[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE UNIVERSITY OF TENNESSEE COLLEGE OF MEDICINE.]

FLUOREMETRY, QUANTITATIVE ANALYSIS BY COMPARATIVE FLUORESCENCE.[1] PRELIMINARY REPORT.

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In connection with a study of the effects of ultra-violet radiation upon biological substances which has been undertaken in this laboratory, it occurred to me that the visible fluorescence which is excited in the solutions of so many substances by these invisible rays might be utilized, under appropriate conditions, as the basis of a new general method for the quantitative determination of such compounds. When visible radiation falls perpendicularly upon the walls of a cylinder with an opaque bottom, containing a solution or liquid, little or no light is transmitted to the eye of an observer placed above the cylinder except such as may be reflected by any suspended particles. A comparatively simple relationship between the amounts of light thus reflected by 2 such suspensions forms the now familiar basis of nephelometry.[2,3,4] I imagined that if 2 such cylinders containing different concentrations of a fluorescent substance in solution were similarly exposed in a nephelometer to a source of ultra-violet radiation, all, or most all, of the visible rays from the latter being excluded by a suitable filter, the heights of the 2 columns required to produce an optical balance in the eye piece of the instrument should bear some readily calculable relationship to the concentrations. Experiment has demonstrated that this assumption is correct.

In the present paper the arrangement of apparatus is described, the conditions essential to concordant determinations are outlined and results are reported in which it is shown that determinations may be made upon acid solutions of quinine sulfate containing from $^1/_2$ to 2 parts per million and alkaline solutions of aniline iodeosine containing 2 to 4 parts per million. A further communication will include a report upon some experiments designed to increase still further the accuracy and delicacy of the method, a study of the influence of added non-fluorescent substances, a further investigation of the form of the curves obtained and certain practical applications.[5] The present results are sufficient, however, to demonstrate that we have here a new method of microanalysis of at least

[1] Presented by title before the Section on Physical and Inorganic Chemistry of the American Chemical Society at the St. Louis meeting, April 15, 1920.

[2] P. A. Kober, *J. Biol. Chem.*, **13**, 485 (1913); **29**, 155 (1917).

[3] Kober and Graves, *J. Ind. Eng. Chem.*, **7**, 843 (1915).

[4] P. A. Kober, *ibid.*, **10**, 556 (1918).

[5] These studies will include the use of comparison cylinders of transparent silica, a determination of the effects of added substances and applications to the determination of urobilin in body fluids and of quinine in the blood of patients treated for malaria.

the same order of sensitiveness as colorimetry and nephelometry and requiring no special technique for the preparation of solutions to be examined, which should prove exceedingly useful in the estimation of small amounts of the numerous substances which are either fluorescent themselves or become so upon the addition of suitable reagents, but which do not readily lend themselves to determination by either of the other methods.

On account of the analogy of the procedure to colorimetry and nephelometry, the natural term by which to designate the new method would be either "fluorometry" or "fluoremetry." The latter is preferred as being less liable to confusion with the process of determining fluorine in an instrument which has been given the name "fluorometer." Even a slight misunderstanding as to meaning of the term "fluoremetry" in the sense now proposed is regarded as less objectionable than the introduction of such a cumbersome word as "fluorescometry."

Arrangement of Apparatus.

The source of radiation employed is a mercury vapor lamp in quartz tube of the type designed for use with a transformer actuated by ordinary 110-volt alternating current,[1] giving an arc about 12 cm. in length. In order to cut off from the eye of the observer the powerful visible radiation from the lamp, the latter must be enclosed in a suitable light-tight box which, on account of the large heat evolution, should not be too small and must be provided with some means of ventilation. Practically I found it convenient to install the lamp in an ordinary chemical hood, the interior of which was painted white, several coats of black paint applied to the glass of the sash windows and all cracks made tight by cotton wool or putty. Beneath one of the partially raised sashes was closely fitted a section of "beaver" wall board in which an opening 8.75 cm. square

[1] This lamp and transformer are manufactured by the Hanovia Chemical Company of Newark, N. J., and billed by them as "A. S. type quartz lamp for 110 volts A. C., with transformer to operate." In a letter from this company it is stated that the lamp emits 3000 candle power visible radiation and that the 110-volt actuating current is stepped up by the transformer to 390 volts. A plug on the transformer is pushed in after the lamp has been in operation some 7 to 10 minutes, thus cutting out part of the external resistance and giving the maximum of radiation both visible and ultra-violet. The current consumption is stated to be 3.5 amperes while operating and somewhat greater at the moment of establishing the arc. These figures I have not personally attempted to verify.

