by subtraction of the free pepsin from the total pepsin. Since the free pepsin is determined by diluting the clear gastric juice after filtration and the total pepsin is determined after diluting the mixture containing bread solids, the concentrations are not exactly equal. Therefore, for an extremely accurate determination of stomach contents, it is necessary also for the determination of the free pepsin to dilute in such a manner that 1 cc. of the mixture is diluted with a hydrochloric acid solution of the same acidity as the stomach contents, so that the total dilution again equals 1 to 60.

For practical purposes no such refinements are needed. It suffices to filter acid stomach contents directly, and subacid contents are filtered after adding a few drops of 0.1 N hydrochloric acid until the contents are acid to Congo red paper. The filtrate then contains the total pepsin and is diluted with distilled water to 1 part in 60. Through this dilution no question can arise of the protein content of the gastric juice, except in case the stomach contents contain pus, etc.

After filtration and dilution the solution is ready for digestion. For this purpose 18 cc. of acid substrate mixture are put into 25-cc. test tubes (a duplicate is treated likewise) and the tubes placed in a water thermostat at 37° C. Also 4.5 cc. of acid substrate mixture are put into each of the first pair of receivers.

Now to each test tube containing 18 cc. of substrate are added 2 cc. diluted gastric juice; the solutions are mixed with a pipette and the time noted. After every ten minutes, 5 cc. of the contents of each test tube are taken out and put into the receivers. Three portions are taken. The receiver which received only 4.5 cc. of substrate solution instead of 5 cc. is treated in this manner to take care of the dilution of the substrate by the ferment solution. The missing 0.5 cc. is supplied by adding that amount of water to the receivers.

Now to the test tubes (receivers) are added 5 cc. of 25 per cent hydrochloric acid, 3.0 cc. of distilled water, and 7.0 cc. of 20 per cent sodium sulfosalicylate and the solutions mixed. The resulting nephelometric cloud is allowed to develop at least three minutes and then within forty-five minutes it is measured against the undigested substrate taken as a standard.

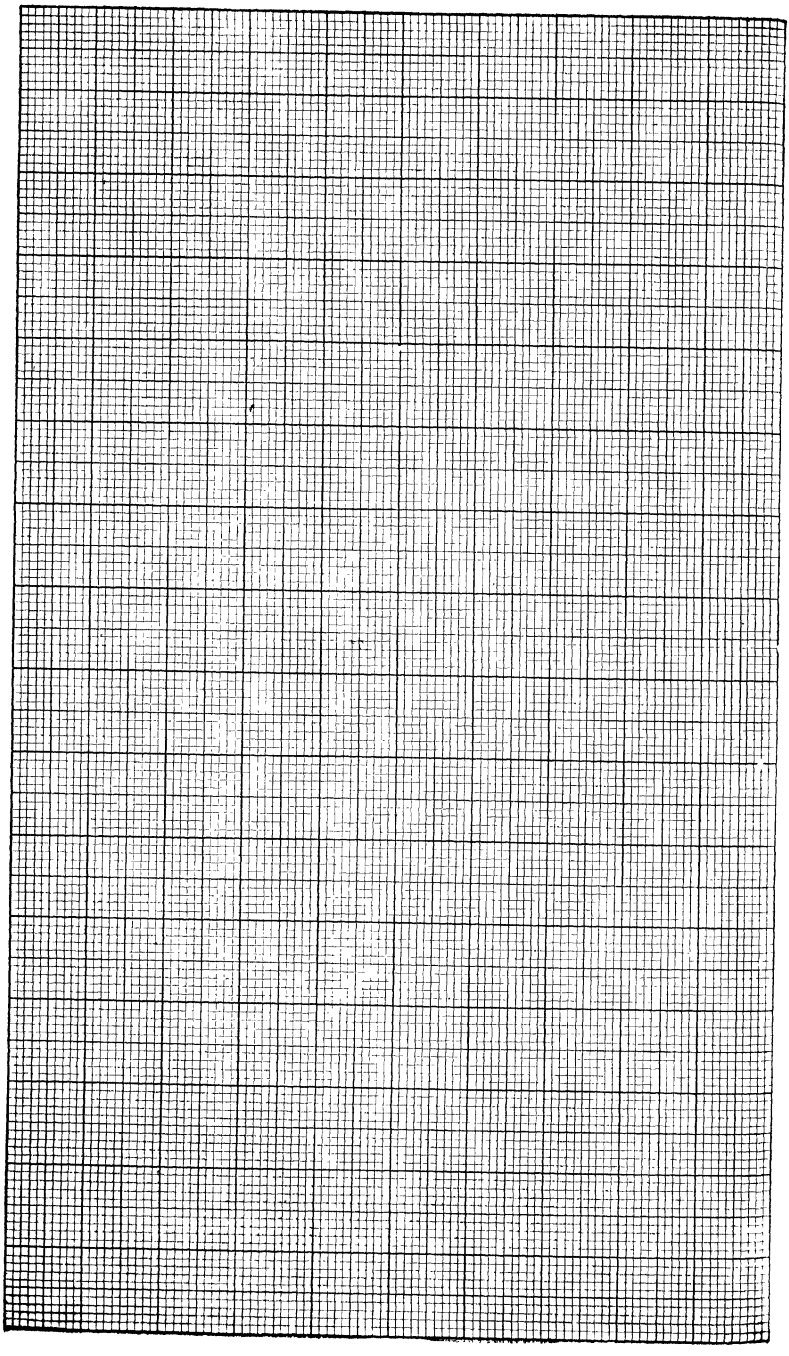
Calculation of Results.—The calculation of the results is made as follows: The protein concentrations are inversely proportional to the nephelometric readings when a Kleinmann instrument is used. If for example the undigested solution is set on 20 mm. and the digested solution on 25 mm., then $20 : 25 = X : 100$. Hence $X = 80$; i.e., the digested solution contains 80 per cent of the protein undigested. The digested protein equals $100 - 80$, or 20 per cent.

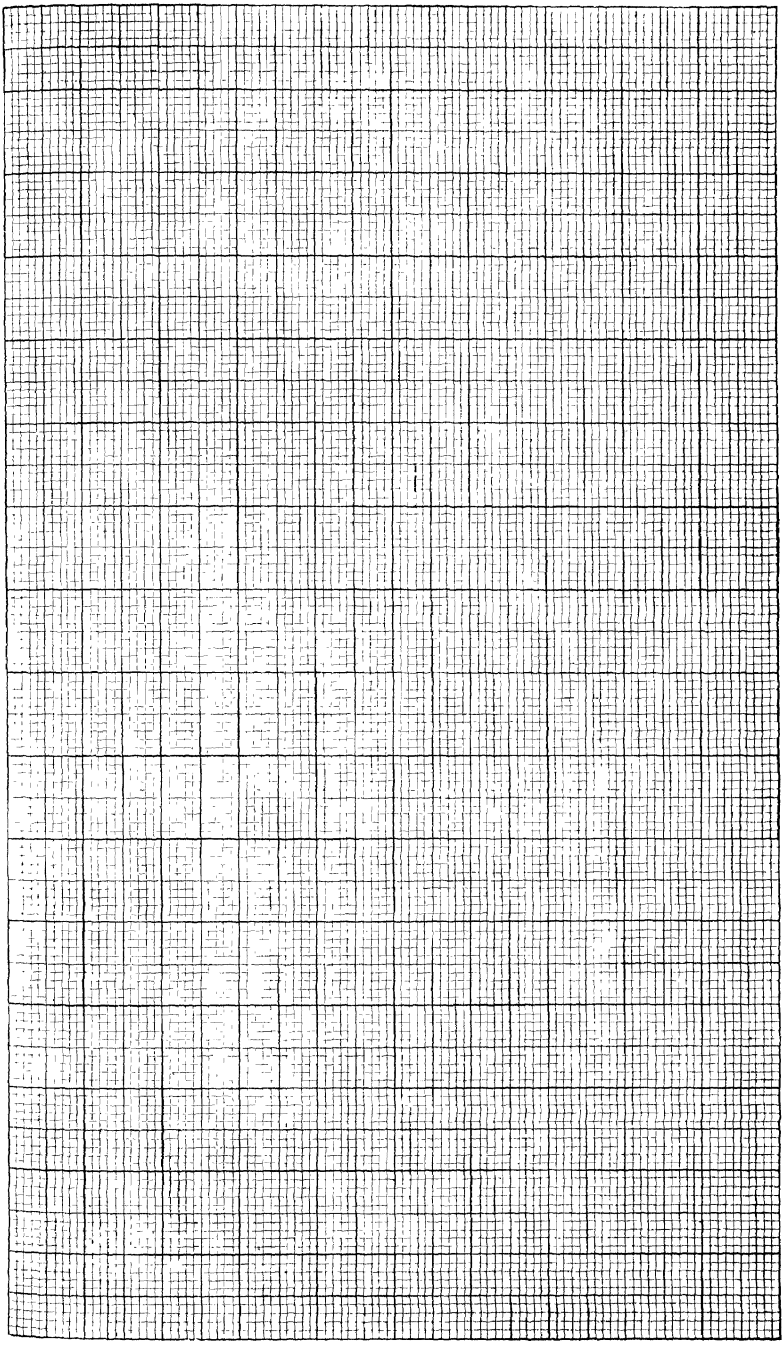
The amount of pepsin at hand in comparison with that of a commercial preparation is obtained by comparing a digestion with gastric juice to that obtained with a diluted commercial preparation. If the digestion with equal time is not too different, the amount of pepsin can be taken as approximately proportional to the amount of protein digested. For example, if a commercial preparation after a definite time in a dilution of 1 to 50,000 has digested 50 per cent, but a gastric juice has digested only 25 per cent, one can assume the diluted gastric juice equal to a commercial preparation diluted to 1 in 100,000.

The total amount of pepsin in gastric juice is obtained by taking the dilution into consideration.

REFERENCE

P. Rona and H. Kleinmann, Klin. Wochschr., **6**, 1174 (1927).





CHAPTER XXI

PROTEINS

DETERMINATION OF PROTEIN

Method of Kober

The quantitative estimation of proteins and other colloidal substances is usually a long, tedious, and often inaccurate process. Such substances form suspensions that are extremely difficult to filter and require much washing to remove the small amount of adsorbed mother-liquor. Moreover, some precipitates are quite soluble in the wash water. By means of the nephelometer such substances can be accurately and rapidly determined quantitatively, provided a suitable precipitant can be found. By "suitable precipitant" is meant a substance that will produce a quantitative precipitation in solutions 0.01 per cent or weaker and in the form of a suspension which does not agglutinate appreciably in less than ten to twenty minutes, i.e., time enough to permit taking two to five readings with the nephelometer. Kober [1] has obtained such precipitants for the proteins. He employs a 9 per cent sodium chloride solution for edestin and a 3 per cent solution of sulfosalicylic acid for albumins, globulins and native proteins, i.e., coagulable proteins.

From two to three days are required for the determination of casein, globulin and albumin in milk when it is done by the usual technique, whereas with the nephelometric method it can be done in twenty to thirty minutes.

Kober's nephelometric method of estimating proteins will show the presence of 1.0 part of protein in a million parts of water.

[1] J. Biol. Chem., **13**, 485 (1912–13); J. Am. Chem. Soc., **35**, 290, 1585 (1913). See also Rona and Kleinmann, Biochem. Z., **140**, 461 (1923).

A. DETERMINATION OF EDESTIN

Reagents.

1. Sodium chloride, 9 per cent.

2. Stock solution of edestin. Place 0.1000 gram of Merck's edestin in a 50-cc. beaker and thoroughly rub it into a thick paste with a little water. Add 3 cc. of 0.1 N hydrochloric acid. This will dissolve the edestin, leaving a trace of suspended matter. Allow the solution to settle for an hour, filter through a well-washed filter paper into a 100-cc. volumetric flask, dilute to the mark, add a few drops of chloroform, thoroughly shake, and let stand twenty-four hours. The solution is then ready for use.

3. Standard solution of edestin. Dilute 10 cc. of the stock solution (2) to 100 cc. and thoroughly mix. This gives a 0.01 per cent solution. One volume of this solution with one, two or more volumes of a 9 per cent sodium chloride solution gives suitable standard suspensions for nephelometric work. The suspensions do not settle for an hour or more depending on the dilution.

Procedure.—Place a measured volume of the edestin solution in a beaker or small Erlenmeyer flask and add the sodium chloride solution, drop by drop, from a carefully standardized burette. The flask or beaker is shaken gently in a rotatory fashion as the precipitant is being added. Compare the resulting suspension in a nephelometer against a standard suspension similarly prepared. From the nephelometer reading and a curve made as directed on p. 46, the edestin content of the sample may be obtained.

Notes.

1. Test tubes should not be used to make the precipitations in for the reason that they make it difficult to shake the solution properly, without using a stopper and introducing minute air-bubbles into the solution.

2. Best results can be obtained when the edestin concentration is between 0.0005 and 0.005 per cent.

B. DETERMINATION OF CASEIN

Reagents.

1. Sulfosalicylic acid, 3 per cent. The commercial product is sufficiently pure for this solution. Since sulfosalicylic acid slowly forms a red color with cellulose, the reagent should be filtered through paper rapidly or through an asbestos filter.

2. Stock solution of casein. Dissolve 0.1000 gram of casein, or its equivalent, in 1 cc. of 0.1 N NaOH. Stir to aid solution. Add about 95 cc. of distilled water and 2 cc. of toluene, mix thoroughly and make up to 100 cc. This solution will keep unchanged for three or four days, or even longer.

3. Standard casein solution. Dilute 10 cc. of the stock solution (2) to 100 cc. with distilled water and thoroughly mix. This gives a 0.01 per cent solution. It should be made up fresh each day. One volume of the standard solution with one or more volumes of the sulfosalicylic acid solution gives suspensions suitable for nephelometric work. These suspensions will not settle under twenty minutes or longer.

Procedure.—Place a measured volume of the casein solution in a small beaker or Erlenmeyer flask and add two volumes of 3 per cent sulfosalicylic acid with gentle rotation of the flask or beaker, so that air bubbles do not form in the solution. Compare the suspension thus obtained with a standard prepared similarly, mixing one volume (5 cc.) of a 0.01 per cent casein solution with two volumes (10 cc.) of 3 per cent sulfosalicylic acid. From the nephelometer reading and a curve constructed as directed on p. 46, calculate the casein content of the sample.

Notes.

1. A small amount of sulfosalicylic acid completely precipitates casein, while a large amount does not cause resolution.

2. Under the conditions given in the above procedure sulfosalicylic acid seems to be characteristic for albumins, globulins and coagulable proteins (with the exception of yeast nucleic acid which gives a precipitate, even in 0.01 per cent solution),

and does not precipitate amino acids, peptides, peptones, and urinary constituents.

C. DETERMINATION OF CASEIN, GLOBULIN AND ALBUMIN IN MILK

Reagents.

1. Sodium hydroxide, 0.1 N.
2. Ether.

For additional reagents, see p. 258.

Procedure.—Carefully measure into a 250-cc. volumetric flask 5 cc. of milk, add 200 cc. of distilled water, 10 cc. of 0.1 N sodium hydroxide solution, and then fill to the mark with water. Thoroughly mix. Place 10 cc. of the solution with exactly 2 cc. of ether in a centrifuge tube, tightly stopper with a cork, and shake vigorously (see Note 1.) Allow the mixture to stand until the layers have separated, or centrifuge a minute or two. Remove the cork and withdraw 5 cc. of the aqueous layer. To withdraw the liquid, close the top of the pipette with the finger and insert it quickly into the centrifuge tube, thus preventing the ether solution from contaminating the sample. The 5 cc. aqueous layer is run into a 50-cc. volumetric flask, diluted to the mark with distilled water, and mixed thoroughly.

Milk treated in this way is quite clear and is ready for nephelometric estimation. To 10 cc. of this solution add 10 cc. of 3 per cent sulfosalicylic acid solution. The resulting suspension is then matched nephelometrically with a standard made by adding two volumes (10 cc.) of 3 per cent sulfosalicylic acid solution to one volume (5 cc.) of a 0.01 per cent casein solution. Calculate results in the usual way.

The protein thus obtained is not all casein. To obtain the exact amount of casein, proceed as follows: Precipitate the casein from a fresh portion of the milk according to the official agricultural method and determine the amount of precipitate obtained in an aliquot part of the filtrate, by adding four volumes of the 3 per cent sulfosalicylic acid solution and estimating nephelometrically. This fraction may be termed the " globulin and albumin

fraction " which, subtracted from the " gross casein," gives the amount of casein precipitated by the " official " method.

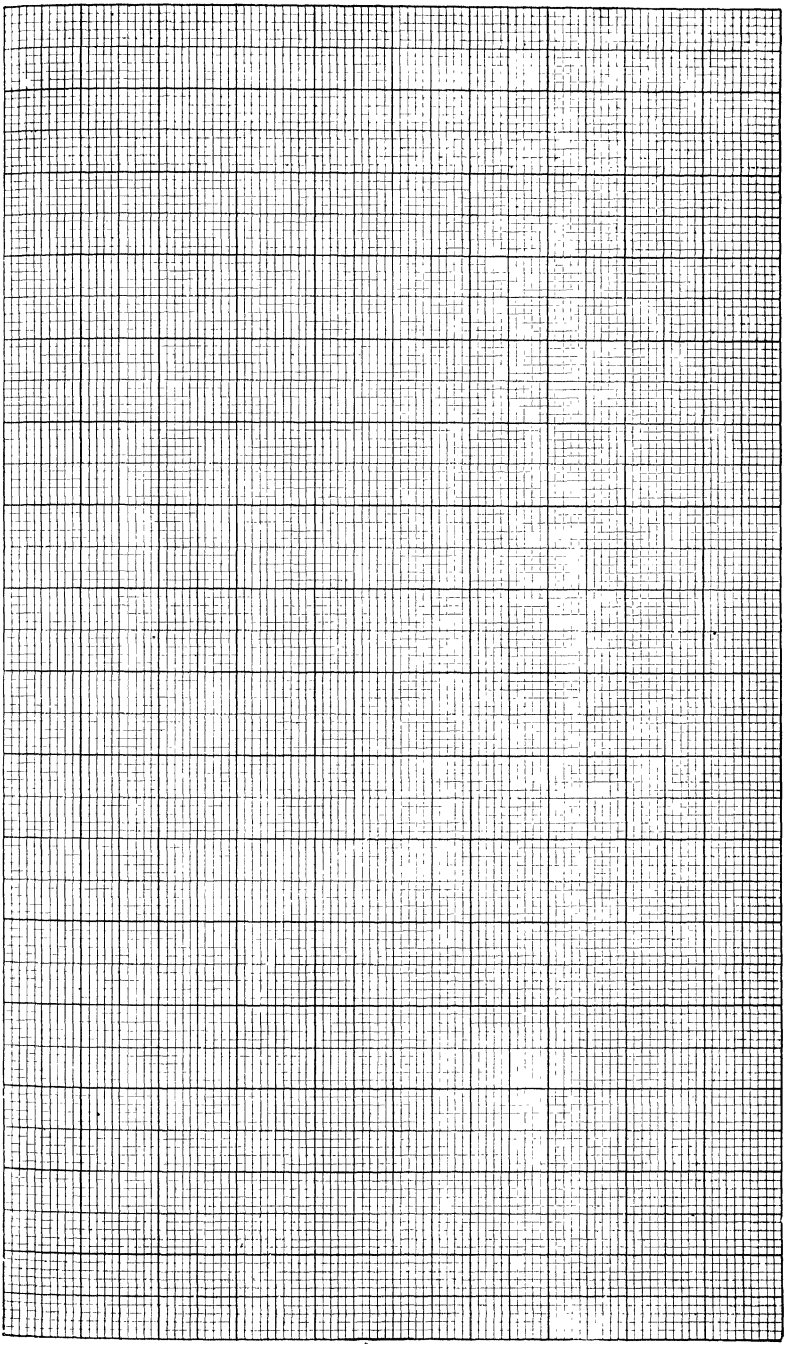
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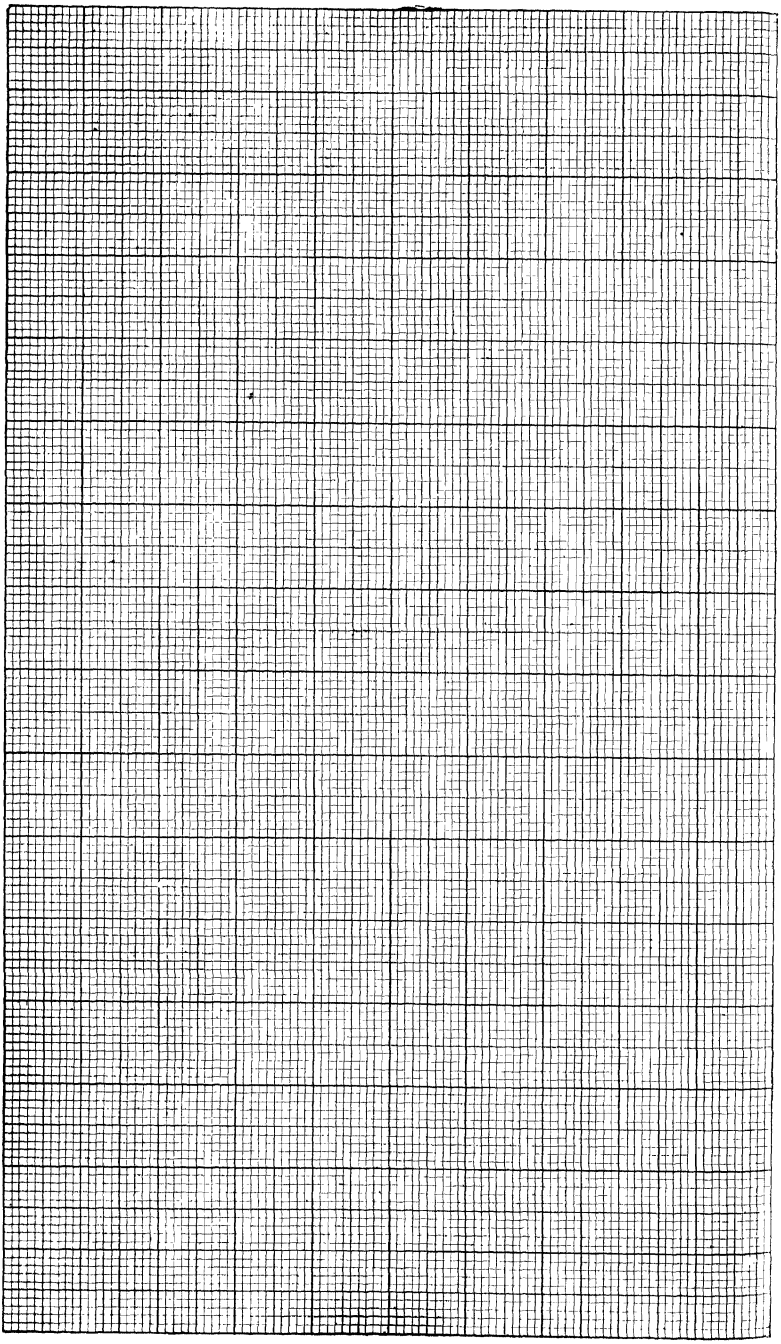
1. The ether used in extracting the fat increases the volume of the solution and, hence, a factor allowing for this must be used. For 10 cc. of the diluted milk and 2 cc. of ether, this factor is 0.910.

