

the hypothesis of positive and negative valences may possibly become a necessary adjunct to the structure theory. This, as I have stated before,<sup>1</sup> must depend upon the extent of its applications and experimental verifications, and upon the part that is played by just criticisms in bringing to light the relative merits and demerits of its *applications*.

From these and the preceding points of view noted in this paper, the general criticisms and conclusions of Brunel have little, if any, bearing upon the *electronic conception of positive and negative valences as a formulative hypothesis*.

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## NEPHELOMETRIC ESTIMATION OF PHOSPHORUS.

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### I. Introduction.

The estimation of phosphorus in biological and industrial sciences, especially of minute amounts, is becoming of increasing importance. A large number of volumetric and colorimetric methods have been proposed but thus far none has been satisfactory for micro-quantitative work. While looking for a suitable nephelometric precipitant<sup>2</sup> for phosphorus in connection with our nuclease technic<sup>3</sup> our attention was called<sup>4</sup> to the reagent developed by Pouget and Chouchak.<sup>5</sup> This reagent consists of a nitric acid solution of strychnine and molybdic acid and produces a very sensitive reaction with phosphates (one milligram of phosphorus in 2 liters giving a very marked suspension of insoluble substance) similar in sensitiveness to the Nessler or the Graves<sup>6</sup> reaction for ammonia. The authors found it will detect 1 part of phosphorus in 20 million parts

<sup>1</sup> THIS JOURNAL, 37, 892 (1915).

<sup>2</sup> Uranium acetate and magnesia mixture were tried as nephelometric precipitants, but as preliminary experiments, especially with the latter, were not successful, these reagents were abandoned.

<sup>3</sup> P. A. Kober and S. S. Graves, THIS JOURNAL, 36, 1304 (1914).

<sup>4</sup> Dr. I. Greenwald of this laboratory, after making an attempt to apply this reagent directly for nephelometric work, abandoned it. Subsequently (see *J. Biol. Chem.*, 21, 29 (1915)) he recommended the original reagent for a colorimeter method, although, according to him, the color of the opalescent liquid is so slight that it is not apparent until it is put into the instrument. It is obvious that any defect in the suspension for nephelometric work will make a defect for colorimetric, and our experiments, as indicated elsewhere, show that the reagent as recommended by the original authors, although usable, is not satisfactory.

<sup>5</sup> *Bull. soc. chim.*, 5, 104 (1909); 9, 649 (1911).

<sup>6</sup> THIS JOURNAL, 37, 1181 (1915).

of water. As the precipitate is slightly yellow and remains in suspension for a long time, the authors recommended it as a colorimetric reagent, but, practically, their estimations were turbidimetric.

On studying the reaction carefully, we found that (1) it was not constant and quantitative<sup>1</sup> and (2) the reagent gradually become yellow and deteriorated, probably because of the action of the nitric acid. Pouget and Chou-chak realized this, as their directions state that the mixing of constituents must be made only just before using. After making many variations of all constituents, no marked improvement was obtained, but *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.*

## II. Reagent.

(a) **Variations of Constituents.**—The insoluble substance formed in this reaction is probably a phosphomolybdic acid complex of strychnine. The important factor is the hydrochloric acid content of the reagent. If the amount of acid is too small, an insoluble strychnine-molybdic acid compound precipitates and if too large, the phosphate precipitate is re-dissolved. The latitude between these extremes is, however, quite satisfactory. Owing to the insolubility of the molybdic acid complex of strychnine, no large variations of strychnine could be made and therefore the solution used was saturated with respect to strychnine.

The amount of hydrochloric acid necessary for the reagent, using commercial grades of molybdic acid or sodium molybdate, was very difficult to determine, for the reason that some grades of molybdic acid obtained contained as much as 11% of ammonia; a few samples of sodium molybdate were soluble in water, and others were not. Merck's<sup>2</sup> sodium molybdate was found satisfactory, but difficult to obtain, so that the preparation of this substance by ourselves was necessary.

To show the effect of varying amounts of strychnine and hydrochloric acid, the following experiments were made:

**Strychnine.**—To 1.5 g. of sodium molybdate, dissolved in 2.5 to 3.25 cc. of water, and 10 cc. of hydrochloric acid (1-1) sp. gr. 1.098, were added 1.0, 0.75, 0.50, 0.25 and 0.00 cc. of a saturated solution of strychnine sulfate (2%), respectively.