The attempt has been made to render fluoremetric methods available to more workers by the substitution of a source of illumination more generally accessible than the quartz-enclosed mercury arc. A marked, though less intense, fluorescence is observed when a 750 watt "Mazda, Type C" lamp is used in connection with the filter G 586 AW. With this arrangement comparisons may be made with a fair degree of accuracy upon fully acidified solutions of quinine sulfate about 10 times more concentrated than those used with the mercury arc.—June 14, 1920.

had been cut at the level of the lamp tube. On the outside of the hood was installed a Kober nephelometer[1] with the end of the lamp house (from which the incandescent bulb, the lens and glass plate had been removed) directly against and surrounding the opening in the "beaver" board. Radiation from the lamp (which is used "side-on") therefore falls upon the walls of the comparison cylinders when placed in their proper holders at a distance of 50 to 80 cm. from the source of illumination, depending upon the position of the lamp with reference to the front of the hood.

When the cylinders are thus exposed to the intense full radiation of the lamp there is so much reflection of visible rays from the walls of the cylinders and other parts of the instrument, that there is considerable light visible in the eye piece when only distilled water is present in the cylinders—or, in fact, when the cylinders are removed. It is, therefore, absolutely essential that the greater part of the visible rays be eliminated by a suitable filter so placed as to tightly close the opening in the beaver board. Plates of glass of the Uviol type are best adapted to this purpose.[2] With one of these ("Violet Ultra") it was possible to reduce the visible radiation to such an extent that, with the lamp at 50 cm. from the cylinders, one of the latter filled with distilled water or N sulphuric acid to a depth of 100 mm. gave a barely discernible haze—of the order produced

[1] This is one of the newer types of the instrument, differing from that described by Kober (*J. Biol. Chem.*, **29**, 155 (1917)) in that the optical arrangement is not such as to produce the Lummer-Brodhun effect (square within a circle) but gives a simple divided circle as in most other instruments. Unlike the Duboscq, however, the *right* field corresponds to the *right* cup and not the reverse. The instrument is also provided with the pin-hole aperture in the observation tube, the importance of which has been emphasized by Kober (*J. Ind. Eng. Chem.*, 10, 556 (1918)).

[2] Two glasses suitable for this purpose may be obtained from the Corning Glass Works, Corning, N. Y. One of these, known as "Red-Purple Ultra, G 586 A," comes in a thickness of about 5 mm. As visually observed with the spectroscope, this glass transmits the 2 faint red lines at the extreme left of the mercury spectrum (in the neighborhood of 7080 and 6920), and a barely distinguishable trace of the brilliant green line at 5461 while all of the lines in the violet from 4078 to the end of the visible spectrum are strongly transmitted. The second glass "Violet Ultra G 586 AW," is about 10 mm. in thickness and transmits no visible radiation of wave-length greater than 3984. Both of these are claimed to be, and apparantly are, extremely transparent to the rays in the neighborhood of 3650, the region of greatest intensity of ultra-violet radiation in the mercury arc. The choice of the one or the other as a filter in fluoremetry will depend somewhat upon the intensity of the source of illumination. The "Red Purple Ultra" appears to transmit somewhat more of the ultra-violet as well as the longer waves than does the "Violet Ultra" but in order to obtain zero illumination when using it with a strong lamp the latter must be removed to a considerably greater distance from the comparison cylinders than when using the "Violet Ultra."

The use of these glasses was suggested to me by Dr. E. K. Carver in connection with the qualitative observation of fluorescence.

by 3 to 4 mm. of a one part to 20 million solution of quinine sulfate in *N* sulfuric acid.

In the early experiments, the laboratory was darkened by curtains for the purpose of making the readings, but when it was found that the experiment was yielding results which would justify considerable work, a small dark room was built against the front of the hood. The greater ease and accuracy with which readings could be made, clearly demonstrated that for this work a dark-room is a practical necessity. A black cloth hung around the instrument, to aid in cutting off such rays as pass the openings of the side doors, may be used without interfering with the operation of adjusting the column heights, and may be lifted for reading the scale. The latter is accomplished by the aid of a very dim light to keep the eyes sensitive for the next setting.

Precautions.

Workers accustomed to the use of the colorimeter and nephelometer will instinctively guard against such sources of error pointed out by Folin, Kober and others, as eye fatigue, bodily strain, dust upon the optical parts, incorrect adjustment of verniers, failure to dry the exterior of cylinders or to rinse these properly and to wash and dry the plungers when solutions are changed. They will also naturally adopt a regular routine under which settings are made alternately from dark to light, and *vice versa*, and the mean of an even number in each direction taken for the average. Special mention should perhaps be made of the recent observations of Lamb, Carleton and Meldrum,[1] who point out the errors which may arise through rotation of the eye piece and suggest the marking of cups to insure their replacement in exactly the same positions. The latter I have found particularly important in the work now reported.

