A NEW FORM OF NEPHELOMETER.

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The nephelometer (Gr. νεφέλη, a cloud), an instrument for the quantitative determination of small amounts of material in suspension, has attracted considerable attention of late, although the principles involved are by no means new. Since the time of Gay-Lussac attempts have been made to estimate small quantities of material by the turbidity or opalescence of their suspensions. This was generally done by comparing the suspension with a graded series of known suspensions prepared in the same way, and the comparison was made by looking through a column of the liquid and noting the turbidity, or by observing the opalescence, that is, the light reflected from the minute particles when the liquid is illuminated by a powerful beam of light. It is evident that matter in smaller quantities or in a finer state of subdivision may be recognized more easily by the opalescence than by the turbidity of its suspension. That even excessively minute particles possess the ability to diffract light has been shown by the ultramicroscope, while by the Faraday-Tyndall convergent beam of light, the optical in-homogeneity of solutions of crystalloids has been detected.

T. W. Richards in the course of atomic weight determinations in 1894[1] devised a simple instrument to enable the opalescence of very dilute suspensions of silver bromide to be more readily observed, and in a measure, quantitatively determined. Ten years later, Richards and Wells[2] improved the instrument optically and suggested its applicability to suspensions of substances other than the silver halides. Their actual determinations, however, seem to have been arrived at by a process of approximation; that is, the unknown was compared in the instrument to a suspension of known concentration, and from these readings a first approximation of its strength was calculated. A new standard of more nearly the same concentration as the unknown was then prepared

[1] *Proc. Am. Acad.*, XXX., 369, 1894.

[2] Richards and Wells, *Am. Chem. Jour.*, XXXI., 235, 1904.

and comparison again made. This process was repeated until a standard was obtained which when precipitated under the same conditions and compared in the instrument with the unknown gave the same amount of opalescence. The postulate involved, that the same quantities of material precipitated under identical conditions give equal opalescences, is undoubtedly correct, but the method is somewhat tedious in application, although good accuracy was obtained in about three approximations.

Wells in 1906[3] published the results of numerous experiments in which silver chloride was precipitated under different conditions, showing the influence of electrolytes both on the maximum opalescence developed and on the time required for this maximum to be reached. He came to the natural conclusion that the amount of light reflected varies not only with the quantity of material in suspension but also with its state of subdivision. In this investigation he used the Richards instrument of 1904 except that for the usual standard suspension he substituted fixed standards of ground glass as reflecting surfaces.

P. A. Kober[4] in 1913 took up the problem of determining quantitatively by the use of the nephelometric method, proteins and other substances occurring in biochemical investigations for which the ordinary gravimetric methods are either very tedious or inadequate. He used an instrument on the principle of the Richards nephelometer but adapted to the framework and optical parts of the Duboscq colorimeter. In comparing the opalescences of suspensions differing considerably in concentration, he observed that the readings were not quite inversely proportional to the concentration of matter in suspension, and from a large number of experiments with suspensions of different substances he developed an empirical formula expressing the relation between scale readings and concentration. This formula holds very well for ratios up to 1 : 3. He has successfully applied his instrument and method to the determination of a number of organic substances such as casein in milk, uric acid, and other purines. The nephelometer in various modifications has been used by W. R. Bloor to determine the fat

[3] Wells, *Am. Chem. Jour.*, XXXV., 99, 1906.

[4] P. A. Kober, *Jour. Biol. Chem.*, XIII., 485, 1913.

in blood, by McKim Marriot for acetone, and by S. S. Graves in ammonia determinations.

A number of instruments and methods have been devised for determining the amount of substance in suspension by the turbidity of its solution and these find considerable use in industrial chemistry. While the theory underlying this method is undoubtedly simpler than the nephelometric theory, it may easily be seen from the following considerations that the turbidimeter cannot equal the nephelometer in delicacy or sensitivity. Let us suppose that a standard as used in the turbidimeter absorbs about 10 per cent. of the light, then an unknown of twice the concentration will absorb about twice that quantity. However, it is not the amount of light absorbed, but the amount transmitted that is observed in this instrument; consequently the quantities measured would be in the ratio of about 9:8. The reflected lights measured in the nephelometer on the other hand would be nearly in the ratio of 1:2. Clouds which may be measured with considerable accuracy in the nephelometer show very slight absorption when observed by transmitted light in the turbidimeter.