2. " In removing fat from milk, hydrochloric acid cannot replace sodium hydroxide to dissolve the membrane from the fat globules, as free hydrochloric acid seems to interfere with the precipitation of casein by sulfosalicylic acid. Furthermore, the residual turbidity with HCl is much greater than with NaOH. The former is appreciable, while the latter is inappreciable." [2]

3. The nephelometric method gives results in good agreement with those obtained by the official agricultural method.

[2] P. A. Kober, J. Am. Chem. Soc., **35**, 1591 (1913).





CHAPTER XXII

PURINE BASES

DETERMINATION OF PURINE BASES, INCLUDING URIC ACID

METHOD OF GRAVES AND KOBER

SALKOWSKI'S reagent for purine bases, which consists of equal volumes of magnesia mixture and ammoniacal silver nitrate (26 grams per liter and sufficient NH_4OH to prevent precipitation of Ag_2O), has been modified by Graves and Kober [1] to meet nephelometric conditions. The ammoniacal silver nitrate precipitates the purine bases as white silver complexes, the presence of chlorides in the reagent preventing the reduction of the silver salt to black metallic silver by uric acid.

The modified reagent contains a lower concentration of silver nitrate, and ammonium chloride alone instead of magnesia mixture. It will precipitate xanthine, hypoxanthine, guanine, adenine, and uric acid quantitatively in solutions as dilute as 0.0002 per cent. By use of egg albumin as a protective colloid the precipitates are kept in suspension and may be estimated nephelometrically.

In a mixture of purine bases, including uric acid, the latter may be oxidized quantitatively in three to five minutes by means of a suspension of manganese dioxide and without any appreciable effect on the four purine bases—xanthine, hypoxanthine, adenine, and guanine. Hence, to estimate uric acid, and the other purine bases separately, it is only necessary to determine (1) the total purine content and (2) the purine bases (excluding uric acid). The difference between (1) and (2) will give the uric acid content.

[1] J. Am. Chem. Soc., **37**, 2430 (1915).

Reagents

1. Hydrochloric acid. One volume of concentrated hydrochloric acid is made up to 100 volumes with water.

2. Ammonium hydroxide, sp. gr. 0.90.

3. Lithium carbonate. Use a saturated solution (about 1 per cent).

4. Lithium carbonate-hydrogen sulfide solution. Saturate a 1 per cent lithium carbonate solution with hydrogen sulfide.

5. Manganese dioxide suspension. Twenty-five grams of potassium permanganate are dissolved in 500 cc. of water, the solution brought to a boil, and treated with 95 per cent alcohol until the permanganate pink has disappeared. Filter, wash with distilled water, and suspend the manganese dioxide in 500 cc. of water. The finely divided suspension will keep indefinitely.

6. Graves and Kober's purine reagent. Mix, in the order named, 50 cc. of ammoniacal silver nitrate solution (26 grams $AgNO_3$ per liter, with sufficient NH_4OH—26 to 27 cc., sp. gr. 0.90—to prevent precipitation of Ag_2O), 8 cc. of ammonium chloride solution (16.5 grams NH_4Cl per 100 cc.), sufficient ammonium hydroxide to redissolve any silver chloride formed (usually 9 cc., sp. gr. 0.90), an excess of 5 cc. of ammonium hydroxide, sp. gr. 0.90, and then water sufficient to make 100 cc. volume. The ammoniacal silver nitrate solution should be filtered before using to free it of reduced silver, or the final reagent may be filtered.

The reagent keeps indefinitely, if stored in well-stoppered bottles.

7. Protective colloid—albumin solution, 2.0 per cent. A weighed amount of egg-white is thoroughly shaken with 0.1 N acetic acid (0.5 cc. per gram of egg-white) and sufficient water added to make a 2.0 per cent albumin solution, the egg-white being calculated as containing 10 per cent of albumin. The solution is then mixed thoroughly and filtered until clean. From 5 to 10 per cent excess of acid sometimes gives a clearer solution, but for the purpose of this method a slight cloud in the albumin is of no consequence so long as it is completely soluble in a little ammonia.

For a long series of estimations, it is best to take the whites from many incubated eggs, thoroughly mix, and keep in an ice-box as stock solution. In this way the albumin seems to keep indefinitely.

Care must be taken to keep the yolk out of the albumin to be used, because it tends to produce cloudy solutions.

8. Standard solutions.

(*a*) Uric acid. A good grade of uric acid may be obtained on the market and may well be adopted for making a standard suspension. The nephelometric values of the different purines (as free bases) in terms of uric acid are:

0.100 gram uric acid = 0.031 gram adenine; 0.079 gram guanine; 0.106 gram hypoxanthine; 0.104 gram xanthine; 0.071 gram purine mixture; 0.067 gram adenine and guanine; 0.104 gram xanthine and hypoxanthine.

Dissolve 0.100 gram of finely divided uric acid in 25 to 30 cc. of saturated lithium carbonate solution in a small beaker. To assist solution, stir and gently press the solid with the flat end of a stirring rod. Transfer the solution to a 100-cc. volumetric flask with a little water, make up to the mark with 0.2 per cent tricresol (see Note 1) and thoroughly mix.

(*b*) Xanthine and hypoxanthine. Prepare as directed for uric acid. Since commercial xanthine is likely to be slightly impure, the solution should be standardized.

(*c*) Adenine sulfate and guanine hydrochloride. Dissolve a weighed amount of the salt in 25 to 30 cc. of dilute hydrochloric acid (1 vol. of the conc. acid made up to 100 vols. with water). If the salts are finely powdered, solution takes place very readily, otherwise gently warm the solution.

A. DETERMINATION OF PURINES IN URINE

Place 5 cc. of the urine in a 15-cc. graduated centrifuge tube, add 5 cc. of Graves and Kober's purine reagent, and centrifuge for one to three minutes. Pour off the supernatant liquid, add 10 cc. of dilute hydrochloric acid (1 volume concentrated acid to 99 volumes water), and place the tube in boiling water for two to five minutes. Cool, and make up the solution to 15 cc.

Allow the larger part of the suspended silver chloride to settle, or centrifuge. The liquid is then drawn off with a pipette and marked Solution A.

I. Total Purines.—To 3 cc. of Solution A add the following in the order named: Seven cubic centimeters of water, 10 cc. of 2 per cent albumin, 0.05 to 0.10 cc. of ammonium hydroxide, sp. gr. 0.90 (to dissolve any AgCl) and 10 cc. of reagent. The resulting suspension is then compared in the nephelometer with a standard made by treating 5 cc. of 0.01 per cent uric acid solution with 5 cc. of water, 10 cc. of 2 per cent albumin and 10 cc. of reagent. This standard is a satisfactory concentration for most normal urines, but may be made stronger if the urine sample is abnormally high in purine bases.

II. Purine Bases (excluding Uric Acid).—To 10 cc. of Solution A add the following in the order named: 2 cc. of 1 per cent lithium carbonate solution saturated with H_2S, 2 cc. of 1 per cent lithium carbonate solution, and 2 cc. of manganese dioxide suspension (see Note 2). Allow the solution to stand three to five minutes, with occasional shaking, and filter until clear. (See Note 3.) Mark Solution B. Add 5 cc. of reagent to 10 cc. of Solution B and compare the cloud with a standard as described above (see Note 4).

III. Uric Acid.—The uric acid is obtained by subtracting the purine bases (II) from the total purines (I).

Calculation of Results.—The readings of the unknown and standard suspensions are made according to the usual nephelometric directions (see Chapter IV) and are then substituted for S and Y in the equation

$$X = \frac{S + SK + \sqrt{(S + SK)^2 - 4SKY}}{2Y}.$$

Taking the value for K as 0.10, the equation is solved for X, the ratio of the suspensions, by means of which the amount of substance in grams may readily be calculated. The slide rule may be used in these calculations.

B. DETERMINATION OF PURINES IN BLOOD

In this determination use is made of Greenwald's [2] discovery of trichloroacetic acid as a reagent for the removal of blood proteins. It gives a water-clear filtrate, free from proteins, and permits the estimation of the purine bases in it directly, after removing the calcium by precipitation with ammonium oxalate and centrifuging.

Reagents.

1. Trichloroacetic acid, 5 per cent.
2. Oxalic acid. Use a saturated solution.
3. Purine reagent. Dissolve 26.0 grams of silver nitrate in 50 cc. of water and add 66 cc. of ammonium hydroxide, sp. gr. 0.90. The reagent contains no ammonium chloride, since blood contains sufficient chlorides to prevent the reduction of silver.

For additional reagents see p. 264.

Procedure. To 20 cc. of the sample of blood add 100 cc. of 5 per cent trichloroacetic acid and filter. To 80 cc. of the clear filtrate add 5 cc. of oxalic acid (saturated) solution and 5 cc. of ammonium hydroxide, sp. gr. 0.90. Centrifuge to remove the oxalate, pipette off 80 cc. of the clear supernatant liquid and add to it 5 cc. of the reagent. Place the resulting suspension in a nephelometer (one which holds at least 100 mm. of the liquid) and match against a standard suspension. Calculate the results as directed above, remembering that two successive aliquot parts were taken in the course of the procedure.

Notes.

1. As a diluting liquid for organic substances liable to bacterial decomposition, Graves and Kober [3] use an aqueous solution of tricresol (usually 0.2 per cent). "This is a much more powerful germicide than chloroform or toluene and causes no trouble in volumetric measurement. Both chloroform and toluene, as is well known, make measuring apparatus unclean and oily, so they are unsuitable for accurate work."

[2] J. Biol. Chem., **21**, 61 (1915).
[3] J. Am. Chem. Soc., **36**, 751 (1914).

2. In the determination of total purine bases the small amount of suspended silver chloride present in the solution is of no consequence, but in the determination of the purines excluding uric acid, the medium for oxidation must be alkaline, in which case the silver chloride forms a soluble silver ammonia complex. By using a solution of lithium carbonate saturated with H_2S, the oxidation medium is made alkaline and the silver chloride is converted into silver sulfide and completely filtered off with the manganese dioxide. Not only is the silver completely removed but the uric acid is completely oxidized by the manganese dioxide, leaving the purine bases practically unaltered.

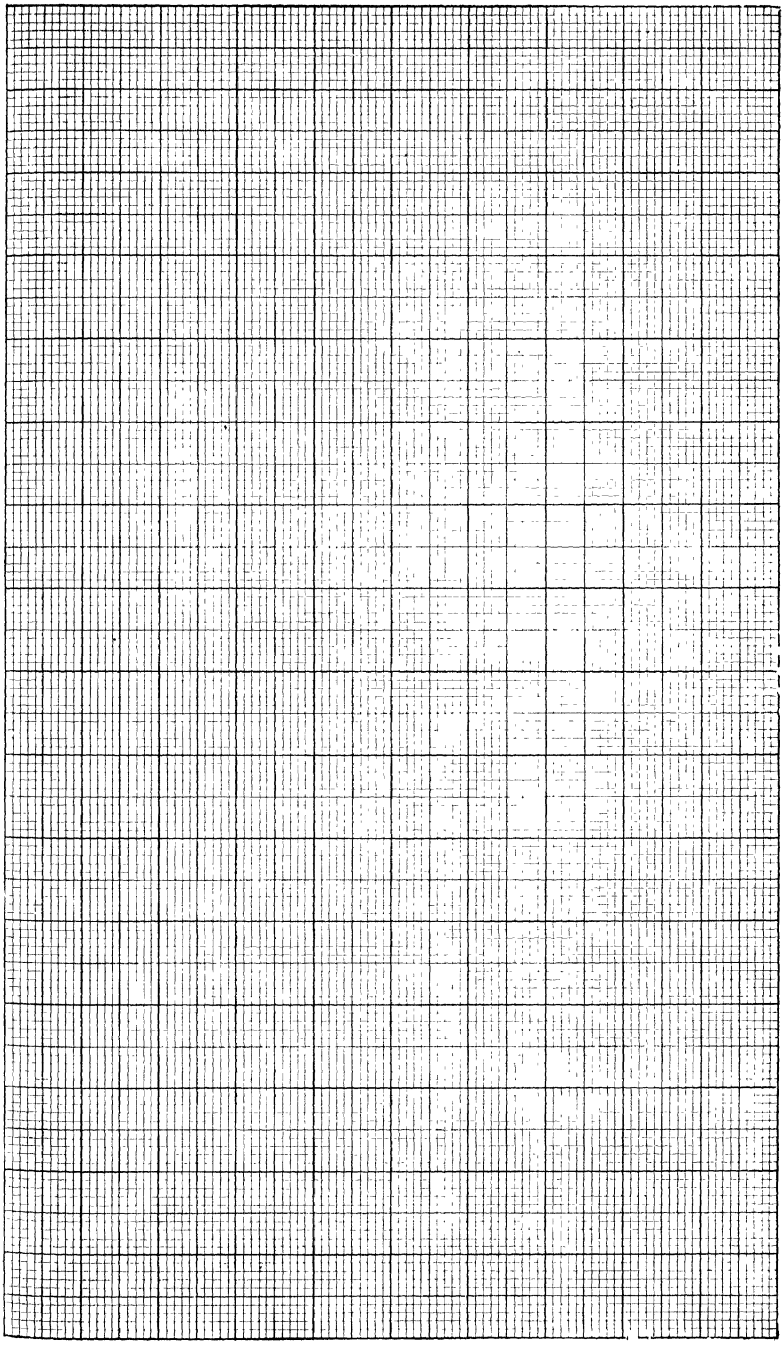
3. Usually two filtrations are sufficient owing to the presence of salts in the urine but in pure solutions some electrolyte should be added, e.g., 1 cc. of a 4 per cent solution of sodium acetate. The electrolyte causes the manganese dioxide to flocculate and thereby increases the efficiency and rate of filtering.

4. Since the purine bases do not agglutinate in eight to ten minutes without the addition of albumin, it is assumed that sufficient protective colloid is present in the urine to keep them in suspension. Only 5 cc. of a 0.2 per cent albumin solution give ample protection to pure solutions of much greater purine content.

5. Upon studying Salkowski's reagent, Graves and Kober found that a white precipitate developed upon standing, possibly magnesium hydroxide and silver either in combination or occluded. The precipitate interfered with the analysis and therefore the reagent had to stand over night and then be filtered. This trouble was avoided by using ammonium chloride alone, instead of the magnesia mixture.

6. Graves and Kober [4] tried Witte's peptone, gelatin, soluble starch and casein as protective colloids and finally adopted a dilute solution of egg albumin. The latter was found to serve quite satisfactorily. Oddly enough, albumin from eggs several months old (cold-storage eggs) is a stronger protective colloid than albumin from fresh eggs. Commercially dried egg albumins seem unsatisfactory.

[4] J. Am. Chem. Soc., **37**, 2432 (1915).



CHAPTER XXIII

TRYPSIN

NEPHELOMETRIC METHOD FOR THE STUDY OF TRYPSIN

By Hans Kleinmann

The nephelometric method for the determination of tryptic action depends upon the splitting of highly diluted solutions of sodium caseinate, which, after the action of the ferment, are transformed into nephelometric suspensions by means of the reagent quinidin hydrochloride. These suspensions are then measured in a nephelometer against a solution which was not subjected to ferment action and the protein content obtained. The diminution of the protein content gives the splitting (digestion) in per cent. This is done with the assumption that quinidin hydrochloride precipitates only undigested protein.

Comments on the Method.—The nephelometric method for the determination of tryptic action exhibits the characteristic properties necessary for nephelometry. It permits working with highly diluted solutions and in this manner avoids secondary disturbing impurities. On account of its sensitivity the method permits the determination of the first stages of digestion which for clinical purposes is of particular interest. The method, moreover, permits the analysis of numerous protein solutions in a short time, and makes possible the study of a whole series. The experimental error is small (about 2 per cent).

Contrary to most methods given for the study of tryptic activity this method does not measure split-products, which are not proportional to the protein digestion, but measures the decrease of the substrate itself.

Reagents.

1. Substrate solution. Five grams of casein (Hammarsten) are dissolved in 12 cc. 1 N sodium hydroxide and some water and the solution finally made up to 2000 cc. The solution, whose *p*H is about 8 to 9 is covered with toluol and preserved in an ice-box. The solutions become cloudy after a time. For a determination a portion is filtered through a quantitative filter (S & S Blue Ribbon or a filter of similar quality) several times; if it does not become perfectly clear it does no harm, as casein will clear up in the determination after the addition of the buffer solution.

2. Buffer solutions. According to the acidity desired, phosphate buffer (of *p*H 5 to 8) or glycine buffer (*p*H 8 to 10) is used. The concentration of the buffers must not be too great. For one thing the salt action upon the ferment must not be too pronounced, but, above all, the acidity must after the digestion be brought to the acidity necessary for the analysis. This is done by the use of a more concentrated buffer. For this purpose an M/15 buffer is used and the final concentration in the solution is about M/45, since it is diluted approximately threefold.

3. Stock buffer. As stock buffer an M/15 phosphate buffer is used. This solution is to bring the digestion media to the desired acidity for analysis. The acidity of the solutions, which for the analysis should be about *p*H 7.8, is kept at a *p*H of 7.8 to 8.0 by the use of a stock buffer of 85 parts by volume of secondary and 15 parts of primary sodium phosphate. The *p*H of this solution is 7.8. If other acidities are desired for the medium of digestion it is necessary to use other stock buffers previously tested out, so that the final acidity of *p*H 7.8 is attained for the determination.

4. Quinidin solution. A saturated solution of quinidin hydrochloride is used for the production of turbidity. The solution is prepared as follows: About 30 grams of quinidin are dissolved in 1000 cc. of warm water (not boiling) and the solution titrated with N hydrochloric acid until it becomes weakly acid to litmus. Then the solution is allowed to stand at least twenty-four hours. During the twenty-four-hour period the solution is shaken frequently, since it crystallizes very slowly.

5. Ferment solution. Ferment solutions are obtained by making an aqueous extract of dry pancreas powder like " Pancreatin Rhenania " or " Pancreas Dispert " in a concentration of 1 to 20. The suspension is allowed to remain standing for ten minutes with an occasional shaking, after which it is filtered and diluted with distilled water. The most suitable concentration for a digestion lasting thirty minutes is 1 to 3000, and for two hours 1 to 8000.

Since the ferment solution is diluted tenfold in the digestion mixture the final concentration is 1 in 30,000 to 1 in 80,000.

Procedure.—The method of procedure is arranged so that a substrate system is made which always has the same amount of substance during the progress of digestion as well as in the period before the ferment is added. After a portion is removed, its ferment digestion is stopped by boiling. The casein solutions do not flocculate during boiling. However, the protein changes its character during the boiling in such a manner that the clouds with quinidin are stronger than those in solutions which are not boiled. This reaction reaches a maximum within six minutes and remains constant. Also, the reactions at different concentrations are proportional so that the proportionality of the clouds with concentration is not changed with the boiling.

Every test is made with two parallel series. In each of several 75 cc. graduates are put 12.0 cc. of casein solution, 15 cc. of M/1 phosphate buffer (for example, with a digestion having a *p*H of 8.02, a buffer consists of 99 parts of secondary and one part of primary phosphate) and then water is added from a pipette until the volume is 46 cc. and the solution mixed. The test is run thirty minutes and seven portions or samples may be taken. For this purpose twenty-five large test tubes (25 cc. capacity) are used. The test tubes should have a mark at 22.5 cc. Five cubic centimeters of water are put in each tube and the latter placed in a boiling water-bath. From each graduate cylinder 10 cc. are removed with a pipette and 4.5 cc. added to each test tube, the remaining 5.5 cc. being discarded. There remain therefore in each cylinder 36 cc. of the substrate solution. The first portion is 4.5 cc. and not 5 cc. as in later sampling, because the system

is diluted with ferment solution. The portion taken out is held exactly six minutes in the water-bath and then cooled in cold water. In this same manner all later samples are treated. They stay in the water-bath six minutes and are then cooled. The reagent must not be added until the temperature has reached an equilibrium. The four cylinders which even before taking the first samples have been preheated in a water thermostat at 37° c. (±0.05°), are increased by the addition of 4 cc. of ferment solution and the time noted. The ferment solution is added by means of a pipette and must be perfectly clear and preheated to 37° C.