In addition to these, and to the lighting conditions already discussed, certain special precautions are to be observed. The source of illumination being neither at an infinite distance, as in the Duboscq colorimeter, not attached to the instrument base as in the regular Kober nephelometer, it is desirable to adjust the position of the lamp from right to left, or *vice versa*, until an approximate balance of the fields has been secured, after which the relative positions of lamp and instrument must be maintained throughout any series of readings. Even so, however, it has not yet been possible so to control conditions that the ratio between standard solutions contained in both cylinders remains exactly the same for many minutes. Thus in a series of readings in which the standard in the left-hand cup remains at the same height and the same solution in the right-hand cup was read against it at intervals of about 20 minutes such values (each an average of 4 settings) as 19.75, 19.1, 18.7 and 19.0, were obtained. Practically, this source of error may be overcome with reasonable

[1] THIS JOURNAL, **42**, 251 (1920).

accuracy by reading the standard both before and after the unknown and taking the mean of these two as the true basis for comparison.

Such a shifting of the relative values of the fields is observed in nephelometry, but according to most reports it occurs less rapidly. I am of the opinion that in the present case it must be due to variations in the intensity of the mercury arc at different points, caused probably by changes in the outside voltage.[1] If this is the case, it would seem possible to correct it by using, instead of the direct radiation, the rays brought to a focus by a suitable quartz lens or system of metallic mirrors, but neither of these has been available. As stated above, the lamp has been so placed that it is radiation from one side of the quartz tube which is utilized; using it in the end-on position results only in diminution of the intensity without improvement in constancy. The shifting is not due, as I at one time imagined, to a photolysis of the fluorescent substance standing in the left-hand cell during a whole series of comparisons. This is shown by the fact that the shift is not always in the same direction and persists even when the solution in the left cylinder is changed after the comparison of each unknown. The possibility of such a photolysis should, however, be borne in mind when dealing with substances which are particularly sensitive to photochemical change.

A further precaution is that of adequate dilution of the solutions examined. We have here a situation much more important than the errors which arise in colorimetry through the comparison of columns widely differing in depth. In fluoremetry we are dealing with a phenomenon in which the light is a function not only of the amount of dissolved fluoregene, but also of the amount of exciting radiation absorbed. It is to be expected, therefore, that when the concentration of a solution is already such that it *completely* absorbs the exciting rays, further increases in concentration will produce little or no increase in the amount of fluorescence observed.[2] Thus it has been found that 3.6 mm. of a 400 mg./liter solution of quinine sulfate is matched by 22 mm. of a solution con-

[1] In this connection it should be noted that this shifting of the fields is more marked soon after lighting the lamp than later on. The lamp does not develop its full intensity until it has been in operation some 20 minutes and it has been found advantageous to allow it to remain lighted for even a longer period before starting an important series of readings.

[2] This result would naturally be anticipated from the reports of numerous investigations which show that the rate of a photochemical reaction does not follow the law of mass-action when the active rays are completely absorbed. Thus Luther and Forbes (THIS JOURNAL, 31, 777 (1909)), found that for the reaction between quinine and chromic acid "in ultra-violet light the velocity appears independent of the concentration of quinine, but in violet light these two quantities are roughly proportional to each other." This is explained by the fact that the ultra-violet light is completely absorbed while the violet light is absorbed only slightly. The fluorescent light with which the present paper is concerned is due entirely (within the limits of our power of

taining 1 mg. per liter (both of the same acid concentration). The assumption that the column heights will be inversely proportional to the concentrations, would call for a ratio of 1 : 400 whereas, in fact, it is only about 1 : 6. Yet, as noted below, when it is a question of comparing among themselves solutions containing from 0.5 to 2 mg. per liter a relationship closely approximating inverse proportionality is found to exist.

As a practical means of securing adequate dilution, it is suggested that the solution of any unknown substance be diluted by known stages until a point is found where further dilution produces a marked reduction in the luminescence of the flask or test-tube when held in the path of the screened ultra-violet radiation. It is better still to dilute the solution of the unknown substance until, when poured into a flat-walled vessel 10 mm. in thickness (spectroscopic cell) placed between the source of radiation and the parent solution contained in an observation cylinder standing in the instrument, enough radiation is transmitted by the solution in the cell to produce a marked fluorescence in the cylinder.[1]

Finally, it is to be observed that for the substances so far examined the reaction of the solution bears an important relationship to the amount of fluorescence as shown by the data in Table I. In this case the marked

TABLE I.