After shaking and standing over night, molybdic acid-strychnine crystallized out in all solutions, except the last. The one to which 0.25 cc. of strychnine sulfate had been added contained the least (only a small amount), while the others contained precipitates in proportion. The reagents were, after filtering, tested for their precipitating power nephelometrically, and it was found that the one without strychnine gave no test for phosphorus and that the others had about the same efficiency. A slight difference was observed, but before the question can be definitely settled the

<sup>1</sup> The suspensions always gave negative constants (see discussion of nephelometric constants in this paper).

<sup>2</sup> Further work with this grade of sodium molybdate was impossible, since we were unable to obtain it owing to the war.

experiments will have to be repeated. As our results below show, the reagent with 1 cc. of strychnine sulfate gave very satisfactory results, and until further data are at hand, it is best to adopt that amount.

**Hydrochloric Acid.**—To 15 cc. of water, 2.5 cc. of reagent (see directions, given below) and 5.00 cc. of  $\text{KH}_2\text{PO}_4$  solution (5 mg. to a liter) were added varying amounts of normal hydrochloric acid and the volume made up to 40 cc. The table gives the nephelometric readings.

Amount of <i>N</i> acid, cc. ....	17.5	10.0	5.00	2.50	1.25	0.00
Mm. ....	19.4	15.5	14.4	13.9	14.0	14.0

Since the amount adopted for regular work is 1.25 cc. *N* under these conditions, almost four times as much may be used without decrease in the amount of precipitate. As may be seen, the results are also good when no acid is added in addition to that contained in the original reagent, thus showing a considerable latitude in the amount of free acid.

(b) **Directions.**—*Preparation of Sodium Molybdate.* Thirty-five and one-half grams of molybdic acid (c. p. free from ammonia, "100%") are boiled for about one hour with 50 cc. sodium hydroxide solution (containing 400 g. of 96% NaOH dissolved in 1 liter). The volume of liquid is brought up to 84 cc. with distilled water and the mixture shaken, until practically all of the solid sodium molybdate is dissolved. After adding a few grams of pure talcum powder and again shaking, the solution is filtered free from any residue, which in some preparations may be very dark. The filtrate, which should not have more than a slight yellowish tint, is evaporated<sup>1</sup> practically to dryness on the steam bath, and the residue washed, first, by grinding it with 40 cc. of 95% ethyl alcohol, filtering, and then by washing it on the filter with several portions of 20 cc. of alcohol. The residue, which should be perfectly white, is then dried in an oven at about 50° or in a vacuum desiccator.

*Strychnine Solution.*—Two grams of pure strychnine sulfate are put into a 100 cc. flask, with 80 cc. of water, and heated to about 90°. When the salt is completely dissolved, the solution is allowed to cool and then made up to the mark with distilled water.

*Hydrochloric Acid (1-1).*—Fifty cubic centimeters of strong HCl (sp. gr. 1.20) are diluted to 100 cc. with water; 5.00 cc. of this acid diluted to 100 cc. can be standardized by titrating with 0.5 *N* sodium carbonate. Twenty-four cc. of acid should be equivalent to 30 cc. of 0.5 *N* alkali.

*Preparation of Reagent.*—One and one-half grams of sodium molybdate (as prepared above) are dissolved in 2.5 cc. of distilled water and 10 cc. of (1-1) hydrochloric acid added while shaking. The precipitate which is formed on the first additions of acid redissolves in the excess of acid. One cubic centimeter of strychnine sulfate solution is then added while shaking, and the solution allowed to stand over night. *After filtering, the solution should be perfectly clear and practically colorless. For this*

<sup>1</sup> After concentrating for sometime the solution forms a crust of sodium molybdate, which must be broken up occasionally with a stirring rod.

*purpose ordinary filter paper cannot be used, as the strongly acid solution extracts from it a substance which gives a very marked cloud on standing. This substance seems to be a phosphorus compound, as S & S No. 575, a hardened paper, and S & S No. 589 a quantitative paper, do not seem to give the slightest trace even on standing for weeks. S & S No. 597 gives a large amount of this suspension.*

*For Making Standard Solution.*—One-tenth of a gram of  $\text{KH}_2\text{PO}_4$  is dissolved in 2 liters of water; 100 cc. of this stock solution diluted to 1 liter will make a standard solution containing 5 mg. to a liter.