At intervals of every five minutes, using the same pipette, 5 cc. are taken from the graduate cylinder, and placed in the heated test tube. After seven samples have been taken (a thirty minute digestion), 5 cc. remain for a *p*H determination. The heated samples can be analyzed immediately or can be stoppered and preserved. However, it is advisable to make the analysis immediately following the digestion. For the determination, 5 cc. of M/15 phosphate buffer are added to each test tube. This brings the acidity of the solution up to about *p*H 7.8. To the mixture are added 7.5 cc. of cold saturated quinidin solution. The volume of the mixture is then brought up to the 22.5 cc. mark by the addition of water and the mixture is allowed to stand ten to fifteen minutes.

The comparison is made in a nephelometer, within about forty-five minutes after the addition of the reagent, against the first sample whose concentration is placed at 100. The standard solution side of the instrument is given a 20-mm. opening. The nephelometric readings are inversely proportional to the concentration if a Kleinmann nephelometer is used; other nephelometers may require a correction or standardization curve.

When the digestion has proceeded over 50 per cent it is in the interest of accuracy not to use the standard solution but to employ one-half the value of the standard.

The method can be tested out by using a hypothetical series, without any ferment added, i.e., the arrangement is the same as described except instead of the active ferment a heat-destroyed

ferment solution is added and the variations in the amounts of substrate are effected by variations in the amounts of the solution taken for samples and not through ferment action.

In the following example the amounts taken as samples were so chosen that they represented 90, 80, 70, 60, 50, 40, 30, 20, and 10 per cent of the original solution.

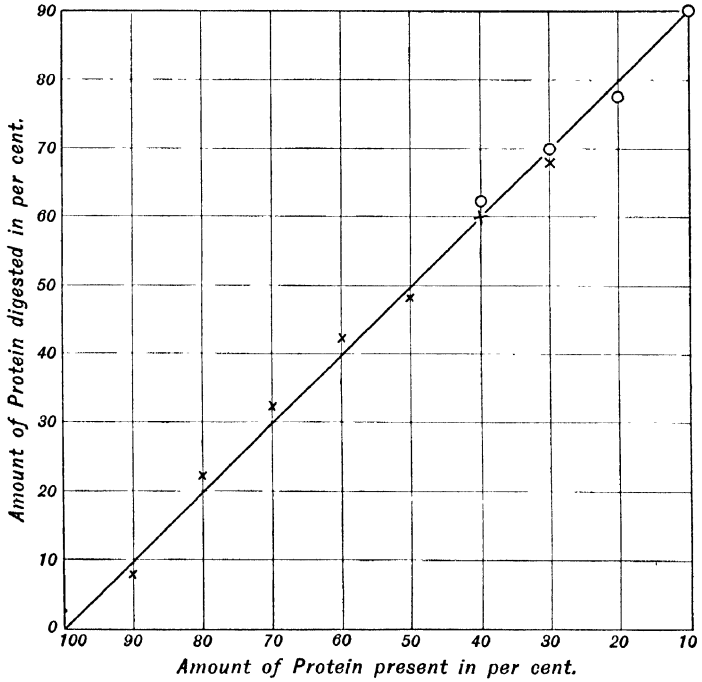


FIG. 43.—A Hypothetical Digestion Experiment.

The hypothetical test, therefore, went from 10 to 90 per cent. The digestion was not only measured against the standard but also against a half-standard. The course of the test is shown in Fig. 43. The straight line represents the theoretical values. The points found do not deviate on an average from the theoretical by more than 1.6 per cent and never beyond an error of 3 per cent. The values of an apparent digestion of 60 per cent are more accurately measured against a half-standard than against a standard solution. These errors also include those

inherent in digestion experiments, such as errors of pipetting, etc. The test shows, therefore, that by means of the method described the course of a tryptic digestion of a 0.025 per cent casein solution can be determined with such an accuracy that the position

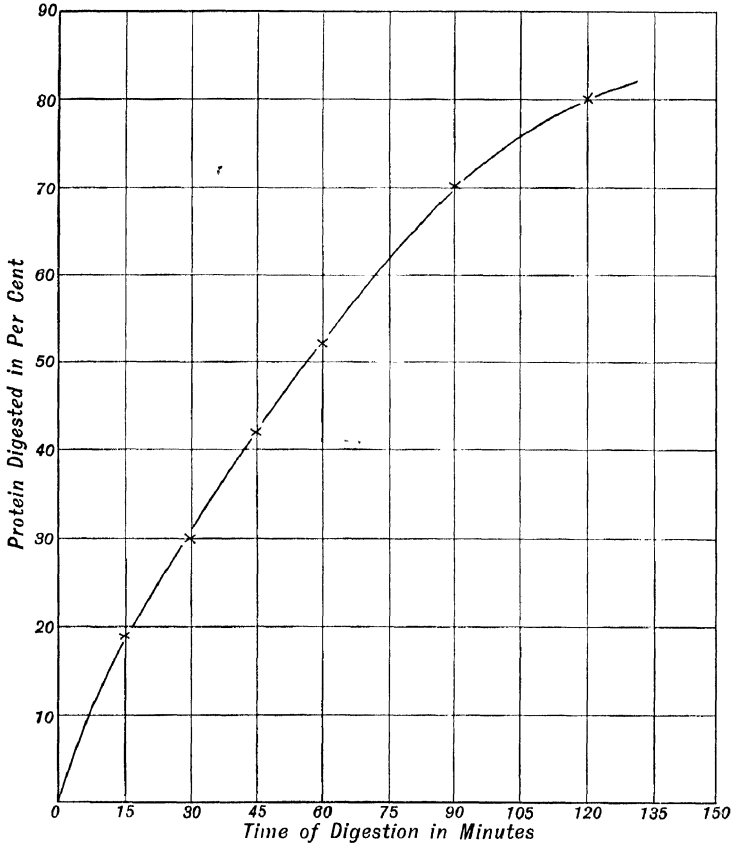


FIG. 44.—Tryptic Digestion of Casein. (Ferment solution 1 to 7000.)

of individual points on the curve are accurate to within an error of approximately 2 per cent.

Calculation of the Analyses.—The calculation of the analyses when a Kleinmann nephelometer is used is very simple. The height of the nephelometric reading is inversely proportional to the concentration. If C is the concentration of the undigested

casein solution, which is equal to 100 per cent protein, and C_1 the protein concentration of the digested solution, H the nephelometric reading of the solution C and H_1 the reading of solution C_1, then the unknown concentration C_1 becomes $C_1 = \frac{C \cdot H}{H_1}$. The protein content of the digested solutions are obtained in per cent. By subtraction from 100 the amount of digested protein in per cent is obtained.

As an example of the method Table XXII and Fig. 44 are given.

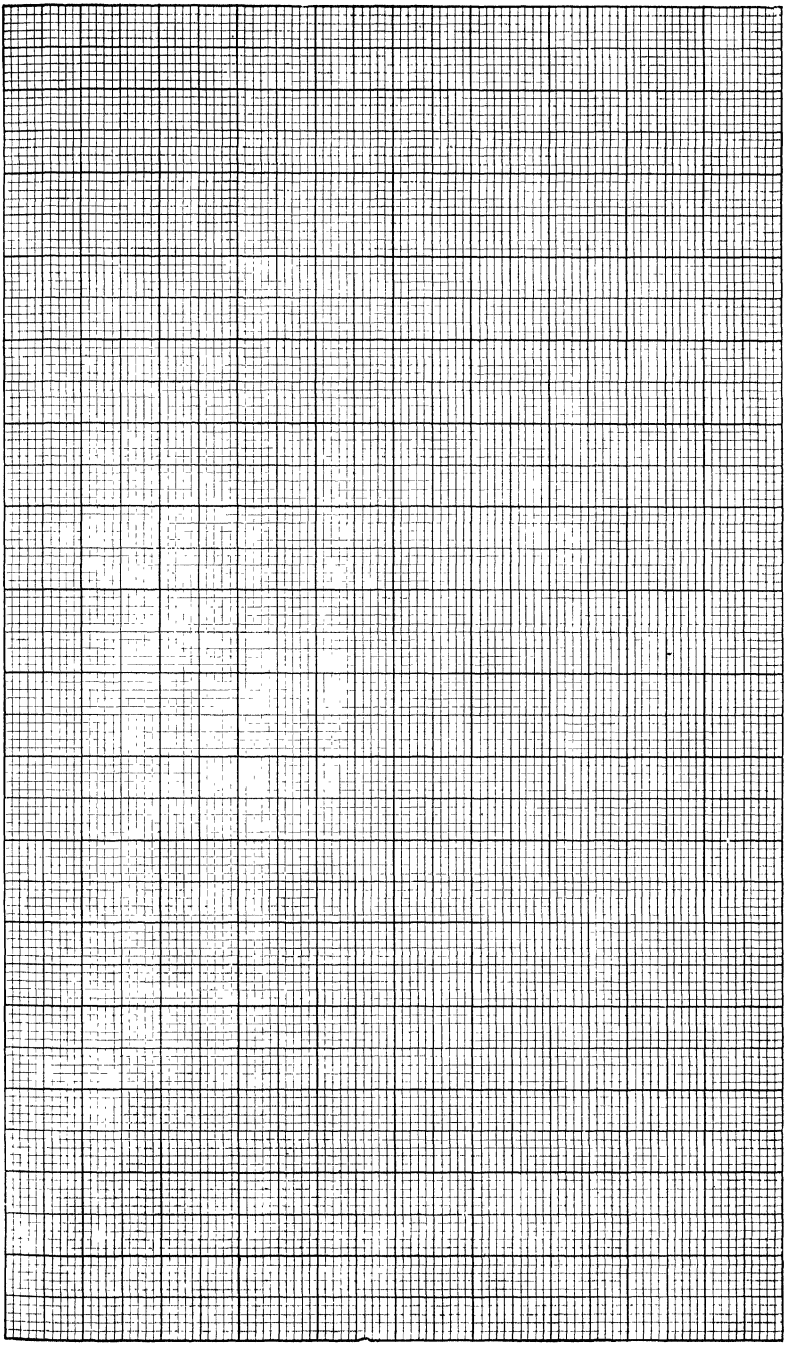
TABLE XXII

Casein Digestion.—Stopped by boiling. Four parallel series I to IV. Each had 12 cc. casein solution, 15 cc. buffer solution, 46 cc. distilled water, and then 4 cc. of ferment solution (1 to 7000). The receiver contained 5 cc. distilled water, 5 cc. of buffer solution, and 5 cc. of quinidin solution.

Minutes		I	II	III	IV	Height of Standard Solution
0	Nephelometric readings, average	Mm 20 0	Mm 20 0	Mm 20 0	Mm 20 0	Mm. 20
	Digestion, in per cent	00 0				
15	Nephelometric readings, average	24 5	24 6	24 3	24 4	20
	Digestion, in per cent	18 0				
30	Nephelometric readings, average	28 5	28 6	28 4	28 0	20
	Digestion, in per cent	30 07				
45	Nephelometric readings, average	41 1	41 0	34 5	34 3	20
	Digestion, in per cent	42 0				
60	Nephelometric readings, average	32 3	32 0	32 2	32 3	15
	Digestion, in per cent	53 2				
90	Nephelometric readings, average	34 6	35 1	32 8	32.5	10
	Digestion, in per cent	69 0				
120	Nephelometric readings, average		18 9	20 5	25 0	5
	Digestion, in per cent	80.0				
150	Nephelometric readings, average	34 6	33 9	34 0	34.1	5
	Digestion, in per cent	85 2				

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CLINICAL METHOD FOR THE DETERMINATION OF TRYPSIN IN DUODENAL JUICES

By Hans Kleinmann

The method depends upon the splitting of very dilute sodium caseinate solutions with diluted intestinal fluids in media of certain acidities. The digested casein solutions are then brought to a certain acidity and through the addition of quinidin hydrochloride precipitated and nephelometrically determined.

The apparatus and solutions required are the same as those used in the study of trypsin (see p. 271).

Preparation of Substrate Solution.—For the production of the substrate solution, the sodium caseinate solution (see described method), which clouds up somewhat while standing in the refrigerator, is filtered several times until it is practically clear and free of toluol. Twelve cubic centimeters of casein solution are diluted with 15 cc. of an M 15 phosphate buffer solution of *p*H 8 and water to make a volume of 46 cc.

Preparation of the Receivers.—For the clinical determination three portions, after ten, twenty, and thirty minutes, are sufficient. Therefore, including the portion taken before digestion, four, or with duplicate determinations, eight receivers are necessary. If test tubes are used, they should hold at least 25 cc. and should have a mark at 22.5 cc. In each of the receivers are put 5 cc. of distilled water.

In the first pair of test tubes, which are to receive the sample of the solution prior to digestion, an additional 0.5 cc. is added, making a total of 5.5 cc. of water in each tube. All solutions used in the determination are freed from shreds by filtering through S & S paper or filter paper of similar quality.

Procedure.—The freshly obtained duodenal juice is filtered. It makes no difference in which way the juice is obtained. If it is obtained by the injection or administration (oral) of ether, the ether is removed from the juice by careful aëration. If the juice is obtained through the administration of magnesium sulfate, the presence of magnesium sulfate does not influence the reaction appreciably, because the dilution is so large that the salt

effect is not appreciable. Also the color of the juice, on account of the large dilution, does not come into consideration.

The clear filtrate of the juice is diluted in a ration of 1 to 50 with distilled water. Usually this dilution effects a suitable digestion in thirty minutes. Should the digestion be too great or too small, other suitable dilutions must be used. For the digestion, 18 cc. of substrate buffer mixture are put in a 25-cc. test tube, and the solution warmed in a thermostat to 37° C.

Likewise 4.5 cc. of the substrate buffer mixture are put into the first test tubes, which serve as receivers.

Before mixing, the receivers containing the 5 cc. of water are heated to boiling in a water-bath. To these are added the receiver which has the 4.5 cc. of substrate solution. The receivers after the addition of the substrate remain in the boiling water-bath exactly six minutes (measured with a watch), and then taken out and cooled in a vessel with water.

Now 2 cc. of diluted duodenal juice are added to the 18 cc. of substrate solution, which has been previously warmed in the thermostat. The mixture is thoroughly stirred with a pipette and the time recorded. After every ten minutes, a 5-cc. portion is taken with a pipette and added to the receivers standing in a boiling water-bath. The receivers, after the portions are added, remain in the boiling water-bath exactly six minutes and, like the first pair of test tubes, are then removed and cooled. After thirty minutes three portions will have been taken.

The purpose of the boiling receivers is to stop the digestion instantly. Through heating the casein is not precipitated but is changed, which is recognized by a difference in the turbidity reaction from that of unheated casein. After six minutes the change in turbidity-producing power is at a maximum and in proportion to the concentration. Therefore it is necessary to treat the standard portion which contains no ferment, by boiling and cooling just as the other portions.

After the portions have been thoroughly cooled, 5 cc. of stock buffer—in this case the same buffer as the digestion buffer—are added. Then 7.5 cc. of quinidin solution are added and the test-tubes made up to the 22.5-cc. mark with distilled water.

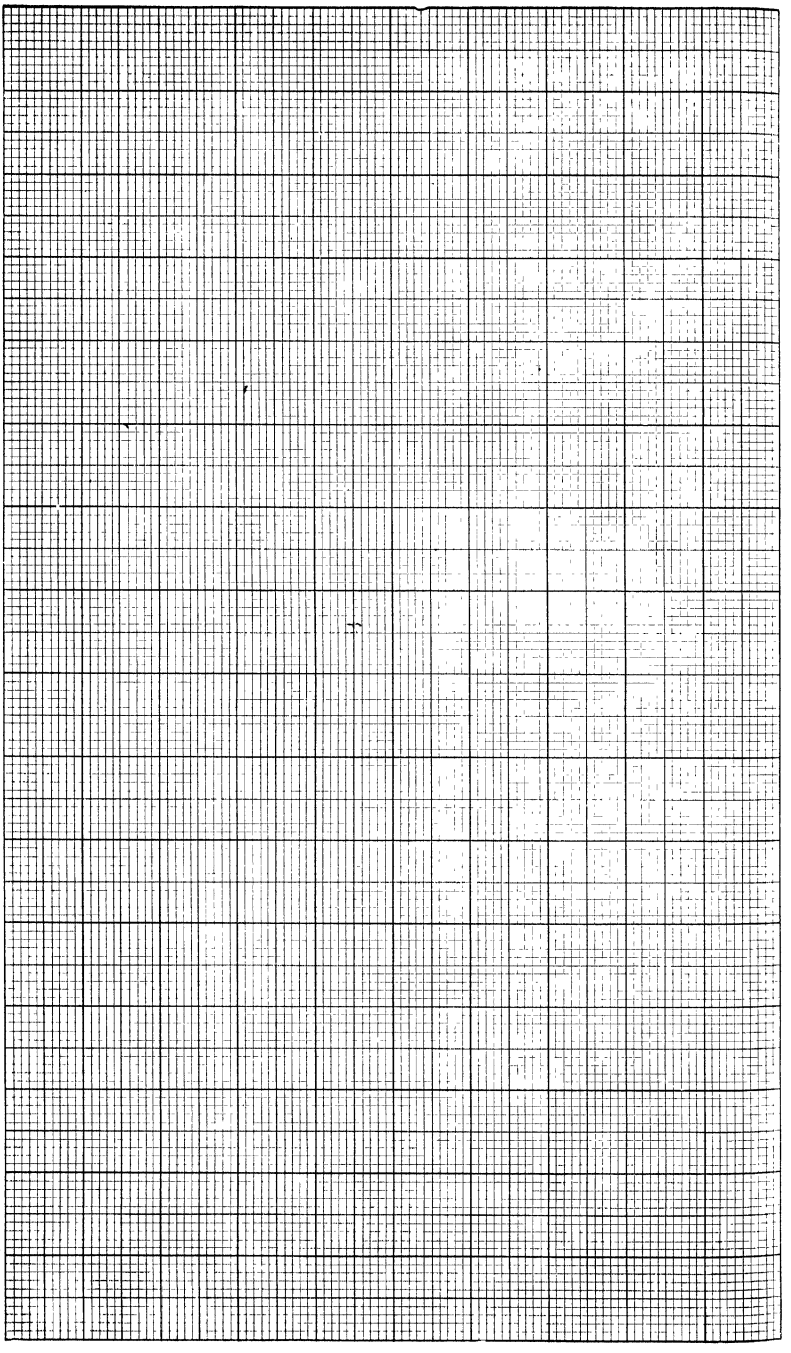
The clouds are allowed to develop five to ten minutes. Then within forty-five minutes the digested portions are measured in a nephelometer against the undigested solution used as a standard and taken as 100 per cent.

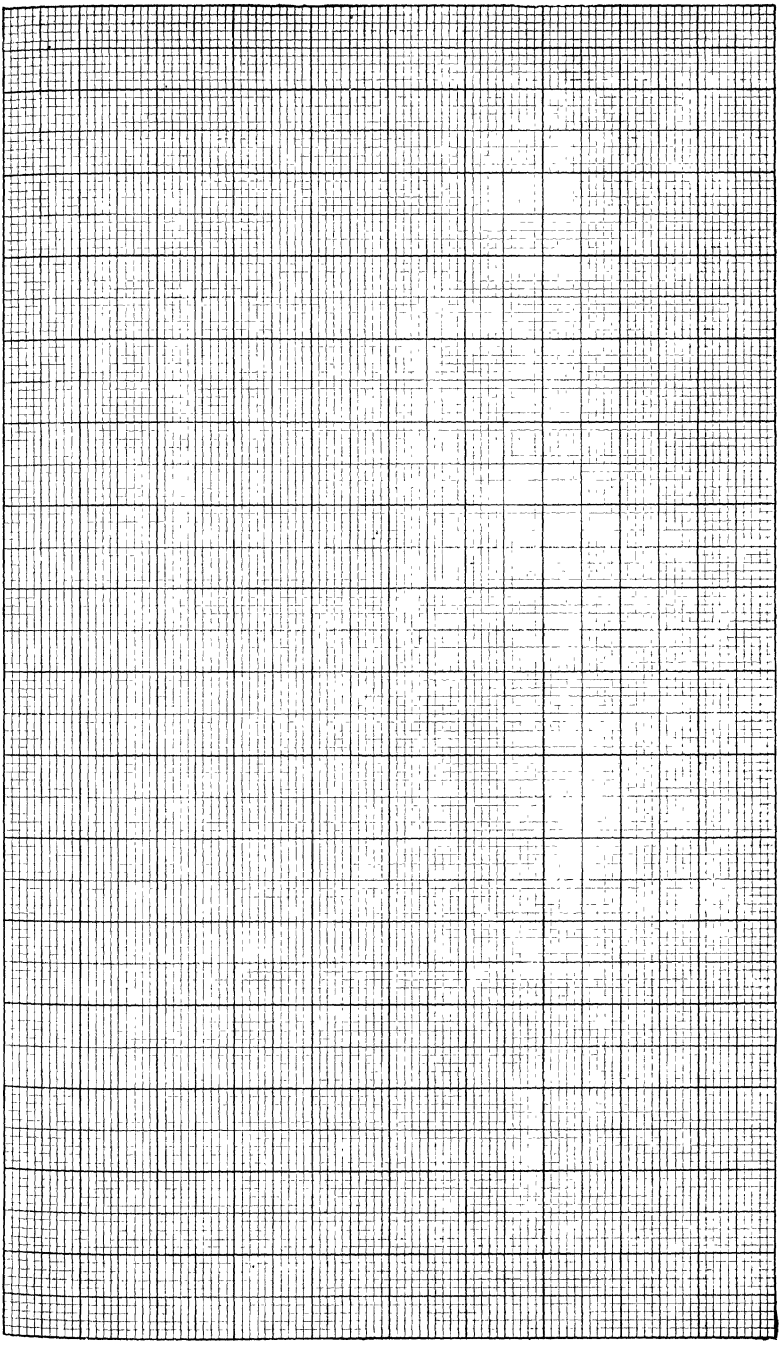
Calculation of the Results.—The casein concentrations vary inversely as the nephelometric readings when a Kleinmann nephelometer is used. By comparison with the casein concentration we get: unknown concentration : 100 = reading of standard solution: reading of unknown solution in per cent. The amount of digested protein is obtained by subtracting from 100 per cent the amount of protein found.