Influence of Hydroxyl Ion Concentration upon Fluorescence of Aniline-Iodeosine Solution Containing 4 Mg. per Liter.

Reaction of solution.	Height of column required to match standard. Mm.	Comparative fluorescence. %.
0.005 *N* H_2SO_4	128.0	13
0.001 *N* H_2SO_4	57.0	29
Neutral	16.7	97
0.1 *N* NaOH	16.2	100
1.86 *N* NaOH	23.0	70

(The standard solution, *i. e.*, the one 0.1 *N* with respect to NaOH, was placed in the left cup of the instrument at 15 mm., which gave a reading of 16.2 for the same solution in the right-hand cup. The other solutions were then read in the right-hand cup, the standard remaining the same on the left, except in the case of the stronger acid where it had to be set at a lower value. The diminution in fluorescence found with the 1.86 *N* NaOH was probably due less to any interference by the excess of alkali as such than to its gradual destruction of the color body, for this solution became colorless after standing for several days.)

increase in fluorescence of aniline-iodeosine solutions in passing from 0.001 *N* acid to neutrality, as compared with the change from neutral to 0.1 *N* alkali, indicates that provided the solutions are distinctly non-acid, such

measurement) to the ultra-violet rays as shown by the fact that no trace of it can be observed when the exciting radiation is made to pass first through 10 mm. of a strong solution of quinine sulfate (1 : 2500) though the latter does not diminish, so far as I have been able to observe, the intensity of any of those visible lines of the mercury arc which are passed by the glass filter "G 586 A."

[1] If comparison cylinders of transparent silica and a cell of the same material

minor differences in alkali concentration as would be incurred in practical determinations would be without appreciable effect. A similar condition was found for quinine sulfate when an acidity about normal is maintained. The presence of considerable quantities of non-fluorescent substances (neutral salts) in solution has also been shown to reduce the fluorescence though the change here is of a much lower order than that caused by variations in the reaction. A more complete study upon these points is in progress.[1] For the present it should be emphasized that for comparable results the concentration of salts and of hydrogen or hydroxyl ion in the standard and unknown solutions should be approximately the same.

Results of Measurements.

Quantitative comparisons have been made with solutions of quinine sulfate and aniline iodeosine. Among the known fluorescent substances which happened to be available, these selections were made for the preliminary work because they exhibit their fluorescence in acid and in alkaline solution, respectively, because an application of the quinine determination in a biochemical connection was contemplated and because the aniline-iodeosine solutions may likewise be studied in the colorimeter, thus affording a direct comparison with that method. No special attention was given to the purification of the original specimens used since, for the present, comparisons were to be made only between different dilutions of the same parent solution.[2] The results of these com-

were used it would be a simple matter to determine what concentration of any substance in the cell when placed in front of *one* of the cylinders would reduce the fluorescence therein to a definite fraction of the former value required to match the companion cylinder not so screened. It is suggested that such measurements may be useful when, in the absence of the difficultly obtainable quartz spectrograph and accessories it is desired to determine in a semi-quantitative way the extinction coefficient for the longer ultra-violet rays.

[1] The referee to whom this paper was submitted by the Editor has kindly called my attention to an observation by Buckingham (*Z. physik. Chem.*, **14**, 129 (1894)), that quinine behaves differently in chloride and in sulfate solutions. In my note book under date of March 3. 1920, there appears a memorandum showing that the addition of conc. hydrochloric instead of conc. sulfuric acid to a certain solution of quinine sulfate gave very little fluorescence. This observation was the basis of the work on the effect of foreign substances here referred to which will be reported in a later communication.

[2] One gram of quinine sulfate, U. S. P., was dissolved in 100 cc. of dil. sulfuric acid and a portion of this still further diluted until a concentration of 2.5 mg. per liter was obtained (the solution having been neutralized in the meanwhile by the addition of the amount of 0.1 *N* sodium hydroxide solution indicated by the titration of a portion, using methyl red as the indicator). To volumes of from 10 to 40 cc. of this neutral stock solution 10 cc. of 5 *N* sulfuric acid was added in each case and the whole made up to 50 cc. The resulting solutions, used for the comparisons, therefore, contained from 0.5 to 2 mg. per liter of quinine sulfate in *N* sulfuric acid.

For the aniline-iodeosine solutions, 0.5 g. of the substance was dissolved in distilled water and diluted to a stock solution containing 20 mg. per liter. Of the latter,

parisons are given in Tables II and III and graphically represented by the continuous curves in the upper parts of Figs. 1 and 2. They will be discussed presently.

TABLE II.