Our reason for devising a new nephelometer may be made more apparent by a brief review of some of the considerations involved in the use of such instruments. The following are the chief factors involved in the amount of light reflected by an opalescent solution. First, the amount of substance in suspension. Second, its physical state, *i. e.*, the number and size of the particles, and their albedo which depends upon their own refractive index and that of the medium in which they are suspended. The amount of light observed is again modified by the fact that the light from any particle is reduced by an amount dependent upon the absorbing power of that part of the liquid above the particle. Thus we receive less light from the bottom layers of the suspension than from those nearer the top. This complex relation between reflection and absorption demands less consideration when the lengths of the illuminated columns are kept equal than when they are varied. As far as we are aware, in the nephelometers hitherto described the light from the two tubes has been equalized by changing the lengths of the illuminated columns of suspension. Although in purely

empirical work the elimination of this factor is not of very great importance, the theoretical consideration of the problem is greatly simplified thereby.

As Wells states, the opalescence of a liquid containing a definite amount of substance in suspension will, owing to the greater total reflecting surface, increase with the continued subdivision of the particles until these reach a limiting size. Rayleigh has pointed out this fact in a mathematical dissertation on the blue color of the sky, stating that as the particles approach the size of a wave length of light their reflecting power decreases. He shows that for very minute particles the amount of light reflected should vary as the sixth power of their radius. The maximum opalescence of the solutions as used in a nephelometer seems, however, to be developed when the particles are much smaller than a wave length of light—in fact of ultramicroscopic size.

The amount of reflected light lost through absorption is also a function of the number and size of the particles.

It is evident that as the refractive index of the medium approaches that of the particles, the amount of light reflected will decrease until, when the two refractive indices become equal, there will be no reflection. This phenomenon may be observed if powdered glass be suspended in a mixture of carbon disulphide and benzol.

With a view to determining some of the underlying laws of opalescent solutions, we undertook to design a nephelometer better adapted both to theoretical and to practical work than those in use at present. By using equal columns of suspension and actually measuring the reflected lights with a suitable photometer, not only is one of the variables eliminated, but also we are enabled to determine the absolute ratio of the lights reflected by various suspensions. The photometric part of the apparatus consists of a wedge of neutral tinted glass by which the light from one of the suspensions may be controlled; and a suitable optical arrangement for observing the two beams of light. A Lummer-Brodhun prism would serve this purpose admirably, but by a simple arrangement of mirrors, a field far more sensitive than that of the Duboscq colorimeter may be obtained.

Briefly the design of the instrument is as follows: The suspensions to be compared are contained in the two cells *A* and *B* shown in the accompanying diagram (Figs. 1 and 2). These consist of cylindrical glass tubes about 4 cm. high and 1 cm. in diameter. A glass plate is sealed into one end, while the other end is covered by a circular plate of glass slightly countersunk and held in place by caps of black fiber. These prevent stray light reflected from the edges

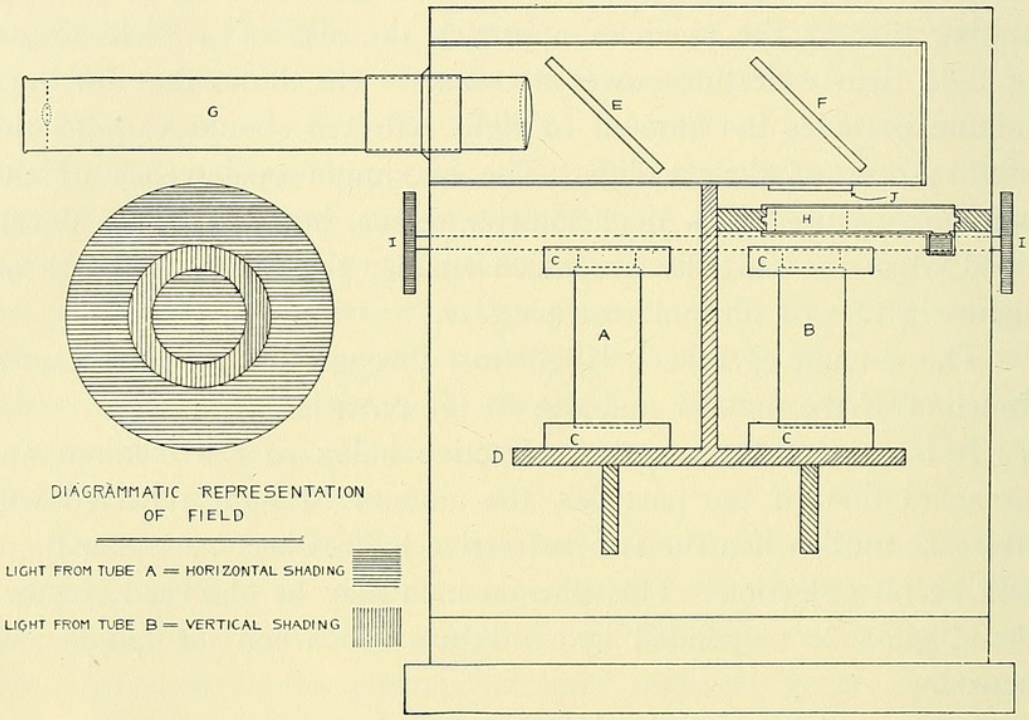

FIG. 1.