Just as with the method for the determination of pepsin in gastric juice, the trypsin content of duodenal fluid may be compared with dilutions of commercial preparations like pancreatin, etc.

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PART IV

BIBLIOGRAPHY

BIBLIOGRAPHY ON NEPHELOMETRY ARRANGED ALPHABETICALLY BY SUBJECT AND CHRONOLOGICALLY UNDER EACH SUBJECT

NEPHELOMETRY is quite a young subject, in fact, with the exception of several references prior to 1900, nephelometry dates from 1912 when Philip A. Kober used it for the first time as an analytical method and not simply as a means of correcting other analytical procedures as it was originally employed by T. W. Richards. About the same time as Kober's first work, but entirely independently, nephelometry was used as a quantitative method by W. R. Bloor, whose work was reported in 1913. The literature on this subject, therefore, has for the most part appeared within the last fifteen years.

It has been the aim of the author to make the following bibliography an accurate and fairly complete survey of the literature on nephelometry. It is possible that some articles have escaped his attention and he will very much appreciate having his attention called to any omissions so that they may be included in a future edition. Also, notice of any errors will be greatly appreciated.

The official abbreviations of Chemical Abstracts have been used. See "List of Periodicals Abstracted by Chemical Abstracts with Key to Library Files," Chemical Abstracts **20**, No. 20, Part 2 (1926).[1]

[1] Compiled by the Research Information Service of the National Research Council under the supervision of C. J. West, director.

In general, the first reference given after an author's name is the journal in which the article originally appeared; succeeding references are abstracts of the article. No attempt has been made to give one or more abstract reference on each article.

The author desires to express his appreciation to the following libraries whose files of periodicals have made this bibliography possible:

Army Medical Library, Washington, D. C.
Chemists' Club, New York City.
Department of Agriculture, Washington, D. C.
Geological Survey, Washington, D. C.
John Crerar Library, Chicago, Ill.
New York Public Library, New York City.
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YABLICK, M., PERROTT, G. ST. J., and FURMAN, N. H., J. Am. Chem. Soc. **42**, 266 (1920); C. A., **14**, 1505 (1920). Determination of traces of dichlorethyl sulfide (mustard gas) in air.

Explosives, Stability of.

POLLARD, A. F. C., Trans. Optical Soc. London, **26**, 63 (1924–25); J. Soc. Chem. Ind., **44**, B614 (1925). Nephelometry and a suggested sensitive test for the stability of explosives. See the abstract of this paper given under **Nephelometry.**

Fat.

BLOOR, W. R., J. Biol. Chem., **17**, 377 (1914). A method for the determination of fat in small amounts of blood. Uses a form of Richards' nephelometer. Sensitive to 1 part of fat in 1 million parts of water.

BLOOR, W. R., J. Am. Chem. Soc., **36**, 1300 (1914). A method for the determination of fat in milk. Sensitive to 1 part of fat in 1 million parts of water.

MENDEL, L. B. and BAUMANN, E. J., J. Biol. Chem., **22**, 165 (1915). The question of fat absorption from the mammalian stomach. Fat was determined by Bloor's method.

LAWS, C. H. and BLOOR, W. R., Am. J. Diseases Children, **11**, No. 3 (1916); J. Am. Med. Assocn., **66**, 918; C. A., **10**, 1539 (1916). Rapid method for determination of fat in feces. A nephelometric method. Extract the stool directly with acidified alcohol and ether, filter the extract, then precipitate the fat in aqueous solution and compare the cloudy suspension so obtained with that of a similarly prepared standard solution.

SHARPE, J. S., Biochem. J., **11**, 96 (1917). Nephelometry of fats and fatty acids.

MURLIN and RICHE, see P. A. KOBER, J. Ind. Eng. Chem., **10**, 561 (1918). M. and R. pour the fat solution into 0.05 per cent gelatin solution (1 gram gelatin in 2 liters of water with 5 cc. glacial HAc). Private communication to Kober.

SPERRY, W. M. and BLOOR, W. R., J. Biol. Chem., **60**, 261 (1924). Fat in feces by Bloor's method.

Fat-cholesterol.

HECKSCHER, H., Biochem. Z., **181**, 444 (1927); C. A., **21**, 2287 (1927). The nephelometric determination of the neutral fat-cholesterol fraction according to the method of Bing and Heckscher. The accuracy of the method and the necessary conditions for its proper use as well as an interpretation of certain technical details.

Fat, Cleavage of.

RONA, P. and KLEINMANN, H., Biochem. Z., **174**, 18 (1926). Untersuchungen über die Beziehung zwischen Substratdispersität und Fermentwirkung mittels einer neuen Methode zur Bestimmung der fermentativen Fettspaltung.

Fatty Acids.

SHARPE, J. S., Biochem. J., **11**, 96 (1917). Nephelometry of fats and fatty acids.

CSONKA, F. A., J. Biol. Chem., **34**, 577 (1918). Nephelometric values of cholesterol and the higher fatty acids.

CSONKA, F. A., J. Biol. Chem., **41**, 243 (1920). A study of the nephelometric values of cholesterol and the higher fatty acids. II. Influenced by saponification, etc.

BLOOR, W. R., PELKAN, K. F. and ALLEN, D. M., J. Biol. Chem., **52**, 191 (1922). Cholesterol separated and determined colorimetrically and the fatty acids determined nephelometrically.

Fibrinogen.

RUSZNYÁK, S. and BARÁT, I., Biochem. Z., **141**, 476 (1923). Die Bestimmung des Fibrinogens im Blutplasma. A nephelometric method.

Fluorescence Apparatus.

TSWETT, M., Z. physik. Chem., **36**, 450 (1901). Vorrichtung zur Beobachtung von Fluorescenz- und Opalescenzerscheinungen.

Globulin.

KOBER, P. A., J. Am. Chem. Soc., **35**, 1585 (1913). Nephelometric determination of proteins; casein, globulin and albumin in milk.

RUSZNYÁK, S., Biochem. Z., **133**, 370 (1922).

Glycogen.

PAECHTNER, J., Biochem. Z., **156**, 249 (1925). Nephelometrische Untersuchungen über Glykogenabbau durch Speicheldiastase.

Guanidine.

RITTMANN, R., Biochem. Z., **172**, 36 (1926); C. A., **21**, 111 (1927). Determination of very minute amounts of guanidine with the aid of a nephelometer. The method depends upon the formation of a bluish to yellow-green turbidity in solutions containing guanidine, upon the addition of Nessler's reagent.

Hardness, Water.

FEIGEL, F. and PAVELKA, F., Mikrochemie, **2**, 85 (1924); J. Chem. Soc., **126**, ii, 784 (1924); J. Soc. Chem. Ind., **43**, B968 (1924). Detection and determination of small quantities of calcium and magnesium by means of ammonium ferrocyanide, and a new nephelometric method for the determination of the hardness of water.

Hydrosol, Colored.

BECHHOLD, H. and HEBLER, F., KOLLOID-Z., **31**, 7 (1922); C. A., **16**, 3782 (1922). Nephelometry of colored hydrosols.

Lead

VAN DER VLUGT, L. S., Chem. Weekblad, **25**, 194 (1928); C. A., **22**, 2338 (1928). Nephelometric determination of small quantities of lead in the presence of zinc by means of potassium chromate. The Pb extract is evaporated to dryness with a drop of strong HNO_3, the residue dissolved in water, filtered and transferred to a colorimeter tube. One drop of N AcOH and one drop of 10 per cent K_2CrO_4 are added and, after shaking, the turbidity is compared with standard turbidities.

DANCKWORTT, P. W. and JURGENS, E., Arch. Pharm., **266**, 374 (1928); C. A., **22**, 3373 (1928). Toxicology of lead and its compounds. Quantitative nephelometric determination of lead. It is possible to determine Pb nephelometrically in amounts as small as 0.006 mg. A turbidity standard produced by 0.05 mg. Pb in an AcOH solution of $K_2Cr_2O_7$ can be used over a period of an hour without appreciable change in particles or danger of precipitation. A series of turbidities is prepared for purposes of comparison and exclusion. NaOAc and Cu are without influence on the turbidity. Large amounts of Fe, however, make the readings somewhat inconstant.

Lecithin.

BLOOR, W. R., J. Biol. Chem., **22**, 133 (1915). A method for the determination of "lecithin" in small amounts of blood.

BLOOR, W. R., J. Biol. Chem., **24**, 450 (1916).

Lipoids.

DUBIN, H., J. Biol. Chem., **33**, 377 (1918). Studies of the blood fat and lipoids of the dog before and after the production of experimental anemia. Nephelometric and colorimetric methods of Bloor.

BLIX, G., Biochem. Z., **167**, 313 (1926); C. A., **20**, 3474 (1926); cf. C. A., **19**, 2218. Nephelometry of blood lipoids.

Magnesium.

DENIS, W., J. Biol. Chem., **41**, 363 (1920). Estimation of magnesium in blood. Estimated indirectly by precipitating as $MgNH_4PO_4$ and determining the phosphate nephelometrically with strychnine molybdate reagent.

KRISS, L., Biochem. Z., **158**, 203 (1925); C. A., **19**, 3442 (1925) and ibid. **20**, 1041 (1926). The nephelometric determination of calcium and magnesium.

KRISS, L., Biochem. Z., **162**, 359 (1925). The nephelometric determination of calcium and magnesium. II.

Manganese.

LODE, G., Arch. Hyg., **97**, 227 (1926). A modification of Croner's method for the determination of manganese in drinking water.

Nephelometer.

MULDER, Die Silberprobiermethode, p. 23, Trans. Grimm, Leipzig, **1859**. Roughly compared opalescent AgCl suspensions obtained in the supernatant liquor, or the filtrates, in his atomic weight determinations. Sometimes used tubes to hold the suspensions but no measuring device was employed. His experiments, however, mark the first step in the development of nephelometry.

STAS, Oeuvres, **1**, 155 (1894). Used a series of tubes with perfectly plain bottoms. The tubes were supported adjacent to each other upon a shelf over holes which were of the same diameter as the tubes and beneath which was an illuminated scale. Everything above the shelf was kept in darkness. When the marks on the scale viewed through two heights of opalescent solution appeared the same, S. assumed there was an equal weight of suspension in the two tubes. This apparatus marks the second step in the development of nephelometry. The disadvantage with this instrument is that transmitted light is observed. In the real nephelometer, first devised by T. W. Richards (1894), the reflected light is observed.

RICHARDS, T. W., Z. anorg. Chem., **8**, 268 (1895). Uses a nephelometric method for determining the amount of strontium in connection with a new determination of its atomic weight. Made the first real nephelometer in 1894.

TSWETT, M., Z. physik. Chem., **36**, 450 (1901). Vorrichtung zur Beobachtung von Fluorescenz und Opalescenzerscheinungen.

RICHARDS, T. W. and WELLS, R. C., Am. Chem. J., **31**, 235 (1904). The nephelometer, an instrument for detecting and estimating opalescent precipitates.

RICHARDS, T. W., J. Chem. Soc., **99**, 1204 (1911). Faraday Lecture. "The fundamental properties of the elements." Mentions the "nephelometer" and gives a brief description of it. "With the nephelometer, minute traces of suspended precipitate may be approximately determined from the brightness of the light they reflect. . . . Traces of substance, which are too attenuated to be caught on any ordinary filter may thus be estimated."

KOBER, P. A., J. Biol. Chem., **13**, 485 (1912–13). Converts a Duboscq colorimeter into a nephelometer.

KOBER, P. A., J. Am. Chem. Soc., **35**, 1585 (1913). Describes an improved model of the nephelometer in J. Biol. Chem., **13**, 485 (1912–13).

DIÉNERT, F., Compt. rend., **158**, 1117 (1914); J. Chem. Soc., **106**, ii, 486 (1914). A new nephelometer for use in analytical chemistry. Uses a Duboscq colorimeter and a projection lamp. Gives method for determining H_2SO_4 in water nephelometrically. $BaSO_4$ suspension formed by adding $BaCl_2$ solution to the water made acid with HCl.

BLOOR, W. R., J. Biol. Chem., **17**, 377 (1914). Uses a form of Richards' nephelometer in the determination of fat in small amounts of blood.

KOBER, P. A. and GRAVES (Miss) S. S., J. Ind. Eng. Chem., **7**, 843 (1915); Chem. Zentr., **2**, 1262 (1915). Nephelometry (Photometric Analysis). I. History of method and development of instruments.

BLOOR, W. R., J. Biol. Chem., **22**, 145(1915). A simple method of converting the Duboscq colorimeter into a nephelometer.

MARSHALL, J. T. W. and BANKS, H. W., 3rd, Proc. Am. Phil. Soc., **54**, 176 (1915); C. A., **9**, 2914 (1915). New form of nephelometer.

KOBER, P. A., J. Biol. Chem., **29**, 155 (1917); J. Soc. Chem. Ind., **36**, 571 (1917). An improved nephelometer-colorimeter.

KOBER, P. A., J. Ind. Eng. Chem., **10**, 556 (1918). Same lecture is reported in J. Soc. Chem. Ind., **37**, 75 (1918), but in less detail. Describes several nephelometers, with figures, and discusses some technical applications of nephelometry.

KLEINMANN, H., Inaugural Dissertation (Berlin, **1919**), "Über die Bestimmung der Phosphorsaure," p. 99.

KLEINMANN, H., Biochem. Z., **99**, 115, 129 (1919); see also his Inaugural-Dissertation, Berlin, **1919**. General principles of nephelometry and a new nephelometer.

CHÉNEVEAU, C. and AUDUBERT, R., Compt. rend., **170**, 728 (1920); J. Chem. Soc., **118**, ii, 327 (1920); C. A., **14**, 1621 (1920). Discuss the theory of nephelometry and describe a simple nephelometer.

KLEINMANN, H., Kolloid-Z., **27**, 236 (1920); J. Soc. Chem. Ind., **40**, 101A (1921). A new nephelometer and the principles of nephelometric measurements.

CHÉNEVEAU, C. and AUDUBERT, R., J. phys. radium, **2**, 19 (1921); C. A., **15**, 2023 (1921). A somewhat more extended discussion of the matter treated in Compt. rend., **170**, 728 (1920).

KOBER, P. A. and KLETT, R. E., J. Biol. Chem., **47**, 19 (1921); J. Soc. Chem. Ind., **40**, 604A (1921). Further improvements in the nephelometer-colorimeter.

WEINBERG, A. A., Biochem. Z., **125**, 292, (1921); C. A., **16**, 943 (1922); J. Soc. Chem. Ind., **41**, 235A (1922). Zur Methodik der Nephelometrie. Ein Nephelometer mit Konstanten Standard.

RUSZNYÁK, S., Biochem. Z., **133**, 365 (1922); C. A., **17**, 1038 (1923). Die Umgestaltung des Chromophotometers von Plesch in ein Nephelometer.

KUGELMASS, I. N., Compt. rend., **175**, 343 (1922); J. Soc. Chem. Ind., **41**, 730A (1922).

FOX, Chem. News, **126**, 327 (1923). Nephelometer with observation tubes of fixed height.

KLEINMANN, H., Biochem. Z., **137**, 144 (1923). Weitere Beiträger zur Nephelometerapparatur und der Methodik nephelometrischen Arbeitens.

KLEINMANN, H., Biochem. Z., **137**, 148 (1923). Über Mikronephelometrie.

KINGSLAKE, R., Trans. Optical Soc. London, **26**, 53 (1924–25); J. Chem. Soc., **128**, ii, 821 (1925); Chem. News, **129**, 372 (1924); J. Soc. Chem. Ind., **44**, B615 (1925); C. A., **20**, 1 (1926).

KLEINMANN, H., Kolloid-Z., **36**, 168 (1925). Reviews nephelometry under two heads: nephelometric methods, and nephelometric apparatus. Describes a micronephelometer. Suggests that a ground glass could be used as a turbidity standard.

KLEINMANN, H., Biochem. Z. **179**, 301 (1926). An improvement of the original apparatus involving the use of a wider nephelometer tube.

KLEINMANN, H., J. Lab. Clin. Med., **12**, 629 (1927). A general discussion on nephelometry and a description of Kleinmann's macro- and micronephelometers.

Nephelometry.

RICHARDS, T. W. and WELLS, R. C., Am. Chem. J., **31**, 235 (1904); cf. Proc. Am. Acad. Arts Sci., **30**, 385 (1894). The nephelometer, an instrument for detecting and estimating opalescent precipitates.

RICHARDS, T. W., J. Chem. Soc., **99**, 1204 (1911). Mentions the "nephelometer" and gives a brief description of it. "With the nephelometer, minute traces of suspended precipitate may be approximately determined from the brightness of the light they reflect. . . . Traces of substance, which are too attenuated to be caught on any ordinary filter, may thus be estimated."

RICHARDS, T. W., Orig. Com. 8th Intern. Congr. Appl. Chem., **1**, 423 (1912); C. A., **6**, 3238 (1912).

RICHARDS, T. W., Discussions 8th Intern. Congr. Appl. Chem., **27**, 24 (1912). Discussion of paper given in ibid., **1**, 423 (1912) as to amounts of material and percentage of accuracy.

KOBER, P. A., J. Biol. Chem., **13**, 497 (1912–13). Helpful suggestions regarding the use of the nephelometer.

KOBER, P. A. and GRAVES, (Miss) S. S., "International Clinics," 24th Series, **3**, 106 (1914). Chief requisite for making nephelometric clouds or colloidal suspensions, and for keeping them as such for a definite time. Substance must be dilute—usually not over 100 mg. per liter.

KOBER, P. A. and GRAVES, (Miss) S. S., J. Ind. Eng. Chem., **7**, 843 (1915); Chem. Zentr., **2**, 1262 (1915). Nephelometry (Photometric Analysis). I. History of method and development of instruments.

KLEINMANN, H., Inaugural Dissertation (Berlin, 1919).

KLEINMANN, H., Biochem. Z., **99**, 115, 129 (1919). General principles of nephelometry and a new nephelometer.

CHÉNEVEAU, C. and AUDUBERT, R., Compt. rend., **168**, 684, 766 (1919). Give a mathematical treatment of the principles of nephelometry.

KLEINMANN, H., Kolloid-Z., **27**, 236 (1920); J. Soc. Chem. Ind., **40**, 101A (1921). A new nephelometer and the principles of nephelometric measurements.

CHÉNEVEAU, C. and AUDUBERT, R., Compt. rend., **170**, 728 (1920); J. Chem. Soc., **118**, ii, 327 (1920); C. A., **14**, 1621 (1920). Discuss the theory of nephelometry and describe a simple nephelometer (no figure given).

SNELL, F. D., Colorimetric Analysis, p. 147, D. Van Nostrand Co., New York, 1921. Makes a few brief remarks on nephelometry.

WELLS, P. V., J. Am. Chem. Soc., **44**, 267 (1922); J. Soc. Chem. Ind., **41**, 310A (1922). A simple theory of the nephelometer.

HEBLER, F., Inaugural Dissertation (Frankfurt, 1922). Über den Nephelometer-effekt Kolloider Systeme.

KLEINMANN, H., Biochem. Z., **137**, 148 (1923). Über Mikronephelometrie.

LEDNICKY, M. U. C. Al., Kolloid-Z., **32**, 12 (1923); C. A., **17**, 1567 (1923). Allgemeine Nephelometrie.

POLLARD, A., Proc. Optical Soc., London, Nov. 13, 1924; Chem. News, **129**, 372 (1924). A note on nephelometry.

POLLARD, A. F. C., Trans. Optical Soc., London, **26**, 63 (1924–25); J. Soc. Chem. Ind., **44**, B614 (1925). Nephelometry, and a suggested sensitive test for the stability of explosives. "The rate of decomposition of propellent explosives at atmospheric temperature is measured by sweeping off the accumulated N peroxides with a current of air. This air is to be passed through a colloidal solution of Ag_2O contained in one tube of a Kingslake nephelometer (Trans. Optical Soc., **26**, 53), and the disappearance of the colloidal particles due to the action of the N peroxides is followed by the reduction of the scattered light, the amount of which is measured by the movement of the standard tube necessary to restore optical equality in the photometric field. The tests are carried out at intervals not exceeding ten hours, the amount of N peroxide accumulated during the interval being thus measured." C. E. Munroe, C. A., **19**, 3594 (1925).