Fluoremetric Readings of Quinine Sulfate Solutions in *N* Acid Compared with Standard (*s*) Containing one mg.of Quinine Sulfate per Liter.

Concentrations in mg./L.	2.0 Mm.	1.7 Mm.	1.4 Mm.	1.2 Mm.	(*s*.) 1.0 Mm.	0.8 Mm.	0.6 Mm.	0.5 Mm
Individual Settings	10.3	12.0	14.7	17.0	19.7	24.6	31.4	....
	10.7	11.95	14.6	17.0	19.6	25.0	32.0	....
	10.6	12.0	14.7	17.3	20.0	24.6	32.1	....
	10.3	...	14.8	17.2	19.1	25.3	31.8	....
Av. 1st series.....	10.45	12.0	14.7	17.1	19.6	24.9	31.8	....
Av. 2nd series.....	10.3	12.1	14.6	16.7	19.8	24.3	31.5	....
Av. 3rd series.....	10.2	11.8	14.3	16.4	19.0	23.0	30.7	36.6
Av. 3 series.....	10.32	11.97	14.53	16.73	19.47	24.07	31.53	36.6
Adjusted av. ([a]).....	10.28	11.98	14.53	16.76	19.5	24.10	31.15	36.6

[a] These "adjusted averages" are the values obtained by averaging the 3 series after the results in each had been calculated (on the assumption of exact proportionality for differences of not more than 0.5 mm.) to what they should have been had the standard (*s*) read in each case 19.5 mm. instead of 19.6, 19.8 and 19.0 as actually found. It will be observed that the results thus obtained differ only inappreciably from those in the line above them, which are the plain averages of actual readings.

TABLE III.

Fluoremetric Readings of Aniline-Iodeosine Solutions in 0.1 *N* Alkali Compared with Standard (*s*) Containing 4 Mg. of Aniline Iodeosine per Liter.

Concentrations in mg./L.	(*s*.) 4 Mm.	3.6 Mm.	(*s*.) 3.2 Mm.	2.6 Mm.	2.0 Mm.
Av. 1st series.....	16.25	18.0	20.1	24.4	31.5
Av. 2nd series.....	16.10	18.0	20.1	24.2	30.6
Av. 3rd series.....	16.05	17.9	20.0	24.7	31.4
Av., 3 series.....	16.10	17.97	20.07	24.43	31.17
Adjusted ([b]) average.....	16.00	17.83	19.90	24.25	30.90

[b] Obtained as in Table II by calculating the figures in each series to a common basis of $s = 16.0$ mm.

The results of comparing the same aniline-iodeosine solutions by colorimetry and fluoremetry (Table IV) are interesting. In addition to showing the divergence in ratios of readings to concentrations for the 2 methods, elsewhere discussed, they indicate that the fluoremetric readings are somewhat more concordant. The latter is not surprising in this case when it is considered that the color compared in the colorimeter is pink,

volumes of from 10 to 20 cc. were measured, 10 cc. of *N* 1 sodium hydroxide solution added in each case and the whole diluted to 100 cc. The resulting solutions thus contained from 2 to 4 mg. of aniline iodeosine per liter in 0.1 *N* sodium hydroxide solution.

to which the eye is much less sensitive than to the greenish blue of the fluorescent light.

TABLE IV.

Fluorometric and Colorimetric Comparisons of the Same Solutions of Aniline Iodeosine.

Fluorometric Readings.		Colorimetric Readings. (Duboscq Colorimeter.)	
Left. (4 mg./L.)	Right. (2 mg./L.)	Left. (4 mg./L.)	Right. (2 mg./L.)
$s = 16.0$[a]	30.6	$s = 16.0$[a]	32.2
	30.6		34.3
	31.5		34.3
	31.5		35.0
	31.6		32.0
	32.0		32.7
	32.0		33.0
	31.5		34.8
16.0	31.4	16.0	33.5

[a] In each instrument the stronger solution was set at such a height in the left hand cup as to give a value of 16.0 mm. in the right-hand cup when the latter was filled with the same. The right cup was then emptied, filled with the weaker solution and readings made as indicated, the left remaining unchanged throughout.

Discussion of Results.

The results obtained with the sets of solutions of quinine sulfate and aniline-iodeosine, equal among themselves in content of acid and alkali, respectively, are expressed graphically by the solid curves in Figs. 1 and 2, in which the readings obtained are plotted as ordinates against the concentrations, in mg. per liter, as abscissas. The dotted curve in each figure represents the colorimetric or theoretical curve drawn on the assumption that $y = s/x$ (s being the reading of the standard).