of the glass from entering the instrument. Difficulties arising from the agitation of the liquid by plungers are also thus avoided by having the cells completely enclosed. The cells rest on a shelf and are illuminated normal to their axes by a parallel beam of light from a 100 Watt lamp. The rays reflected from the suspended particles pass upward to the two mirrors *E* and *F* whence they are reflected into the magnifying eyepiece *G*. This is focused on mirror *E*. A circle cut through the silvering of mirror *E* permits the juxtaposition of the light from tubes *A* and *B* thus giving the eyepiece a field which is represented diagrammatically in the accompanying illustration. Photometric balance is effected by changing the intensity of the light from tube *B* by means of the sliding wedge of

neutral tinted glass *H*. This adjustment is made by the thumb-screw *I* and the position of the wedge is read on a scale mounted alongside (not shown in the diagram). A compensating wedge wedge may be placed at *J*, but unless the sliding wedge *H* is of fairly steep pitch, this is unnecessary, as the illumination of the field is sufficiently uniform without it. All parts of the instrument from which extraneous light may be reflected are painted a dead black.

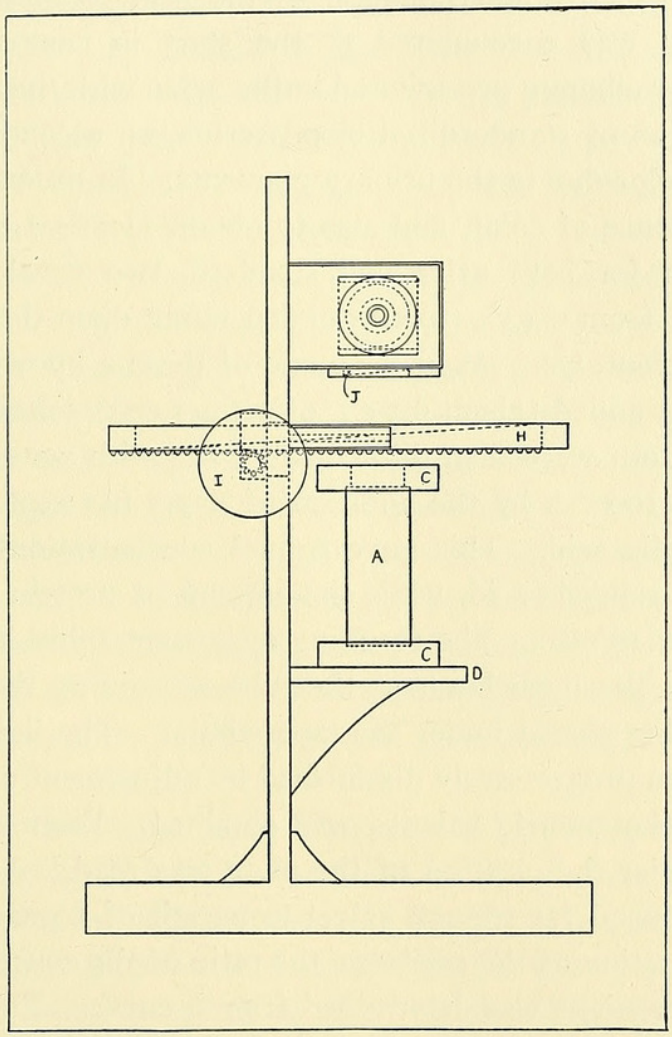

FIG. 2.

The construction of this instrument was delayed owing to difficulties encountered in securing neutral tinted glass. While awaiting its completion we decided to improvise a nephelometer in which several minor changes have been made. Among these may be mentioned the substitution for the glass wedge of a metal plate in which was cut a tapered slot. With this instrument we undertook some

work of rather an empirical nature along biochemical lines. Kober in one of his papers suggested the possibility of a nephelometric determination of albumin in urine, and a turbidimetric method for the same has been developed by Folin and Denis.[5] We therefore decided to apply our instrument to this problem. The standard was prepared from fresh normal human serum as recommended by Folin and Denis, and was standardized by nitrogen determinations and also by gravimetric determination of the heat coagulable proteins.