KLEINMANN, H., Kolloid-Z., **36**, 168 (1925). Reviews nephelometry under two heads: nephelometric methods, and nephelometric apparatus. Describes a micronephelometer. Suggests that a ground glass could be used as a turbidity standard.

WELLS, P. V., Chem. Reviews, **3**, 331 (1927). The present status of turbidity measurements. An article of 52 pp. treating the following

topics: (1) Solutions and dispersions, (2) optics of dispersions, and (3) instruments and standards.

KLEINMANN, H., J. Lab. Clin. Med., **12**, 629 (1927). A general discussion on nephelometry and a description of Kleinmann's macro- and micro-nephelometers.

RITTER, F. H., Biochem. Z. **192**, 337 (1928); C. A., **22**, 2699 (1928). Nephelometric studies on starch hydrosols. A contribution to the question of the nephelometric dilution law. A discussion of the deviations from Kleinmann's rule, observed with a highly dispersed starch solution. These follow a strictly formulated mathematical law.

Nephelometry, Technical Applications of.

KOBER, P. A., J. Soc. Chem. Ind., **37**, 75T (1918); C. A., **12**, 1158 (1918). Technical applications of nephelometry.

KOBER, P. A., J. Ind. Eng. Chem., **10**, 556 (1918). Technical applications of nephelometry. Same lecture reported in J. Soc. Chem. Ind., **37**, 75T (1918), but in more detail. Discusses the application of nephelometry to the determination of NH_3, P, Ca, acetone, oils and fats, and proteins. Describes several nephelometers, with figures.

Nucleases.

KOBER, P. A., J. Biol. Chem., **13**, 485 (1912–13). Nephelometry in the study of proteases and nucleases.

KOBER, P. A. and GRAVES (Miss) S. S., J. Am. Chem. Soc., **36**, 1304 (1914). Nephelometry in the study of nucleases. Use a 0.2 per cent solution of acid egg albumin as a precipitant for yeast nucleic acid and estimate the resulting suspension nephelometrically. The reagent is not appreciably affected in dilute solutions by most substances met with in physiological work and will easily detect one part of yeast nucleic acid in 1,000,000 parts of water.

Oil.

MURLIN and RICHE. Fats and Oils. Private communication to P. A. Kober. See P. A. Kober, J. Ind. Eng. Chem., **10**, 561 (1918). M. and R. pour the fat solution into 0.05 per cent gelatin solution (1 g. gelatin in 2 liters of waters with 5 cc. glacial HAc).

Oil, Essential.

WOODMAN, A. G., GOOKIN, R. T. and HEATH, L. J., J. Ind. Eng. Chem., **8**, 128 (1916). The nephelometric determination of small amounts of essential oils.

Opalescence.

HEBLER, F., Pharm. Ztg., **72**, 485 (1927). Preparation of comparative turbidities in the nephelometric determination of turbidity or opalescence via the German Pharmacopœia.

Opalescence Apparatus.

TSWETT, M., Z. physik. Chem., **36**, 450 (1901). Vorrichtung zur Beobachtung von Fluorescenz- und Opalescenzerscheinungen.

β-Oxybutyric Acid.

MARRIOTT, W. McK., J. Biol. Chem., **16**, 293 (1913). Nephelometric determination of β-oxybutyric acid in blood and tissues.

FOLIN, O. and DENIS, W., J. Biol. Chem., **18**, 263 (1914).

Particle Size, Effect of.

BECHHOLD, H. and HEBLER, F., Kolloid-Z., **31**, 70 (1922); C. A., **16**, 4104 (1922). The nephelometer effect of colloid systems of different particle sizes.

Particle Size, Relation to Intensity.

TOLMAN, R. C., GERKE, R. H., BROOKS, A. P., HERMAN, A. G., MULLIKEN, R. S., and SMYTH, H. D., J. Am. Chem. Soc., **41**, 575 (1919). Relation between intensity of Tyndall beam and size of particles.

Pepsin.

VOIGHT, K., Biochem. Z., **142**, 101 (1923). A nephelometric method for pepsin using sulfosalicylic acid.

RONA, P. and KLEINMANN, H., Klin. Wochschr., **6**, 1174 (1927); C. A., **22**, 792 (1928); *Cf.* C. A., **21**, 1285. Nephelometric methods for the determination of trypsin and pepsin in intestinal and in gastric juice. Observations on the stability of trypsin.

Peptic Digestion.

RONA, P. and KLEINMANN, H., Biochem. Z., **140**, 481 (1923). Versuche über Peptische Verdauung mittels nephelometrischer Methode. Variation der Fermentkonzentrationen bei gleicher Substrat-Konzentration.

KLEINMANN, H. and ASADA, K., Klin. Wochschr., **3**, 572 (1924). Untersuchungen über Peptische Verdauung.

Peptones.

SURMONT, H. and PROVINO (Mlle.), R., Bull. soc. chim. biol., **10**, 406 (1928); C. A., **22**, 2588 (1928). Nephelometric estimation of peptones in a solution of sodium chloride at 1 to 1000. A 30 per cent solution of CCl_3CO_2H precipitates a maximum of albuminoid substances. This precipitate is stable at the end of 20 minutes, and remains constant for 4 hours after the precipitation. The index of diffusion is proportional to the concentration of proteins only when the latter is greater than 1 in 1000. The concentration of the solution should not be greater than 3 in 1000. By taking account of these

statements, it is possible to make a gross quantative analysis of commercial samples of peptone by the nephelometric method and by aid of $(NH_4)_2SO_4$ to determine the quantity of its separate constituents.

Phosphate (see also **Phosphoric Acid** and **Phosphorus**).

FEIGL, J., Biochem. Z., **102**, 131 (1920); C. A., **14**, 2650 (1920). Presence of phosphates in human blood. X. The nephelometry of phosphoric acid in analyses which deal with quantities of the order of magnitude employed in Bang's method as a means of studying the distribution of phosphorus, especially in lecithinemia. A detailed discussion of the technique.

BLOOR, W. R., Bull. soc. chim. biol., **3**, 451 (1921); J. Chem. Soc., **122**, ii, 84 (1922). Nephelometric method for phosphate in blood. An adaptation of the Kober and Egerer method.

PINCUSSEN, L. and JULIUSBURGER, F., Biochem. Z., **177**, 140 (1926). A nephelometric determination of phosphates. "An aliquot portion of the exactly neutralized solution of the ashed material is placed in a 50-cc. flask, and in another similar flask 5 cc. of the P standard. With a pipette 5 cc. of dilute HNO_3 (1 part acid + 2 parts H_2O) is now added and the flasks are filled up to about 45 cc. with H_2O. Two cubic centimeters of the molybdic-strychnine reagent are added quickly to each flask, and the flasks are filled to the mark, well shaken and left standing thirty minutes. Then 2 cc. of a 10 per cent gum arabic solution is added, the contents of the flasks are well mixed and compared in the nephelometer any time within 2½ hours." S. Morgulis, C. A., **21**, 1285 (1927).

Phosphoric Acid (see also **Phosphate** and **Phosphorus**).

SERGER, H., Chem. Ztg., **39**, 613 (1915); C. A., **9**, 2745 (1915); J. Chem. Soc., **108**, ii, 698 (1915); J. Soc. Chem. Ind., **34**, 1027 (1915); see Z. anal. Chem., **55**, 208 (1916), for a correction of one of the factors. The nephelometric determination of small quantities of phosphoric acid. Uses NH_4 molybdate in dilute HNO_3 and strong $(NH_4)_2SO_4$. Original paper states that 1 cc. of a 0.002 N P_2O_5 solution contains 0.0000372 g. P_2O_5. The abstract in Z. anal. Chem., **55**, 208 (1916), calls attention to the error. One cubic centimeter of 0.002 N P_2O_5 contains 0.00474 mg. P_2O_5.

KLEINMANN, H., Inaugural Dissertation (Berlin, **1919**). Über die Bestimmung der Phosphorsuäre.

KLEINMANN, H., Biochem. Z., **99**, 150 (1919). Special phosphoric acid nephelometry and a new form of strychnine-molybdic acid reagent. Method is applicable for amounts from 0.1 to 0.0005 mg. P_2O_5, with an error of 0.5 per cent.

SJOLLEMA, B., J. Biol. Chem., **57**, 255 (1923). The influence of cod liver oil upon calcium and phosphorus metabolism. Determines phosphorus by Briggs' method and nephelometrically by Bloor's method, revised by Kleinmann.

KLEINMANN, H., Biochem. Z., **174**, 43 (1926). Zur Methodik der nephelometrischen Phosphorsäurebestimmung.

Phosphorus (see also **Phosphate** and **Phosphoric Acid**).

KOBER, P. A. and EGERER, G., J. Am. Chem. Soc., **37**, 2373 (1915); C. A., **9**, 2855 (1915); J. Soc. Chem. Ind., **34**, 1170 (1915); C. A., **9**, 2855 gives a correction to C. A., **9**, 2855; Chem. Zentr., **1**, 233 (1916). Nephelometric estimation of phosphorus. 0.005 mg. P in 10 cc. of solution, or 1 part of P in 2 million parts of water, is easily determined quantitatively with a nephelometer. Method is sensitive to one part in 333 million parts of water. *Cf.* Kober, J. Ind. Eng. Chem., **10**, 563 (1918).

MEIGS, E. B., J. Biol. Chem., **36**, 335 (1918); J. Soc. Chem. Ind., **38**, 58A (1919). Estimation of phosphorus by the nephelometric method. Uses the Kober and Egerer strychnine-moylbdate-reagent.

Photoelectric Cell.

REIMANN, S. P., Proc. Soc. Exptl. Biol. Med., **23**, 520 (1926); C. A., **21** 1034 (1927). The photoelectric cell as a colorimeter. Methods are described for using the photoelectric cell in colorimetric and nephelometric determinations. The advantages are extreme accuracy and simplicity.

Photometer.

LEROY, D. and TAILLANDIER, M., Compt. rend. soc. biol., **97**, 557 (1927); C. A., **22**, 100 (1928). Nephelometric micro-estimation by means of the Vernes-Bricq-Yvon photometer. The technic is given for the micro-estimation of chlorides, reducing sugars, urea and uric acid in blood, urine and cerebrospinal fluid.

Plasma.

KIYOTAKI, U., Biochem. Z., **128**, 354 (1922). Nephelometrische Studien über den Einfluss der Temperaturerhöhung auf Serum und Plasma.

Proteases.

KOBER, P. A., J. Biol. Chem., **13**, 485 (1912–13). Nephelometry in the study of proteases and nucleases.

KOBER, P. A., J. Am. Chem. Soc., **35**, 290 (1913). Nephelometry in the study of proteases, II.

Protein.

KOBER, P. A., J. Am. Chem. Soc., **35**, 1585 (1913). Nephelometric determination of proteins; casein, globulin and albumin in milk.

FOLIN, O. and DENIS, W., J. Biol. Chem., **18**, 273 (1914).

PFEIFFER, J. A. F., KOBER, P. A. and FIELD, C. W., Proc. Soc. Exptl. Biol. Med., **12**, 153 (1915); C. A., **10**, 481 (1916). Nephelometric study of the proteins of cerebrospinal fluids. I. Relation of euglobulin, total globulin and total protein to the Wassermann reaction.

DUPUY, L., Presse med., **28**, 104 (1920); Ber. ges. Physiol. exptl. Pharmakol., **2**, 568 (1920).

DENIS, W. and AEYER, F. B., Arch. Inter. Med., **26**, 436 (1920); Ber. ges. Physiol. exptl. Pharmakol., **6**, 91 (1921). Protein determination in spinal fluids.

RONA, P. and KLEINMANN, H., Biochem. Z., **140**, 461 (1923). Eine Methode zur nephelometrischen Bestimmung Kleinster Eiweissmengen.

RUSZNYÁK, S., Biochem. Z., **141**, 479 (1923). Eine mikromethode zur quantitativen Bestimmung der Eiweissfraktionen im Plasma. A nephelometric method.

RUSZNYÁK, S., Biochem. Z. **144**, 147 (1924). Bemerkungen über die Nephelometrie von Eiweisslösungen.

Protein Cleavage.

RONA, P. and KLEINMANN, H., Biochem. Z., **140**, 478 (1923). Nephelometrische Untersuchungen über fermentative Eiweissspaltung.

RONA, P. and KLEINMANN, H., Biochem. Z., **150**, 444 (1924). Nephelometrische Untersuchungen über fermentative Eiweissspaltung. II. Der Einfluss von Ionen auf die peptische Verdauung.

RONA, P. and KLEINMANN, H., Biochem. Z., **155**, 34 (1925). Nephelometrische Untersuchungen über fermentative Eiweissspaltung. III. Eine Methode zur Bestimmung der peptischen und tryptischen Verdauung von Casein.

RONA, P. and KLEINMANN, H., Biochem. Z., **159**, 146 (1925). Nephelometrische Untersuchungen über fermentative Eiweissspaltung. IV. Untersuchungen über die Kinetik der peptischen Spaltung von Serumalbumin.

RONA, P. and KLEINMANN, H., Biochem. Z., **169**, 320 (1926). Nephelometrische Untersuchungen über fermentative Eiweissspaltung, V.

KLEINMANN, H., Biochem. Z., **177**, 89 (1926). Nephelometrische Untersuchungen über fermentative Eiweissspaltung. VI. Zur Kinetik der tryptischen Spaltung.

Purine Bases.

GRAVES, (Miss) S. S. and KOBER, P. A., J. Am. Chem. Soc., **37**, 2430 (1915); J. Soc. Chem. Ind., **34**, 1114 (1915); Proc. Am. Soc. Biol. Chem. J., Biol. Chem., **20**, xx (1915).

Quinine.

RAMSDEN, W. and LIPKIN, I. J., Ann. Trop. Med., **11**, 443 (1918); J. Chem. Soc., **114**, ii, 251 (1918). Detection and estimation of quinine in blood and urine. Use $KHgI_3$.

RAMSDEN, W., LIPKIN, I. J. and WHITLEY, E., Ann. Trop. Med., **12**, 223 (1918); cf. Acton and King, Biochem. J., **15**, 53 (1921). Nephelometric estimation of traces of quinine in tissues and body fluids. Method consists essentially in matching the turbidity produced in the quinine solution of unknown strength by the addition of potassium mercuric iodide, with the turbidity produced in a series of standard solutions.

LIPKIN, I. J., Ann. Trop. Med., **13**, 149 (1919). Quinine nephelometrically. Uses $KHgI_3$.

RAMSDEN, W., Brit. Med. J., **2**, 117 (1920). Quinine nephelometrically. Uses $KHgI_3$.

ACTON, H. W. and KING, H., Biochem. J., **15**, 53 (1921); C. A., **15**, 2458 (1921). The nephelometric estimation of quinine in blood. Use $KHgI_3$ (Tauret's reagent) The sensitiveness of the method is increased by producing the turbidity in saturated $(NH_4)_2SO_4$ solutions. Modify the Ramsden and Lipkin technique. Requires three to four hours and "scrupulous care."

Scopometry.

EXTON, W. G., Arch. Path. Lab. Med., **5**, 49 (1928). "By means of a new instrument, the *scopometer*, measurements of dispersions can be easily and quickly made by several different optic methods without disturbing the sample. These include a measurement of diffuse density (turbidity), several of Tyndall beam intensity (nephelometry, polarized and unpolarized) and new extinction methods for both turbidimetry and colorimetry based on correct spectrophotometric principles. The new extinction method estimates concentration more directly and with better reproducibility than previous turbidity methods.

"Correlation of observations by the various methods available yields information about particle size and other structural characteristics of inorganic and biologic dispersions. The repetition of observations after their initiation gives data on the velocity and mechanism of reactions, and permits one to follow the transformations which take place in living processes.

"The various measurements afforded by the instrument, when suitably related, constitute a new system of analysis which it is proposed to call *scopometry*."

Serum.

KIYOTAKI, U., Biochem. Z., **128**, 354 (1922). Nephelometrische Studien über den Einfluss der Temperaturerhöhung auf Serum und Plasma.

KABELIK, J., Kolloid-Z., **37**, 274 (1925); C. A., **20**, 1822 (1926). The nephelometry of serums.

Silver (see also **Chlorine**).

MULDER, Die Silberprobiermethode, p. 23, Trans. Grimm, Leipzig, **1859**, Roughly compared opalescent AgCl suspensions obtained in the supernatant liquor, or the filtrates, in his atomic weight determinations. Sometimes used tubes to hold the suspensions but no measuring device was employed. His experiments, however, mark the first step in the development of nephelometry.

RICHARDS, T. W., Proc. Am. Acad. Arts Sci., **29**, 74 (1893). One part of AgCl may be detected in 30,000,000 parts of water, by careful comparison in strong sunlight after treatment with excess of $AgNO_3$.

RICHARDS, T. W. and WELLS, R. C., Am. Chem. J., **31**, 235 (1904).

RICHARDS, T. W. and WELLS, R. C., J. Am. Chem. Soc., **27**, 484 (1905). Studied the progressing precipitation of very dilute silver chloride solutions and found several unexpected irregularities. These observations were applied to the nephelometric analysis of very dilute silver chloride solutions. The rate of development of opalescence is decidedly variable in pure water and several hours are required for maximum opalescence to develop. By adding a considerable amount of nitric acid to the solution under comparison, the maximum opalescence is quickly reached by each suspension, and remains unchanged in relative intensity for hours.

WELLS, R. C., Am. Chem. J., **35**, 99 (1906). The estimation of opalescent silver chloride precipitates.

WELLS, R. C., Am. Chem. J., **35**, 508 (1906). Note on "The estimation of opalescent silver chloride precipitates." Wells calls attention to an incorrect conclusion in his paper under the above title in regard to time effects in nephelometry. "The time factor must be carefully regulated for intense opalescences, but it ceases to be a major variable with weak ones."

RICHARDS, T. W., Am. Chem. J., **35**, 510 (1906). Note concerning the use of the nephelometer. Makes some additional remarks on R. C. Wells paper: "The estimation of opalescent silver chloride precipitates," Am. Chem. J., **35**, 99 (1906).

RICHARDS, T. W., Orig. Com. 8th Intern. Congr. Appl. Chem., **1**, 423 (1912); C. A., **6**, 3238 (1912). Ag nephelometrically by precipitation with HCl. Error about 0.0015 mg. Ag. Conditions of precipitation must be same. For details, see Am. Chem. J., **31**, 235 (1904) and **35**, 510 (1906).

SCOTT, W. W., Standard Methods of Chemical Analysis, 4 ed., p. 463, D. Van Nostrand Co., New York, **1925**. Method is based upon the formation of a colloidal suspension of AgCl. Cf. Wells, Am. Chem. J.,

35, 99, 508; Richards, Am. Chem. J., **35**, 510; Diénert, Compt. rend. **158**, 1117.

HEIDLBERG, T. v., Biochem. Z., **192**, 238 (1928); C. A. **22**, 1557 (1928). Nephelometric determination of chloride and silver. A modification of the Richards-Wearn nephelometric procedure for determining chloride. This is precipitated with a definite amount of 0.001 N $AgNO_3$, the precipitate is centrifuged off and the excess Ag determined nephelometrically by treating the solution with H_2S.

Spectrophotometer

JABLCZYN'SKI, K. and STANKIEWICZ, W., Roczniki Chem., **7**, 534 (1927); C. A., **22**, 4079 (1928). Nephelometric analysis using a spectrophotometer.

Starch

RITTER, F. H., Biochem. Z., **192**, 337 (1928); C. A., **22**, 2699 (1928). See this reference under **Nephelometry.**

Strontium.

RICHARDS, T. W., Z. anorg. Chem., **8**, 268 (1895). New determination of the atomic weight of strontium. Uses a nephelometric method for determining the amount of strontium in connection with a new determination of its atomic weight. Made the first real nephelometer in 1894.

Sugar.

LEROY D. and TAILLANDIER, M., Compt. rend. soc. biol., **97**, 557 (1927); C. A., **22**, 100 (1928). Nephelometric micro-estimation by means of the Vernes-Bricq-Yvon photometer. The technic is given for the micro-estimation of chlorides, reducing sugars, urea and uric acid in blood, urine and cerebrospinal fluid.

Sulfate.

DENIS, W., J. Biol. Chem., **49**, 311 (1921). A nephelometric method for organic sulfates in blood or plasma is described.

LORBER, L., Biochem. Z., **163**, 476 (1925). Einfache und schnelle nephelometrische Bestimmung der verschiedenen Substanzen. Zugleich einfache Sulfatbestimmung im Horn.

HAWK, P. B. and BERGEIM, P., Practical Physiological Chemistry, 9 ed., P. Blakiston's Son & Co., Philadelphia, **1926.** Determination of inorganic sulfate in blood nephelometrically, p. 406.

Sulfur.

DENIS, W., and REED, L., J. Biol. Chem., **71**, 191 (1926); C. A., **21**, 751 (1927). Methods for the determination of some of the non-protein sulfur compounds in blood. Nephelometric micro-methods for the

determination of inorganic sulfur, total sulfates and total non-protein sulfur in blood are described. The distribution of these sulfur compounds in the blood of various animal species is given.

DENIS, W. and REED, L., J. Biol. Chem., **71**, 205 (1926); C. A., **21**, 752 (1927). Nephelometric methods for the determination of some sulfur compounds in urine. Methods similar in principle to those employed in blood analysis are described for urine analysis.