The relationships of these 2 sets of curves naturally suggest that existing between the colorimetirc and nephelometric curves which has been discussed by Kober[1] and it was considered interesting to inquire whether the formula developed by him for the nephelometric curve would represent the results of fluorometry.

Kober's formula is as follows

$$y = (s/x) - (1 - x)sk/x^2)$$

in which y is the height of the column of unknown solution, x the ratio of its concentration to that of the standard, s the height of the standard and k a constant to be determined (for any particular instrument and set of solutions) by substituting in the formula the standardization values of y, x and s.

In Col. 4 of Table V will be found the values obtained by solving for k, $(k = x\,(/1 - x) - (x^2y/(1 - x)s)$, when the values for y, s and x in fluorometric measurements (Tables II and III and Cols. 2 and 3 of Table

[1] *Loc. cit.*

V) are substituted. These values for k do not possess the same claim to constancy as those found in nephelometric work,[1] a condition which may or may not be altered by the elimination of further sources of error in the measurements. More significant than this is the fact that in the case of quinine sulfate where solutions both stronger than the standard and more dilute are compared with it, 2 distinctly different sets of values are encountered for k, the one corresponding to the range of higher concentrations, the other to the lower. The divergences are not of a type to indicate that they are due primarily to errors in measurements and it has, therefore, been considered preferable to use the average value of k as actually found for each range of concentration in determining the correction factor (Col. 6) for the solutions included within that range.

TABLE V.

COMPARISON OF FLUOREMETRIC READINGS WITH COLORIMETRIC AND NEPHELOMETRIC CURVES.

1. Conc. mg./L.	2. Ratio to Standard. x.	3. Fluorometric Readings. %Fluor.	4. Constant $\left(\frac{x}{1-x}\frac{x^2y}{(1-x)s}\right) = k$.	5. $y = \frac{s}{x}$ %Color.	6. Neph. Correc'n. $\left(\frac{(1-x)\ sk}{x^2}\right)$	7. %Neph.	8. %Color. / %Fluor.	9. %Neph. / %Fluor.
Quinine Sulfate in N 1 H_2SO_4. Standard contains one mg. per liter; s = 19.5 mm.								
		Mm.		Mm.	(k = 0.139). Mm.	Mm.	%	%
2.0	2.0	10.28	0.109	9.75	+0.68	10.43	94.8	101.5
1.7	1.7	11.98	0.107	11.47	+0.66	12.13	95.7	101.3
1.4	1.4	14.53	0.151	13.86	+0.55	14.41	95.4	99.2
1.2	1.2	16.76	0.188	16.25	+0.38	16.63	97.0	99.2
			Av. 0.139					
1.0	1.0	19.5 (s)	...	19.5	0.00	19.5	...	...
					(k = 0.056).			
0.8	0.8	24.10	0.045	24.38	—0.34	24.04	101.2	99.8
0.6	0.6	31.15	0.062	32.50	—1.21	31.29	104.3	100.4
0.5	0.5	36.60	0.062	39.00	—2.18	36.82	106.6	100.6
			Av. 0.056					
Aniline Iodeosine in 0.1 N NaOH. Standard contains 4 mg. per liter; s = 16.0 mm.								
					(k = 0.024).			
4.00	1.0	16.00 (s)	...	16.00	—0.00	...	...	...
3.6	0.9	17.83	0.024	17.78	—0.05	17.73	99.7	99.4
3.2	0.8	19.90	0.010	20.00	—0.12	19.88	100.5	99.9
2.6	0.65	24.25	0.027	24.62	—0.32	24.30	101.5	100.2
2.0	0.50	30.90	0.034	32.00	—0.77	31.23	103.6	101.3
			Av. 0.024					

Column 5 gives the colorimetric or inverse proportionality curve, $y = s/x$, to which the nephelometric correction (Col. 6) is added algebraically to obtain the values for the nephelometric curve, Col. 7. The

[1] Compare S. S. Graves, THIS JOURNAL, 37, 1176 (1915).

figures in Cols. 8 and 9 show, respectively, the percentage of the fluorescent substance actually present which would be indicated if the results of fluoremetric comparisons were read off directly from the colorimetric and nephelometric curves. The errors thus occasioned are represented graphically by the curves in the lower parts of Figs. 1 and 2. It is obvious that the nephelometric curve, as modified by the use of different constants for the 2 ranges of concentration, expresses the results of fluoremetric comparisons with an average error of about $^1/_5$ the magnitude which is encountered in the use of the inverse proportionality curve. This gives ground for the hope that work with other substances will demonstrate the possibility of a further modification which may render the nephelometric formula applicable to fluoremetry.