Difficulty was encountered at the start in comparing in the nephelometer albumin precipitated in the urine with that precipitated in the solution of standardized blood serum, on account of the difference in color due to the urinary pigments. In order to eliminate this interference of color, and also to obtain identical conditions of precipitation for both urine and standard, two equal portions of the urine of from 0.3 c.c. to 10 c.c. depending upon the quantity of albumen present, were taken. To one of these a known amount of standard was added (about 0.5 c.c. of 0.4 per cent. solution of serum protein). Both were then diluted to 75 c.c. with water and finally made up to 100 c.c. by the addition of a 7.5 per cent. solution of sulpho-salicylic acid. This gave a final concentration of 1.87 per cent. sulpho-salicylic acid, while the amount of protein varied from 2 to 5 mg. in 100 c.c. The resulting opalescent solutions were then compared in the nephelometer, the tube containing the urine plus standard being placed under the tapered slot. The light from this tube was then progressively diminished by adjustment of the slotted plate until photometric balance was obtained. From a scale with suitable vernier the position of the plate was read. As the theory has not advanced far enough as yet to permit of a purely formulative interpretation of the readings, the ratio of the concentrations of the two suspensions was determined from a curve. This curve had been obtained by plotting against the concentrations the scale readings obtained when known ratios of serum, made up with albumin free urine and precipitated with sulpho-salicylic acid under identical conditions, were compared. From the ratio R determined by means of the curve, the amount X of albumin originally present in the urine was found by the formula $R = \frac{X}{X+n}$ where n is the amount of

[5] Folin and Denis, *Jour. Biol. Chem.*, XVIII., 273, 1914.

serum albumin added. Quantities of urine and of standard were so taken that *R* would be in the neighborhood of one half. Urines containing large amounts of albumin (1 per cent. or over) were, after suitable dilution, compared directly with standard serum solution. In the case of such urines the high dilution necessary to obtain suitable nephelometric clouds eliminated the differences of color mentioned above. The results were compared with gravimetric determinations made according to Scherer's method. The clear filtrates from the coagulated protein were tested with sulpho-salicylic acid to make sure that none of the protein remained in solution. Duplicate gravimetric determinations gave good agreement. It was immediately evident that the nephelometric determinations were considerably higher than the gravimetric. Moreover, in the case of determinations on daily specimens of urine from one patient, the nephelometric results were consistently about 25 per cent. higher than the gravimetric, while in a similar series from another individual the ratio between nephelometric and gravimetric determinations was very variable, ranging from 1 to about 1.5. This at once suggested that the different proteins of the serum, while closely related chemically and equally precipitable by sulpho-salicylic acid, might give, in the nephelometer, clouds of different intensities. It is a significant fact that in the case of patient *R* where the ratio of nephelometric to gravimetric was variable, half saturation of the urine with ammonium sulphate gave a considerable precipitate of globulin.

In order to determine what differences might exist between the opalescences produced by equal amounts of the various serum proteins on precipitation with sulpho-salicylic acid under identical conditions, albumin, euglobulin, and pseudoglobulin were prepared from horse serum. Solutions of these when compared in the nephelometer gave surprisingly different results. The albumin gave about two and one half times as great an opalesence as the euglobulin and about three times as great as the pseudoglobulin. Compared with casein[6] suspensions, the following ratios, expressing the light reflect-

[6] As standard solutions of casein are easily prepared and also give very satisfactory clouds on precipitation with sulpho-salicylic acid, this substance forms a very convenient standard of reference in nephelometric work with various proteins.

ing power of equal amounts of these proteins, were found: Casein = 0.67 albumin; euglobulin = 0.63 casein; pseudoglobulin = 0.51 casein.

From the results experimentally obtained with various urines and from the differences in the clouds produced by equal amounts of the serum proteins, it may be seen that the nephelometric comparison of urine, in which these proteins may occur in varying amounts, with any definite standard such as serum cannot give a determination of the total protein. We hope by the use of specific precipitants to apply the nephelometric method to the separate determination of albumin and globulin in urine. This may be of value in diagnosis.

As the object of this paper has been to consider mainly the design of the instrument and the reasons for this design, the discussion of its application to the determination of albumin in urine has of necessity been hardly more than a suggestion of the work along that line. The results of the investigation of this particular problem with the experimental details, will be published shortly.

Summary.

1. The previous work in nephelometry has been briefly reviewed and the underlying principles of the nephelometric and turbidometric methods have been compared.

2. A new form of nephelometer has been described in which columns of suspension of equal lengths are used. The lights reflected are equalized and compared by means of a movable wedge of neutral tinted glass. Juxtaposition of the two emergent beams is secured by mirrors.

3. The variations found in preliminary experiments on the nephelometric determination of albumin in urine indicated that equal amounts of the various serum proteins might give different opalescences. Investigation showed that upon precipitation with 1.87 per cent. of sulphosalicylic acid, the same concentrations of serum albumin and serum globulins gave widely different clouds.

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