MAXWELL, L. C., BISCHOFF, F. and BLATHERWICK, N. R., J. Biol. Chem. **72**, 51 (1927). Micro-methods for the determination of labile and total sulfur in proteins. "The methods are accurate to 10 per cent for amounts of S ranging from 0.1 to 0.3 mg. *Estimation of labile S*—The sample is heated in 0.1 N Na_2CO_3 or 10 per cent NaOH in a current of N_2 for a specified time after which the solution is acidified, the liberated H_2S is swept into a NaOBr solution where it is oxidized and the S estimated as $BaSO_4$ with a nephelometer. *Estimation of total S*—A Na_2O_2 fusion is made, after which the S as $BaSO_4$ is estimated with the nephelometer." A. P. Lothrop, C. A., **21**, 1826 (1927).

Sulfuric Acid.

HINDS, J. I. D., J. Am. Chem. Soc., **22**, 271 (1900). Table for photometric determination of lime and sulfuric acid.

DIENERT, F., Compt. rend., **158**, 1117 (1914); J. Chem. Soc., **106**, ii, 486 (1914). A new nephelometer for use in analytical chemistry. Gives method for determining H_2SO_4 in water nephelometrically. $BaSO_4$ suspension formed by adding $BaCl_2$ solution to the water made acid with HCl.

Trypsin.

RONA, P. and KLEINMANN, H., Biochem. Z., **177**, 107 (1926). Nephelometric method for trypsin determination. "A modification of the authors' method which deals primarily with the mode of preparation of the reagent. If a hot saturated solution is prepared from quinidine-HCl, new solutions, being prepared by boiling the material which crystallizes out on standing, variable results are obtained with these solutions added to a Na caseinate solution. To secure reliable nephelometric results it is necessary to acidify to litmus the hot saturated quinidine solution with a few drops of diluted HCl, and to leave the solution to crystallize out at least twenty-four hours. Of the solution thus prepared, 7.5 cc. should be used instead of 5 cc. as in the original method, the final volume of the solution examined being also made up to 22.5 instead of 20 cc." S. Morgulis, C. A., **21**, 1285 (1927).

RONA, P. and KLEINMANN, H., Klin. Wochschr. **6**, 1174 (1927); C. A., **22**, 792 (1928); cf. C. A., **21**, 1285. Nephelometric methods for the

determination of trypsin and pepsin in intestinal and in gastric juice., Observations on the stability of trypsin.

Turbidity.

HEBLER, F., Pharm. Ztg., **72**, 485 (1927). Preparation of comparative turbidities in the nephelometric determination of turbidity or opalescence via the German Pharmacopœia.

WELLS, P. V., Chem. Reviews, **3**, 331 (1927). The present status of turbidity measurements. An article of fifty-two pages treating the following topics: (1) Solutions and dispersions, (2) optics of dispersions, and (3) instruments and standards.

Turbidity Curves.

HINDS, J. I. D., J. Am. Chem. Soc., **18**, 661 (1896). In connection with Hinds' photometric method for the quantitative determination of lime and sulfuric acid. Gives the "probable error" and "sources of error." Has used the method in sanitary water analysis and in the analysis of urine. Results very satisfactory. Says he sees no reason why his method cannot be successfully used with all fine white precipitates that do not settle rapidly or gather into flakes. Did not try colored precipitates.

Turbidity Standard.

BECHHOLD, H. and HEBLER, F., Kolloid-Z., **31**, 132 (1922). Ein Trübungs-Standard.

KLEINMANN, H., Biochem. Z., **137**, 150 (1923). Ein Neuer Trübungsstandard bei nephelometrischen Untersuchungen.

Tyndall Meter.

MECKLENBERG, W., Kolloid. Z., **15**, 149 (1914); ibid., **16**, 97 (1915). Tyndall meter. Attempting to derive laws for it.

Urea.

AUGUSTE, C., Compt. rend. soc. biol., **89**, 991 (1923); J. Chem. Soc., **126**, ii, 430 (1924). Nephelometric micro-determination of urea in biological fluids.

AUGUSTE, C. and S., Compt. rend. soc. biol., **93**, 639 (1925); cf. C. A., **18**, 3616. Nephelometric determination of urea applicable to biological and clinical material. "The method consists in precipitating the urea in the biological fluid (the urea concentration should be not less than 0.01 and not more than 0.1 per cent) with xanthydrol in a medium containing 66 per cent of acetic acid. After leaving the precipitate to form for an hour the concentration of the acetic acid is brought up to 90 per cent and the precipitate is compared in a nephelometer with a standard." S. M., C. A., **19**, 3506 (1925).

LEROY, D. and TAILLANDIER, M., Compt. rend. soc. biol., **97**, 557 (1927); C. A., **22**, 100 (1928). Nephelometric micro-estimation by means of the Vermes-Bricq-Yvon photometer. The technique is given for the micro-estimation of chlorides, reducing sugars, urea and uric acid in blood, urine and cerebrospinal fluid.

TAILLANDIER, M. and LEROY, D., Compt. rend. soc. biol., **97**, 706 (1927). Nephelometric estimation of urea and of uric acid by means of the Vernes-Bricq-Yvon photometer. The technique is described.

Uric Acid.

GRAVES, (Miss) S. S. and KOBER, P. A., J. Am. Chem. Soc., **37**, 2430 (1915); J. Soc. Chem. Ind., **34**, 1114 (1915); Proc. Am. Soc. Biol. Chem., J. Biol. Chem., **20**, xx (1915). The nephelometric estimation of purine bases, including uric acid, in urine and blood.

LEROY, D. and TAILLANDIER, M., Compt. rend. soc. biol., **97**, 557 (1927); C. A., **22**, 100 (1928). Nephelometric micro-estimation by means of the Vernes-Bricq-Yvon photometer. The technique is given for the micro-estimation of chlorides, reducing sugars, urea and uric acid in blood, urine and cerebrospinal fluid.

TAILLANDIER, M. and LEROY, D., Compt. rend. soc. biol., **97**, 706 (1927). Nephelometric estimation of urea and of uric acid by means of the Vernes-Bricq-Yvon photometer. The technique is described.

PART V

TABLES

ANALYSES OF CHEMICAL GLASSWARE[1]

The marks on both beakers and flasks were identical in the case of all the wares examined except Jena, in which an "N" appeared below the main body of the trade mark on the flasks but did not appear on the beakers. Therefore, with the Jena ware analyses were made of both beakers and flasks, but with the other wares the flasks were not analyzed. It is evident from the results that there is no difference in composition between the Jena beakers and flasks. Table XXIII shows the analyses of the wares tested by the Bureau of Standards.

TABLE XXIII

Ware	Kavalier beaker	M. E. G. Co. beaker	Pyrex beaker	Jena beaker	Jena flask	Nonsol beaker	Fry beaker	Libbey beaker
Al_2O_3.	0 14	1 0	2 0	4 2	4 2	2 5	2 7	2 1
Fe_2O_3.	0 08	0 35	0 25	0 25	0 27	0 23	0 22	0 44
ZnO. .		5 6		10 9	10 9	7 8	3 6	....
PbO.	.	.	.	.	. . .	. . .	.....	1 0
MnO	0 02	0 02	0 01	0 01	0 01	0 01	0.03	0.03
CaO.	8 7	0 66	0 29	0 63	0 56	0 79	2 6	0 42
MgO. .	0 17	4 3	0 06	0 21	0 25	3 4	2 6	0.08
Na_2O. .	7 1	10 8	4 4	7 5	7 8	10 9	9 8	8 2
K_2O. .	7.9	0 30	0 20	0 37	0 31	0 30	1.5	0 67
SiO_2 .	75 9	73 0	80 5	64 7	64.7	67.3	68.6	75.9
B_2O_3 .	.	3 6	11 8	10 6	10 6	6.2	8.1	10.8
P_2O_5.	0 08	. . .	.	.	. . .	.	.....	. . .
So_3. . .	0 20	0 02	. . .	. . . .	.....	.....	.....	.....
As_2O_5	Trace	0 02	0 70	0 14	0 19	Trace	0.18	0.36
Sb_2O_3 . . .	.....	0 60	.....	.....	.....	0.62	.....	.....
Total.....	100.29	100.27	100.21	99 81	99.79	100.05	99.93	100.00

Selenium and fluorine were not found, but lithium was detected spectroscopically by Paul W. Merrill in all the samples.

[1] P. H. Walker and F. W. Smither, Bur. Standards Tech. Papers No. 107 (1918).

GENERAL SUMMARY OF TESTS ON CHEMICAL GLASSWARE [1]

Table XXIV gives a general summary of the resistance to various solutions and to mechanical and heat shock of chemical glassware tested by the Bureau of Standards. In this table the numerical exponents indicate the minor differences in resistance, the lowest number being the most resistant. The absence of an exponent indicates that the differences in resistance are too small to justify any differentiation between the wares graded in the same group. In the rating of resistance to caustic alkalis boiling tests only have been considered.

TABLE XXIV

Ware	Resistance to						
	Water	Mineral acids	Carbonated alkalies	Caustic alkalies	Ammonia and ammonium salts	Heat shock	Mechanical shock
Kavalier	Poor	Good	Poor	Good[2]	Good[2]	Poor	Poor
M. E. G. Co.	Good[3]	Good	Good[1]	Good[1]	Good	Poor	Poor
Pyrex	Good[2]	Good	Good[3]	Fair	Good	Good[1]	Good *
Jena	Good[4]	Good	Good[2]	Fair	Good	Good[3]	Fair
Nonsol	Good[3]	Good	Good[1]	Fair	Good	Good[2]	Fair
Fry	Good[4]	Good	Good[2]	Fair	Good	Poor	Good
Libbey	Good[1]	Good	Good[3]	Fair	Good	Good[2]	Good

* Far superior to any of the other wares

TABLE XXV.—DATA ON THE STRENGTH OF AQUEOUS SOLUTIONS OF SOME COMMON ACIDS AND AMMONIA

Substance	sp. gr. 15.5° C.	Per Cent by Wt.	Normal	Molar
Ammonium hydroxide	0.90	28 5 NH_3	15.1	15.1
Ammonium hydroxide	0.957	10.7 NH_3	6	6
Hydrochloric acid	1.19	37.23 HCl	12	12
Hydrochloric acid	1 10	20.0 HCl	6	6
Nitric Acid	1 42	69.96 HNO_3	15.8	15.8
Nitric acid	1.20	32.2 HNO_3	6	6
Sulfuric acid	1.84	95.6 H_2SO_4	36	18
Sulfuric acid	1 19	26 0 H_2SO_4	6	3
Phosphoric acid	1 809*	93.67 H_3PO_4	5.2	1.7

* At 17.5° C.

[1] P. H. Walker and F. W. Smither, Bur. Standards Tech. Papers No. 107 (1918)

TABLE XXVI.—SOLUBILITY OF SOME GASES IN WATER, GRAMS PER LITER OF SOLVENT

When Partial Pressure of the Gas + Vapor Pressure of the Liquid = 760 mm. at the Respective Temperatures

T.	Oxygen*	Chlorine†	Carbon Dioxide‡	Hydrogen Sulfide§	Sulfur Dioxide‖
0	0.0695	. . . . .	3 347	7.066	228.3
10	.0537	9.972	2 319	5.112	162.1
20	.0434	7.293	1.689	3.846	112.9
30	.0359	5.723	1 259	2.983	78.1
40	.0308	4.590	0 974	2.361	54.1
50	.0266	3.925	0 762	1 883	.
60	.0227	3.295	0.577	1.480	.
70	.0186	2.793	. . .	1.101	. .
80	.0138	2.227	.	0.765	. .
90	.0079	1.270	. .	0 410	
100	.0000	0.000	.	0 000	. . . .

(From H. A. Fales, Inorganic Chemical Analysis, The Century Co., New York, 1925.)

* L. W. Winkler, Ber. **22,** 1772 (1889).
† L. W. Winkler, Math. és Termeszettudományi Ertesitö, **25,** 86 (1907).
‡ C. Bohr, Wied. Ann. **68,** 504 (1899).
§ L. W. Winkler, Math. és Termeszettudományi Ertesitö, **25,** 86 (1907).
‖ F. Schonfeld, Liebig's Ann. **95,** 1 (1855).

TABLE XXVII.—CONVERSION TABLE OF UNITS OF LIQUID CAPACITY

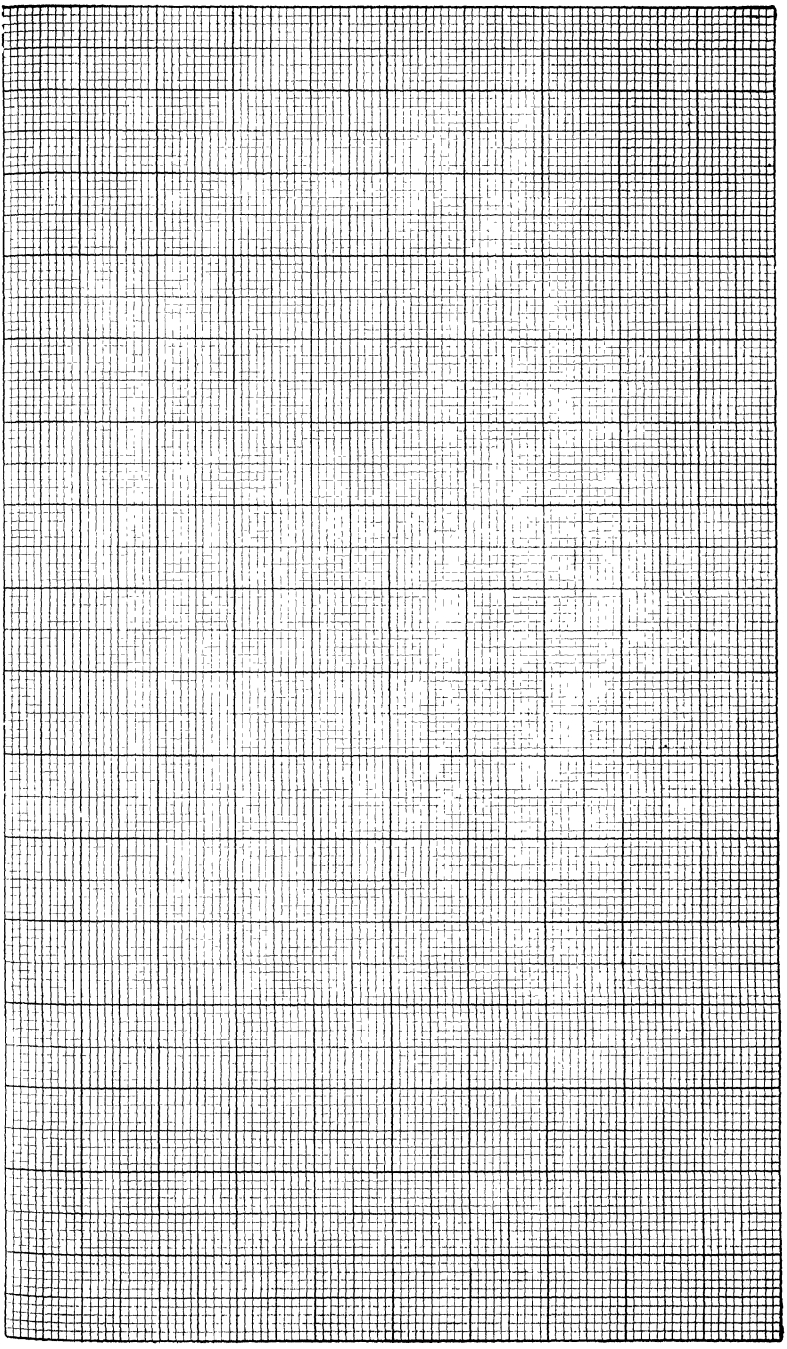
Unit	Cubic Inches	Pounds of Water At 16.7°	U. S. Gallons	Imp. Gallons	Liters	Fluid Ounces
1 U. S. Gallon =	231 00	8.335	1 000	0.83	3.785	128.0
1 Imp. Gallon =	277.27	10.000	1.200	1.00	4.543	160.0
1 Liter =	61.03	2.200	0 264	0.22	1.0	33.8
1 Fluid Ounce =	1.80	0.065	0 008	0 006	0 029	1.0

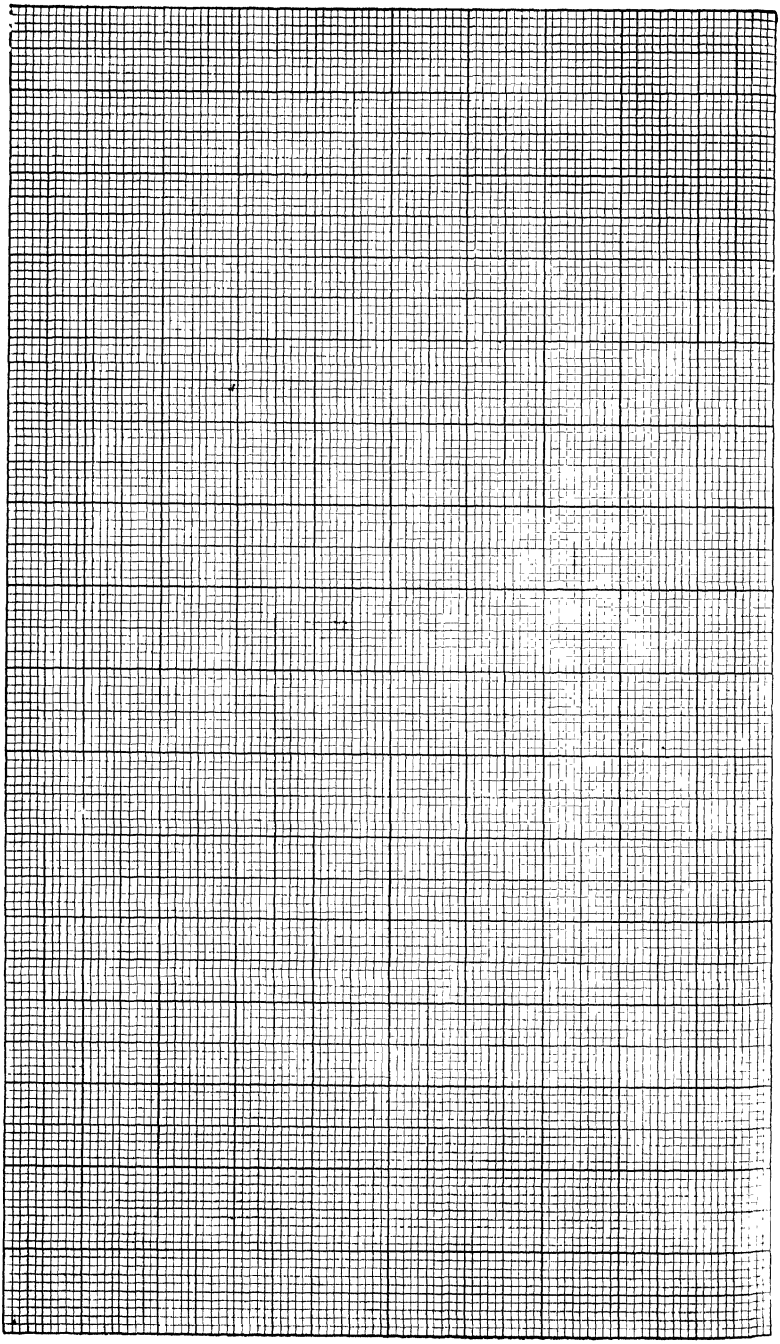
(From H. A. Fales, Inorganic Chemical Analysis, The Century Co., New York, 1925)

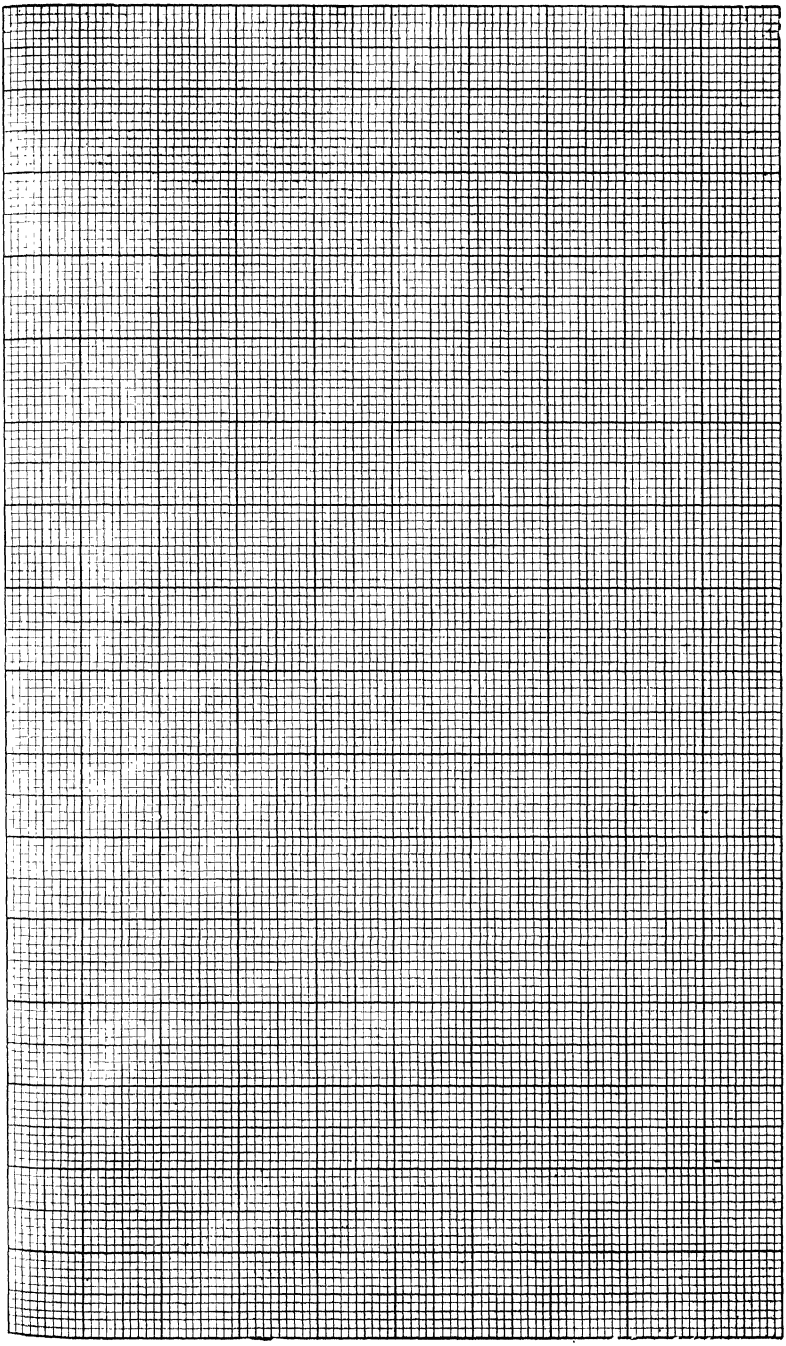
TABLE XXVIII.—INTERNATIONAL TABLE OF ATOMIC WEIGHTS OF THE CHEMICAL ELEMENTS,* 1925