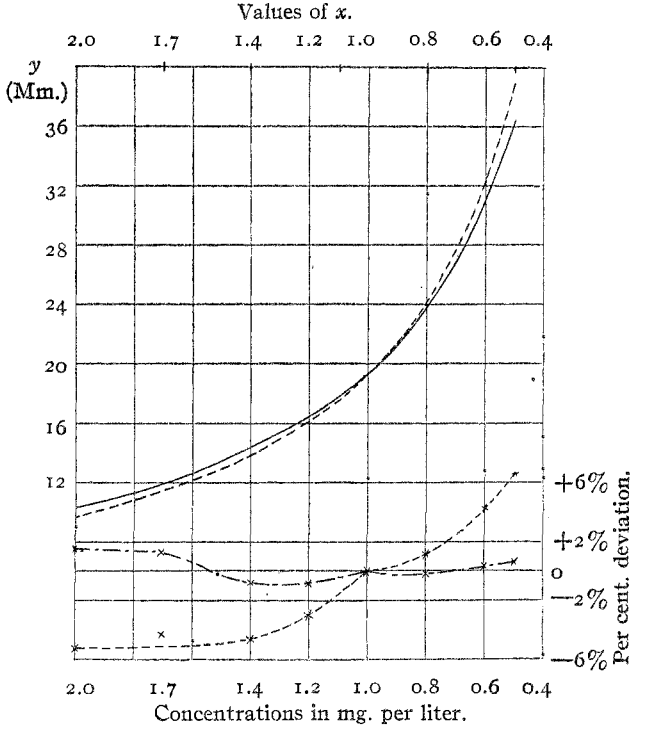

Fig. 1.—Relationship between Fluorescence and Concentration. Quinine sulfate in N H_2SO_4. Upper curves: $x = \frac{\text{Conc. sol'n compared}}{\text{Conc. standard (1 mg./L)}}$; y = height of column required for fluoremetric match with 19.5 mm. of standard; solid line is the curve of observed relationships; broken line is the inverse proportionality (colorimetric) curve. Lower curves: dash line shows the error (%) in actual concentrations of solutions resulting from interpreting fluoremetric readings from colorimetric curve; dot-dash line shows the error (%) from interpretation by modified nephelometric curve.

In further justification of the use of separate constants for the ranges

of concentration greater and less than the standard of comparison, it seems desirable to call attention to certain facts regarding the magnitude and sign of nephelometric correction. It will be observed that in the formula

$$y = (s/x) - ((1 - x)sk/x^2)$$

the expression $-((1 - x)sk/x_2)$ represents the correction which must be applied to the colorimetric curve as given by the term s/x. In all of the curves published by Kober and his co-workers (so far as they have come

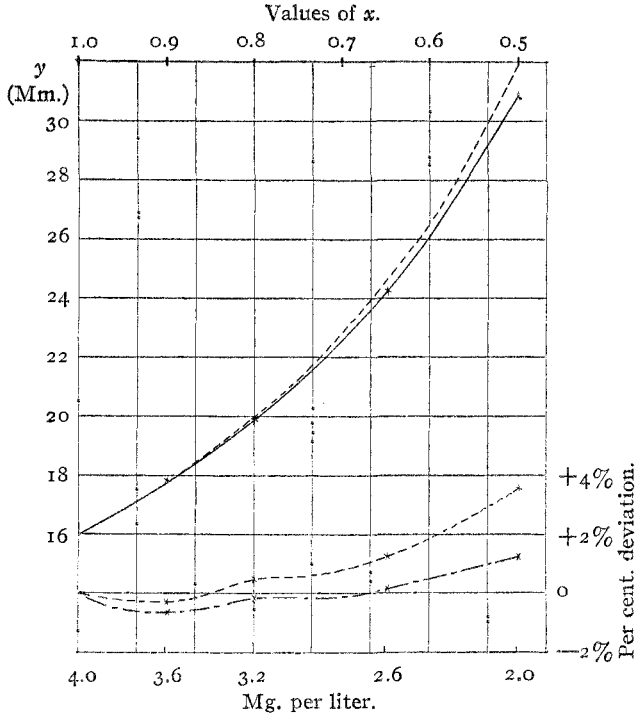

Fig. 2.—Relationship between Fluorescence and Concentration. Aniline Iodeosine in 0.1 *N* NaOH. Upper curves: $x = \frac{\text{Conc. sol'n compared}}{\text{Conc. standard (4 mg./L)}}$; y = height of column required for fluoremetric match with 16.0 mm. of standard. The several curves have the same significance as those in Fig. 1.