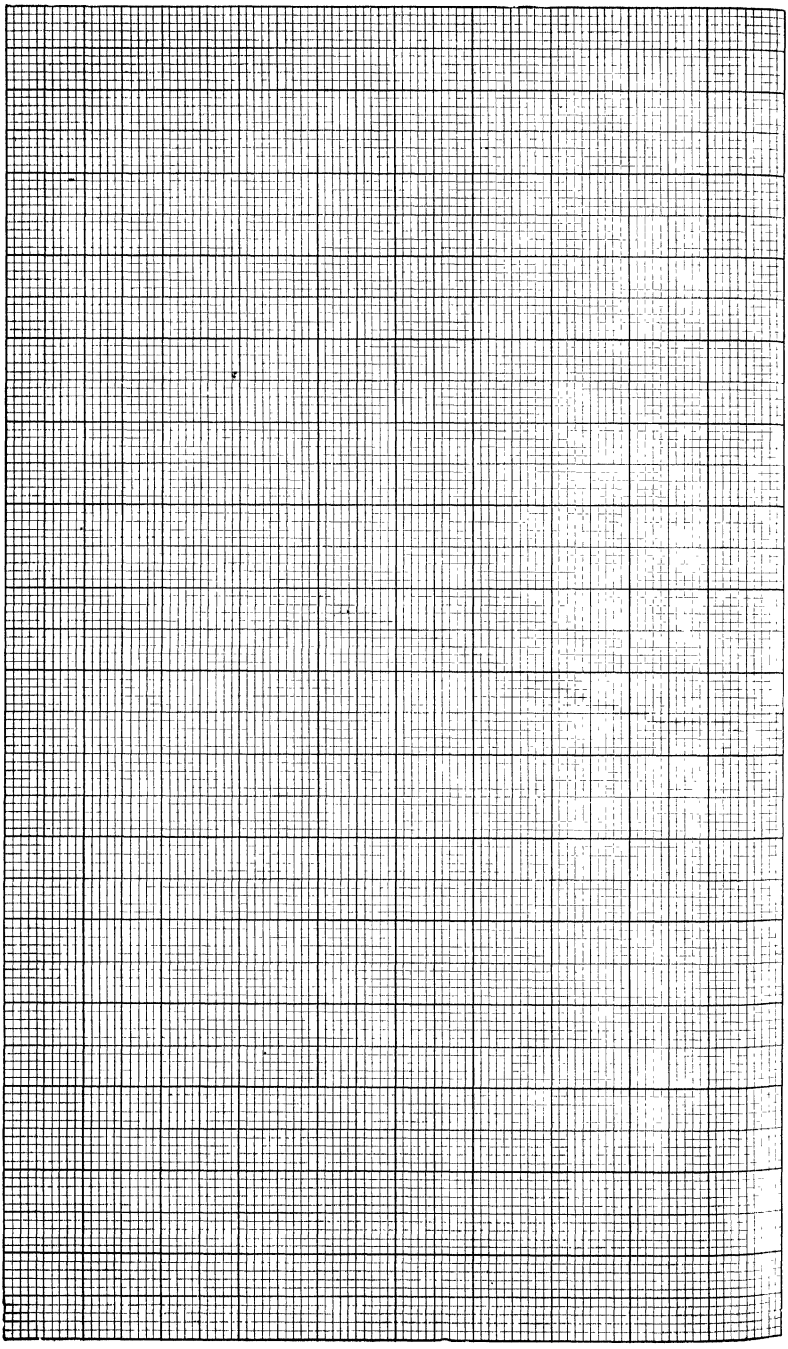
	Symbol	Atomic Number	Atomic Weight		Symbol	Atomic Number	Atomic Weight
Aluminum	Al	13	26 97	Molybdenum	Mo	42	96.0
Antimony	Sb	51	121 77	Neodymium	Nd	60	144.27
Argon	A	18	39 91	Neon	Ne	10	20 2
Arsenic	As	33	74 96	Nickel	Ni	28	58 69
Barium	Ba	56	137 37	Nitrogen	N	7	14 008
Beryllium	Be	4	9 02	Osmium	Os	76	190.8
Bismuth	Bi	83	209.00	Oxygen	O	8	16.000
Boron	B	5	10.82	Palladium	Pd	46	106 7
Bromine	Br	35	79 916	Phosphorus	P	15	31.027
Cadmium	Cd	48	112.41	Platinum	Pt	78	195.23
Calcium	Ca	20	40.07	Potassium	K	19	39.096
Carbon	C	6	12 000	Praseodymium	Pr	59	140 92
Cerium	Ce	58	140 25	Radium	Ra	88	225.95
Cesium	Cs	55	132 81	Radon	Rn	86	222.
Chlorine	Cl	17	35 457	Rhodium	Rh	45	102.91
Chromium	Cr	24	52 01	Rubidium	Rb	37	85.44
Cobalt	Co	27	58 94	Ruthenium	Ru	44	101.7
Columbium	Cb	41	93 1	Samarium	Sm	62	150.43
Copper	Cu	29	63 57	Scandium	Sc	21	45.10
Dysprosium	Dy	66	162 52	Selenium	Se	34	79.2
Erbium	Er	68	167 7	Silicon	Si	14	28.06
Europium	Eu	63	152 0	Silver	Ag	47	107.880
Fluorine	F	9	19 00	Sodium	Na	11	22.997
Gadolinium	Gd	64	157 26	Strontium	Sr	38	87 63
Gallium	Ga	31	69 72	Sulfur	S	16	32 064
Germanium	Ge	32	72 60	Tantalum	Ta	73	181 5
Gold	Au	79	197 2	Tellurium	Te	52	127.5
Helium	He	2	4 00	Terbium	Tb	65	159.2
Holmium	Ho	67	163 4	Thallium	Tl	81	204.39
Hydrogen	H	1	1.008	Thorium	Th	90	232.15
Indium	In	49	114 8	Thulium	Tm	69	169.4
Iodine	I	53	126 932	Tin	Sn	50	118.70
Iridium	Ir	77	193 1	Titanium	Ti	22	48 1
Iron	Fe	26	55 84	Tungsten	W	74	184.0
Krypton	Kr	36	82 9	Uranium	U	92	238.17
Lanthanum	La	57	138.90	Vanadium	V	23	50 96
Lead	Pb	82	207 20	Xenon	Xe	54	130.2
Lithium	Li	3	6.940	Ytterbium	Yb	70	173 6
Lutecium	Lu	71	175.0	Yttrium	Y	39	88 9
Magnesium	Mg	12	24.32	Zinc	Zn	30	65.38
Manganese	Mn	25	54 93	Zirconium	Zr	40	91.
Mercury	Hg	80	200.61				

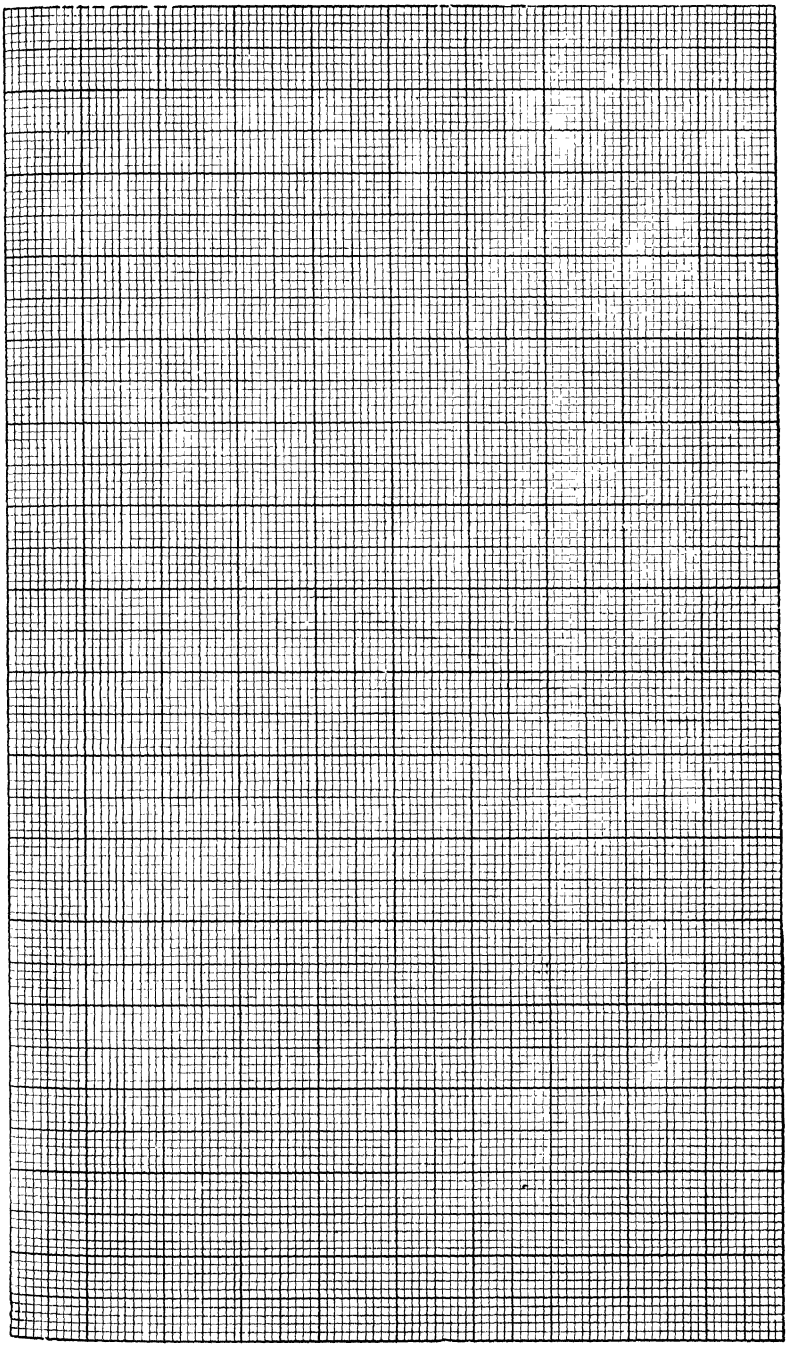
* *Note by the Author.*—The table of Atomic Weights as issued by the International Committee lists the elements alphabetically according to their symbol. In the above table the elements are arranged alphabetically according to their names. The table does not include element No. 72, Hafnium or Celtium, atomic weight 180.8. For information regarding this atomic weight, and also for more recent information regarding other atomic weights, see "Annual Report of the Committee on Atomic Weights," G. P Baxter, J. Am. Chem. Soc. **47**, 601 (1925); *ibid.* **48**, 541 (1926); *ibid.* **49**, 583 (1927); *ibid*, **50**, 603 (1928).

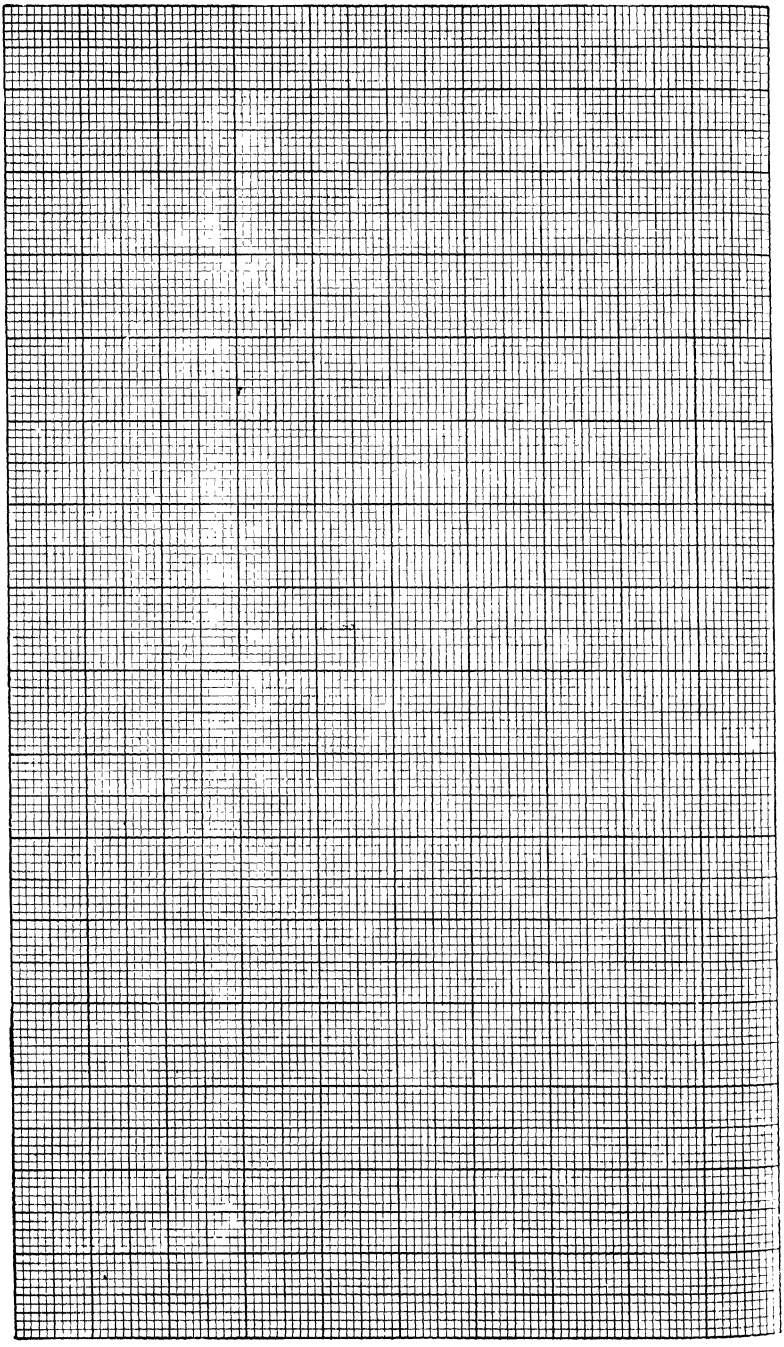


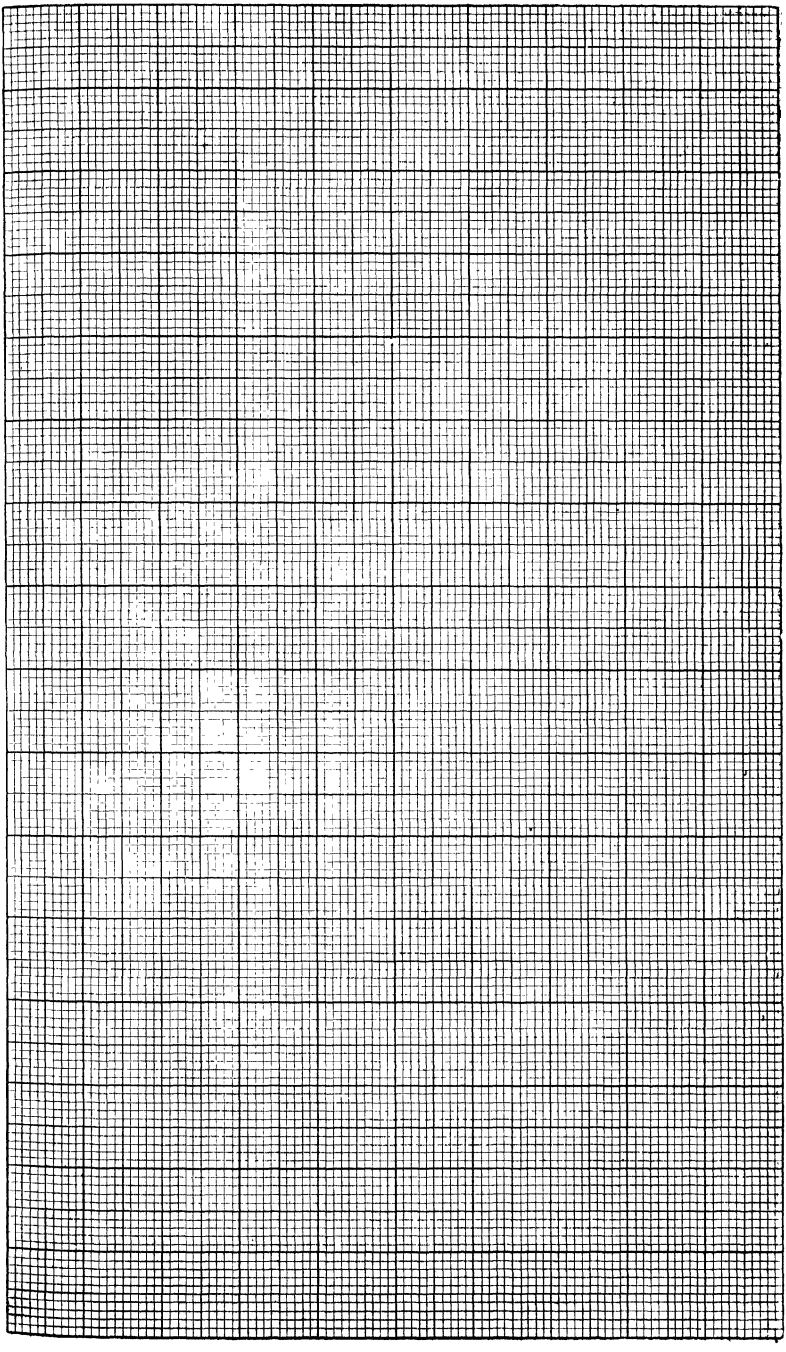












LOGARITHMS OF NUMBERS

Natural numbers.	0	1	2	3	4	5	6	7	8	9	Proportional Parts 1	2	3	4	5	6	7	8	9
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11	0414	0453	0492	0531	0569	0607	0645	0682	0719	0755	4	8	11	15	19	23	26	30	34
12	0792	0828	0864	0899	0934	0969	1004	1038	1072	1106	3	7	10	14	17	21	24	28	31
13	1139	1173	1206	1239	1271	1303	1335	1367	1399	1430	3	6	10	13	16	19	23	26	29
14	1461	1492	1523	1553	1584	1614	1644	1673	1703	1732	3	6	9	12	15	18	21	24	27
15	1761	1790	1818	1847	1875	1903	1931	1959	1987	2014	3	6	8	11	14	17	20	22	25
16	2041	2068	2095	2122	2148	2175	2201	2227	2253	2279	3	5	8	11	13	16	18	21	24
17	2304	2330	2355	2380	2405	2430	2455	2480	2504	2529	2	5	7	10	12	15	17	20	22
18	2553	2577	2601	2625	2648	2672	2695	2718	2742	2765	2	5	7	9	12	14	16	19	21
19	2788	2810	2833	2856	2878	2900	2923	2945	2967	2989	2	4	7	9	11	13	16	18	20
20	3010	3032	3054	3075	3096	3118	3139	3160	3181	3201	2	4	6	8	11	13	15	17	19
21	3222	3243	3263	3284	3304	3324	3345	3365	3385	3404	2	4	6	8	10	12	14	16	18
22	3424	3444	3464	3483	3502	3522	3541	3560	3579	3598	2	4	6	8	10	12	14	15	17
23	3617	3636	3655	3674	3692	3711	3729	3747	3766	3784	2	4	6	7	9	11	13	15	17
24	3802	3820	3838	3856	3874	3892	3909	3927	3945	3962	2	4	5	7	9	11	12	14	16
25	3979	3997	4014	4031	4048	4065	4082	4099	4116	4133	2	3	5	7	9	10	12	14	15
26	4150	4166	4183	4200	4216	4232	4249	4265	1281	4298	2	3	5	7	8	10	11	13	15
27	4314	4330	4346	4362	4378	4393	4409	4425	1440	4456	2	3	5	6	8	9	11	13	14
28	4472	4487	4502	4518	4533	4548	4564	4579	4594	4609	2	3	5	6	8	9	11	12	14
29	4624	4639	4654	4669	4683	4698	4713	4728	4742	4757	1	3	4	6	7	9	10	12	13
30	4771	4786	4800	4814	4829	4843	4857	4871	4886	4900	1	3	4	6	7	9	10	11	13
31	4914	4928	4942	4955	4969	4983	4997	5011	5024	5038	1	3	4	6	7	8	10	11	12
32	5051	5065	5079	5092	5105	5119	5132	5145	5159	5172	1	3	4	5	7	8	9	11	12
33	5185	5198	5211	5224	5237	5250	5263	5276	5289	5302	1	3	4	5	6	8	9	10	12
34	5315	5328	5340	5353	5366	5378	5391	5403	5416	5428	1	3	4	5	6	8	9	10	11
35	5441	5453	5465	5478	5490	5502	5514	5527	5539	5551	1	2	4	5	6	7	9	10	11
36	5563	5575	5587	5599	5611	5623	5635	5647	5658	5670	1	2	4	5	6	7	8	10	11
37	5682	5694	5705	5717	5729	5740	5752	5763	5775	5786	1	2	3	5	6	7	8	9	10
38	5798	5809	5821	5832	5843	5855	5866	5877	5888	5899	1	2	3	5	6	7	8	9	10
39	5911	5922	5933	5944	5955	5966	5977	5988	5999	6010	1	2	3	4	5	7	8	9	10
40	6021	6031	6042	6053	6064	6075	6085	6096	6107	6117	1	2	3	4	5	6	8	9	10
41	6128	6138	6149	6160	6170	6180	6191	6201	6212	6222	1	2	3	4	5	6	7	8	9
42	6232	6243	6253	6263	6274	6284	6294	6304	6314	6325	1	2	3	4	5	6	7	8	9
43	6335	6345	6355	6365	6375	6385	6395	6405	6415	6425	1	2	3	4	5	6	7	8	9
44	6435	6444	6454	6464	6474	6484	6493	6503	6513	6522	1	2	3	4	5	6	7	8	9
45	6532	6542	6551	6561	6571	6580	6590	6599	6609	6618	1	2	3	4	5	6	7	8	9
46	6628	6637	6646	6656	6665	6675	6684	6693	6702	6712	1	2	3	4	5	6	7	7	8
47	6721	6730	6739	6749	6758	6767	6776	6785	6794	6803	1	2	3	4	5	5	6	7	8
48	6812	6821	6830	6839	6848	6857	6866	6875	6884	6893	1	2	3	4	4	5	6	7	8
49	6902	6911	6920	6928	6937	6946	6955	6964	6972	6981	1	2	3	4	4	5	6	7	8
50	6990	6998	7007	7016	7024	7033	7042	7050	7059	7067	1	2	3	3	4	5	6	7	8
51	7076	7084	7093	7101	7110	7118	7126	7135	7143	7152	1	2	3	3	4	5	6	7	8
52	7160	7168	7177	7185	7193	7202	7210	7218	7226	7235	1	2	2	3	4	5	6	7	7
53	7243	7251	7259	7267	7275	7284	7292	7300	7308	7316	1	2	2	3	4	5	6	6	7
54	7324	7332	7340	7348	7356	7364	7372	7380	7388	7396	1	2	2	3	4	5	6	6	7

LOGARITHMS OF NUMBERS (*Continued*)

Natural numbers.	0	1	2	3	4	5	6	7	8	9	Proportional Parts.								
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55	7404	7412	7419	7427	7435	7443	7451	7459	7466	7474	1	2	2	3	4	5	5	6	7
56	7482	7490	7497	7505	7513	7520	7528	7536	7543	7551	1	2	2	3	4	5	5	6	7
57	7559	7566	7574	7582	7589	7597	7604	7612	7619	7627	1	2	2	3	4	5	5	6	7
58	7634	7642	7649	7657	7664	7672	7679	7686	7694	7701	1	1	2	3	4	4	5	6	7
59	7709	7716	7723	7731	7738	7745	7752	7760	7767	7774	1	1	2	3	4	4	5	6	7
60	7782	7789	7796	7803	7810	7818	7825	7832	7839	7846	1	1	2	3	4	4	5	6	6
61	7853	7860	7868	7875	7882	7889	7896	7903	7910	7917	1	1	2	3	4	4	5	6	6
62	7924	7931	7938	7945	7952	7959	7966	7973	7980	7987	1	1	2	3	3	4	5	6	6
63	7993	8000	8007	8014	8021	8028	8035	8041	8048	8055	1	1	2	3	3	4	5	5	6
64	8062	8069	8075	8082	8089	8096	8102	8109	8116	8122	1	1	2	3	3	4	5	5	6
65	8129	8136	8142	8149	8156	8162	8169	8176	8182	8189	1	1	2	3	3	4	5	5	6
66	8195	8202	8209	8215	8222	8228	8235	8241	8248	8254	1	1	2	3	3	4	5	5	6
67	8261	8267	8274	8280	8287	8293	8299	8306	8312	8319	1	1	2	3	3	4	5	5	6
68	8325	8331	8338	8344	8351	8357	8363	8370	8376	8382	1	1	2	3	3	4	4	5	6
69	8388	8395	8401	8407	8414	8420	8426	8432	8439	8445	1	1	2	2	3	4	4	5	6
70	8451	8457	8463	8470	8476	8482	8488	8494	8500	8506	1	1	2	2	3	4	4	5	6
71	8513	8519	8525	8531	8537	8543	8549	8555	8561	8567	1	1	2	2	3	4	4	5	5
72	8573	8579	8585	8591	8597	8603	8609	8615	8621	8627	1	1	2	2	3	4	4	5	5
73	8633	8639	8645	8651	8657	8663	8669	8675	8681	8686	1	1	2	2	3	4	4	5	5
74	8692	8698	8704	8710	8716	8722	8727	8733	8739	8745	1	1	2	2	3	4	4	5	5
75	8751	8756	8762	8768	8774	8779	8785	8791	8797	8802	1	1	2	2	3	3	4	5	5
76	8808	8814	8820	8825	8831	8837	8842	8848	8854	8859	1	1	2	2	3	3	4	5	5
77	8865	8871	8876	8882	8887	8893	8899	8904	8910	8915	1	1	2	2	3	3	4	4	5
78	8921	8927	8932	8938	8943	8949	8954	8960	8965	8971	1	1	2	2	3	3	4	4	5
79	8976	8982	8987	8993	8998	9004	9009	9015	9020	9026	1	1	2	2	3	3	4	4	5
80	9031	9036	9042	9047	9053	9058	9063	9069	9074	9079	1	1	2	2	3	3	4	4	5
81	9085	9090	9096	9101	9106	9112	9117	9122	9128	9133	1	1	2	2	3	3	4	4	5
82	9138	9143	9149	9154	9159	9165	9170	9175	9180	9186	1	1	2	2	3	3	4	4	5
83	9191	9196	9201	9206	9212	9217	9222	9227	9232	9238	1	1	2	2	3	3	4	4	5
84	9243	9248	9253	9258	9263	9269	9274	9279	9284	9289	1	1	2	2	3	3	4	4	5
85	9294	9299	9304	9309	9315	9320	9325	9330	9335	9340	1	1	2	2	3	3	4	4	5
86	9345	9350	9355	9360	9365	9370	9375	9380	9385	9390	1	1	2	2	3	3	4	4	5
87	9395	9400	9405	9410	9415	9420	9425	9430	9435	9440	0	1	1	2	2	3	3	4	4
88	9445	9450	9455	9460	9465	9469	9474	9479	9484	9489	0	1	1	2	2	3	3	4	4
89	9494	9499	9504	9509	9513	9518	9523	9528	9533	9538	0	1	1	2	2	3	3	4	4
90	9542	9547	9552	9557	9562	9566	9571	9576	9581	9586	0	1	1	2	2	3	3	4	4
91	9590	9595	9600	9605	9609	9614	9619	9624	9628	9633	0	1	1	2	2	3	3	4	4
92	9638	9643	9647	9652	9657	9661	9666	9671	9675	9680	0	1	1	2	2	3	3	4	4
93	9685	9689	9694	9699	9703	9708	9713	9717	9722	9727	0	1	1	2	2	3	3	4	4
94	9731	9736	9741	9745	9750	9754	9759	9763	9768	9773	0	1	1	2	2	3	3	4	4
95	9777	9782	9786	9791	9795	9800	9805	9809	9814	9818	0	1	1	2	2	3	3	4	4
96	9823	9827	9832	9836	9841	9845	9850	9854	9859	9863	0	1	1	2	2	3	3	4	4
97	9868	9872	9877	9881	9886	9890	9894	9899	9903	9908	0	1	1	2	2	3	3	4	4
98	9912	9917	9921	9926	9930	9934	9939	9943	9948	9952	0	1	1	2	2	3	3	4	4
99	9956	9961	9965	9969	9974	9978	9983	9987	9991	9996	0	1	1	2	2	3	3	3	4

ANTILOGARITHMS

Logarithms.	0	1	2	3	4	5	6	7	8	9	Proportional Parts.								
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.01	1023	1026	1028	1030	1033	1035	1038	1040	1042	1045	0	0	1	1	1	1	2	2	2
.02	1047	1050	1052	1054	1057	1059	1062	1064	1067	1069	0	0	1	1	1	1	2	2	2
.03	1072	1074	1076	1079	1081	1084	1086	1089	1091	1094	0	0	1	1	1	1	2	2	2
.04	1096	1099	1102	1104	1107	1109	1112	1114	1117	1119	0	1	1	1	1	2	2	2	2
.05	1122	1125	1127	1130	1132	1135	1138	1140	1143	1146	0	1	1	1	1	2	2	2	2
.06	1148	1151	1153	1156	1159	1161	1164	1167	1169	1172	0	1	1	1	1	2	2	2	2
.07	1175	1178	1180	1183	1186	1189	1191	1194	1197	1199	0	1	1	1	1	2	2	2	2
.08	1202	1205	1208	1211	1213	1216	1219	1222	1225	1227	0	1	1	1	1	2	2	2	3
.09	1230	1233	1236	1239	1242	1245	1247	1250	1253	1256	0	1	1	1	1	2	2	2	3
.10	1259	1262	1265	1268	1271	1274	1276	1279	1282	1285	0	1	1	1	1	2	2	2	3
.11	1288	1291	1294	1297	1300	1303	1306	1309	1312	1315	0	1	1	1	2	2	2	2	3
.12	1318	1321	1324	1327	1330	1334	1337	1340	1343	1346	0	1	1	1	2	2	2	2	3
.13	1349	1352	1355	1358	1361	1365	1368	1371	1374	1377	0	1	1	1	2	2	2	3	3
.14	1380	1384	1387	1390	1393	1396	1400	1403	1406	1409	0	1	1	1	2	2	2	3	3
.15	1413	1416	1419	1422	1426	1429	1432	1435	1439	1442	0	1	1	1	2	2	2	3	3
.16	1445	1449	1452	1455	1459	1462	1466	1469	1472	1476	0	1	1	1	2	2	2	3	3
.17	1479	1483	1486	1489	1493	1496	1500	1503	1507	1510	0	1	1	1	2	2	2	3	3
.18	1514	1517	1521	1524	1528	1531	1535	1538	1542	1545	0	1	1	1	2	2	2	3	3
.19	1549	1552	1556	1560	1563	1567	1570	1574	1578	1581	0	1	1	1	2	2	3	3	3
.20	1585	1589	1592	1596	1600	1603	1607	1611	1614	1618	0	1	1	1	2	2	3	3	3
.21	1622	1626	1629	1633	1637	1641	1644	1648	1652	1656	0	1	1	2	2	2	3	3	3
.22	1660	1663	1667	1671	1675	1679	1683	1687	1690	1694	0	1	1	2	2	2	3	3	3
.23	1698	1702	1706	1710	1714	1718	1722	1726	1730	1734	0	1	1	2	2	2	3	3	4
.24	1738	1742	1746	1750	1754	1758	1762	1766	1770	1774	0	1	1	2	2	2	3	3	4
.25	1778	1782	1786	1791	1795	1799	1803	1807	1811	1816	0	1	1	2	2	2	3	3	4
.26	1820	1824	1828	1832	1837	1841	1845	1849	1854	1858	0	1	1	2	2	3	3	3	4
.27	1862	1866	1871	1875	1879	1884	1888	1892	1897	1901	0	1	1	2	2	3	3	3	4
.28	1905	1910	1914	1919	1923	1928	1932	1936	1941	1945	0	1	1	2	2	3	3	4	4
.29	1950	1954	1959	1963	1968	1972	1977	1982	1986	1991	0	1	1	2	2	3	3	4	4
.30	1995	2000	2004	2009	2014	2018	2023	2028	2032	2037	0	1	1	2	2	3	3	4	4
.31	2042	2046	2051	2056	2061	2065	2070	2075	2080	2084	0	1	1	2	2	3	3	4	4
.32	2089	2094	2099	2104	2109	2113	2118	2123	2128	2133	0	1	1	2	2	3	3	4	4
.33	2138	2143	2148	2153	2158	2163	2168	2173	2178	2183	0	1	1	2	2	3	3	4	4
.34	2188	2193	2198	2203	2208	2213	2218	2223	2228	2234	1	1	2	2	3	3	4	4	5
.35	2239	2244	2249	2254	2259	2265	2270	2275	2280	2286	1	1	2	2	3	3	4	4	5
.36	2291	2296	2301	2307	2312	2317	2323	2328	2333	2339	1	1	2	2	3	3	4	4	5
.37	2344	2350	2355	2360	2366	2371	2377	2382	2388	2393	1	1	2	2	3	3	4	4	5
.38	2399	2404	2410	2415	2421	2427	2432	2438	2443	2449	1	1	2	2	3	3	4	4	5
.39	2455	2460	2466	2472	2477	2483	2489	2495	2500	2506	1	1	2	2	3	3	4	5	5
.40	2512	2518	2523	2529	2535	2541	2547	2553	2559	2564	1	1	2	2	3	4	4	5	5
.41	2570	2576	2582	2588	2594	2600	2606	2612	2618	2624	1	1	2	2	3	4	4	5	5
.42	2630	2636	2642	2649	2655	2661	2667	2673	2679	2685	1	1	2	2	3	4	4	5	6
.43	2692	2698	2704	2710	2716	2723	2729	2735	2742	2748	1	1	2	3	3	4	4	5	6
.44	2754	2761	2767	2773	2780	2786	2793	2799	2805	2812	1	1	2	3	3	4	4	5	6
.45	2818	2825	2831	2838	2844	2851	2858	2864	2871	2877	1	1	2	3	3	4	5	5	6
.46	2884	2891	2897	2904	2911	2917	2924	2931	2938	2944	1	1	2	3	3	4	5	5	6
.47	2951	2958	2965	2972	2979	2985	2992	2999	3006	3013	1	1	2	3	3	4	5	5	6
.48	3020	3027	3034	3041	3048	3055	3062	3069	3076	3083	1	1	2	3	4	4	5	6	6
.49	3090	3097	3105	3112	3119	3126	3133	3141	3148	3155	1	1	2	3	4	4	5	6	6

ANTILOGARITHMS (*Continued*)

Logarithms.	0	1	2	3	4	5	6	7	8	9	Proportional Parts.								
											1	2	3	4	5	6	7	8	9
.50	3162	3170	3177	3184	3192	3199	3206	3214	3221	3228	1	1	2	3	4	4	5	6	7
.51	3236	3243	3251	3258	3266	3273	3281	3289	3296	3304	1	2	2	3	4	5	5	6	7
.52	3311	3319	3327	3334	3342	3350	3357	3365	3373	3381	1	2	2	3	4	5	5	6	7
.53	3388	3396	3404	3412	3420	3428	3436	3443	3451	3459	1	2	2	3	4	5	6	6	7
.54	3467	3475	3483	3491	3499	3508	3516	3524	3532	3540	1	2	2	3	4	5	6	6	7
.55	3548	3556	3565	3573	3581	3589	3597	3606	3614	3622	1	2	2	3	4	5	6	7	7
.56	3631	3639	3648	3656	3664	3673	3681	3690	3698	3707	1	2	3	3	4	5	6	7	8
.57	3715	3724	3733	3741	3750	3758	3767	3776	3784	3793	1	2	3	3	4	5	6	7	8
.58	3802	3811	3819	3828	3837	3846	3855	3864	3873	3882	1	2	3	4	4	5	6	7	8
.59	3890	3899	3908	3917	3926	3936	3945	3954	3963	3972	1	2	3	4	5	5	6	7	8
.60	3981	3990	3999	4009	4018	4027	4036	4046	4055	4064	1	2	3	4	5	6	6	7	8
.61	4074	4083	4093	4102	4111	4121	4130	4140	4150	4159	1	2	3	4	5	6	7	8	9
.62	4169	4178	4188	4198	4207	4217	4227	4236	4246	4256	1	2	3	4	5	6	7	8	9
.63	4266	4276	4285	4295	4305	4315	4325	4335	4345	4355	1	2	3	4	5	6	7	8	9
.64	4365	4375	4385	4395	4406	4416	4426	4436	4446	4457	1	2	3	4	5	6	7	8	9
.65	4467	4477	4487	4498	4508	4519	4529	4539	4550	4560	1	2	3	4	5	6	7	8	9
.66	4571	4581	4592	4603	4613	4624	4634	4645	4656	4667	1	2	3	4	5	6	7	9	10
.67	4677	4688	4699	4710	4721	4732	4742	4753	4764	4775	1	2	3	4	5	7	8	9	10
.68	4786	4797	4808	4819	4831	4842	4853	4864	4875	4887	1	2	3	4	6	7	8	9	10
.69	4898	4909	4920	4932	4943	4955	4966	4977	4989	5000	1	2	3	5	6	7	8	9	10
.70	5012	5023	5035	5047	5058	5070	5082	5093	5105	5117	1	2	4	5	6	7	8	9	11
.71	5129	5140	5152	5164	5176	5188	5200	5212	5224	5236	1	2	4	5	6	7	8	10	11
.72	5248	5260	5272	5284	5297	5309	5321	5333	5346	5358	1	2	4	5	6	7	9	10	11
.73	5370	5383	5395	5408	5420	5433	5445	5458	5470	5483	1	3	4	5	6	8	9	10	11
.74	5495	5508	5521	5534	5546	5559	5572	5585	5598	5610	1	3	4	5	6	8	9	10	12
.75	5623	5636	5649	5662	5675	5689	5702	5715	5728	5741	1	3	4	5	7	8	9	10	12
.76	5754	5768	5781	5794	5808	5821	5834	5848	5861	5875	1	3	4	5	7	8	9	11	12
.77	5888	5902	5916	5929	5943	5957	5970	5984	5998	6012	1	3	4	5	7	8	10	11	12
.78	6026	6039	6053	6067	6081	6095	6109	6124	6138	6152	1	3	4	6	7	8	10	11	13
.79	6166	6180	6194	6209	6223	6237	6252	6266	6281	6295	1	3	4	6	7	9	10	11	13
.80	6310	6324	6339	6353	6368	6383	6397	6412	6427	6442	1	3	4	6	7	9	10	12	13
.81	6457	6471	6486	6501	6516	6531	6546	6561	6577	6592	2	3	5	6	8	9	11	12	14
.82	6607	6622	6637	6653	6668	6683	6699	6714	6730	6745	2	3	5	6	8	9	11	12	14
.83	6761	6776	6792	6808	6823	6839	6855	6871	6887	6902	2	3	5	6	8	9	11	13	14
.84	6918	6934	6950	6966	6982	6998	7015	7031	7047	7063	2	3	5	6	8	10	11	13	15
.85	7079	7096	7112	7129	7145	7161	7178	7194	7211	7228	2	3	5	7	8	10	12	13	15
.86	7244	7261	7278	7295	7311	7328	7345	7362	7379	7396	2	3	5	7	8	10	12	13	15
.87	7413	7430	7447	7464	7482	7499	7516	7534	7551	7568	2	3	5	7	9	10	12	14	16
.88	7586	7603	7621	7638	7656	7674	7691	7709	7727	7745	2	4	5	7	9	11	12	14	16
.89	7762	7780	7798	7816	7834	7852	7870	7889	7907	7925	2	4	5	7	9	11	13	14	16
.90	7943	7962	7980	7998	8017	8035	8054	8072	8091	8110	2	4	6	7	9	11	13	15	17
.91	8128	8147	8166	8185	8204	8222	8241	8260	8279	8299	2	4	6	8	9	11	13	15	17
.92	8318	8337	8356	8375	8395	8414	8433	8453	8472	8492	2	4	6	8	10	12	14	15	17
.93	8511	8531	8551	8570	8590	8610	8630	8650	8670	8690	2	4	6	8	10	12	14	16	18
.94	8710	8730	8750	8770	8790	8810	8831	8851	8872	8892	2	4	6	8	10	12	14	16	18
.95	8913	8933	8954	8974	8995	9016	9036	9057	9078	9099	2	4	6	8	10	12	15	17	19
.96	9120	9141	9162	9183	9204	9226	9247	9268	9290	9311	2	4	6	8	11	13	15	17	19
.97	9333	9354	9376	9397	9419	9441	9462	9484	9506	9528	2	4	7	9	11	13	15	17	20
.98	9550	9572	9594	9616	9638	9661	9683	9705	9727	9750	2	4	7	9	11	13	16	18	20
.99	9772	9795	9817	9840	9863	9886	9908	9931	9954	9977	2	5	7	9	11	14	16	18	20

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