under my observation) the *strongest* solution in a series is the one uniformly taken as the standard. In consequence, no value of x greater than one is encountered. When, however, an intermediate concentration is used for the standard of measurement, ratios greater than one will appear and, from the form of the expression $-((1 - x)sk/x^2)$ it will be seen that whenever x becomes greater than one, the sign of the resulting correction must necessarily change. That is to say, if the nephelometric

curve lies below the colorimetric for the region where $x < 1$ it should lie *above* the other for any values of x greater than one. As stated, I have seen no nephelometric curves which could be used for ascertaining whether this relationship holds good and I have not yet had the time to make appropriate measurements myself. But in plotting the observed results of fluoremetric measurements I was at once struck with the regular reversal of the positions of the actual and inverse proportionality curves whenever I passed from solutions weaker than the standard to those more concentrated. This observation led me to calculate the magni-

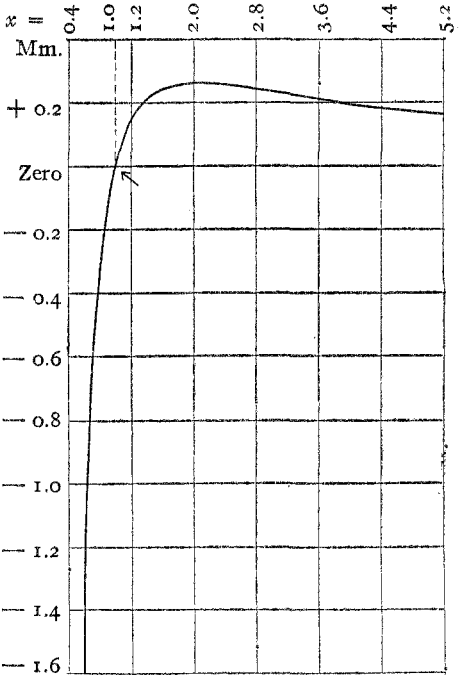

Fig. 3.—Variations in the sign and magnitude of the nephelometric correction factor, $\frac{(1-x)\,s\,k}{x^2}$, (ordinates of this curve) with changes in ratio of concentrations on both sides of $x = 1$. Calculated for standard (s) set at 20 mm. and $k = 0.052$.

tude of the nephelometric correction, $-((1-x)sk/x^2)$, for considerable variations in x on both sides of $x = 1$. The results of such a calculation for the case in which $k = 0.052$ and $s = 20.0$ mm. are plotted in Fig. 3. In addition to the change in sign of the correction on the two sides of $x = 1$, it is noted that the absolute value for the interval between $x = 1$ and $x = 2$ is only about $^1/_{10}$ of that between $x = 1$ and $x = ^1/_2$; that a maximum occurs at $x = 2$ and that thereafter the value is practically constant. So far, therefore, from expecting exactly the same form of

expression to hold good on both sides of $x = 1$, it would be rather surprising if this were found to be the case.[1] In a practical way, the comparison of unknown stronger solutions with a fairly tall column of a weaker standard might give some interesting results.

Summary.

A new method of micro-analysis is described, of the same order of sensitiveness as colorimetry and nephelometry, which should prove generally applicable to the determination of minute quantities of the considerable number of substances which are either fluorescent themselves or may be rendered so by the addition of a suitable reagent.

Ultra-violet rays from a quartz-enclosed mercury arc, filtered from most of the visible radiation, are used to excite fluorescence in solutions of such substances contained in the comparison cylinders of the Kober nephelometer. The intensity of the fluorescent light thus produced, as observed in the eye piece of the instrument, is equalized in the usual manner by altering the heights of the exposed columns.

For sufficiently dilute solutions the curves obtained by plotting the scale readings against concentrations are quite regular. These calibration curves are drawn for solutions containing 0.5 to 2 mg. per liter of quinine sulfate in N sulfuric acid and for solutions of 2 to 4 mg. per liter of aniline iodeosine in 0.1 N alkali.

Such a curve differs from that of inverse proportionality (the colorimetric curve) much more than from one drawn according to the nephelometric formula. Some peculiarities of the latter are indicated and it is shown that when 2 constants are used (one for solutions stronger than the standard and the other for those more dilute) the values corresponding to the fluoremetric readings may be taken from the nephelometric curve with an average error of less than 1%. It is expected that this can be reduced by the further elimination of errors in measurement or a further modification of the formula, or by both together.

Various phases of the work, including certain applications, are receiving attention.

MEMPHIS, TENN.

[1] This is in no wise intended as a criticism of Kober's formula which is remarkably accurate for the particular purpose and region of concentrations for which it was designed and used. On the contrary, the present suggestions are only intended to indicate a possible widened usefulness.