*For Precipitating.*—To 30 cc. of distilled water and 5 cc. of 0.5 N HCl, 5.00 cc. of reagent are added and the solution shaken thoroughly. Ten cc. of phosphate solution (standard or unknown) are then added slowly with a pipet; the mixture is shaken by gentle rotation of the flask, and after 3 minutes' standing it is ready for reading in the nephelometer.<sup>1</sup>

*For Reading the Instrument.*—As these directions have already been given in detail, there is no need for repeating them here.<sup>1</sup>

(c) **Results with New Reagent.**—As Pouget and Chouchak studied the effect of different salts on their nitric acid reagent, the experiments with the new reagent were limited to pure solutions of mono-potassium phosphate. In series of liquids, containing 5.0, 4.5, 4.0, 3.5, 3.0 and 2.5 mg. of phosphate, respectively, precipitates were produced as described in the directions and the readings plotted, as shown below.

Curve A (Fig. 1) was made September 11, 1914, with a reagent containing Merck's sodium molybdate and Curve B (Fig. 2) was made May 20, 1915, with a reagent containing a preparation of our own sodium molybdate, as indicated on page 2375.

TABLE I.

Solution of $\text{KH}_2\text{PO}_4$ . 10 cc. sample contained		Curve A.		Curve B.	
Mg. $\text{KH}_2\text{PO}_4$ .	Mg. P.	Readings. Min.	k.	Readings. Min.	k.
0.0300	0.01140	13.92	..	14.74	..
0.0450	0.01025	14.95	0.29	15.92	0.24
0.0400	0.00911	16.40	0.23	17.26	0.23
0.0350	0.00797	18.16	0.21	19.00	0.22
0.0300	0.00683	19.90	0.21	20.90	0.22
0.0250	0.00569	22.00	0.21	23.00	0.22
		Average, 0.23		0.23	

The figures represent the average of 2 to 3 readings upon two or three solutions of each concentration and the relatively large constant indicates that the reaction is practically complete. (See Discussion.)

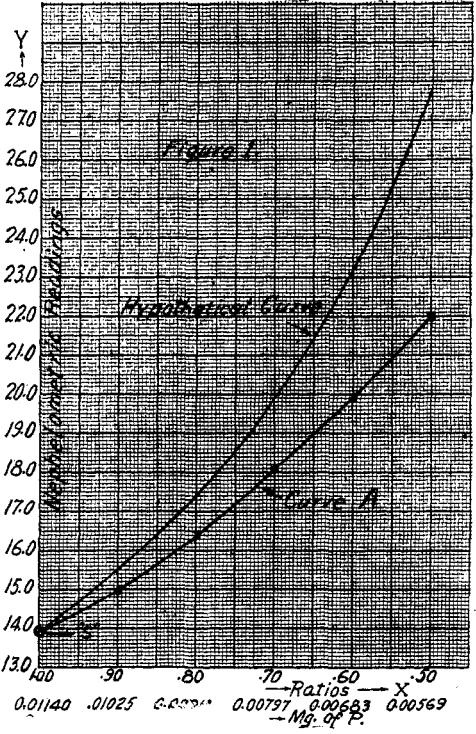
### III. Discussion.

In nephelometric work published from the Harriman Laboratory thus far, considerable stress has been laid on the position of the curves obtained

<sup>1</sup> Kober, *J. Biol. Chem.*, 13, 485 (1913).

from a gradationed series of known solutions. These suspensions represent such small amounts of material that no other technic can be conveniently employed to determine the completeness of precipitation, and so far we have relied only on the position of these curves, relative to the colorimetric curves, to tell us whether the precipitation is complete or not. As these curves are very important for rapid and accurate work, a recapitulation of their meaning and use will not be out of place here.

Taking Fig. 1 as an example, when the standard solution, after precipitation, was put on both sides of the instrument, the right side was set at 15.0 mm. and when the light in the eye-piece was matched, the left side read 13.92 (this reading, 13.92, is denoted by "s" in the nephelometric formula and curve). Now, leaving the standard solution<sup>1</sup> on the right set at 15.0 mm., another solution (0.900 as strong as the standard) was put in place of the standard on the left side, which

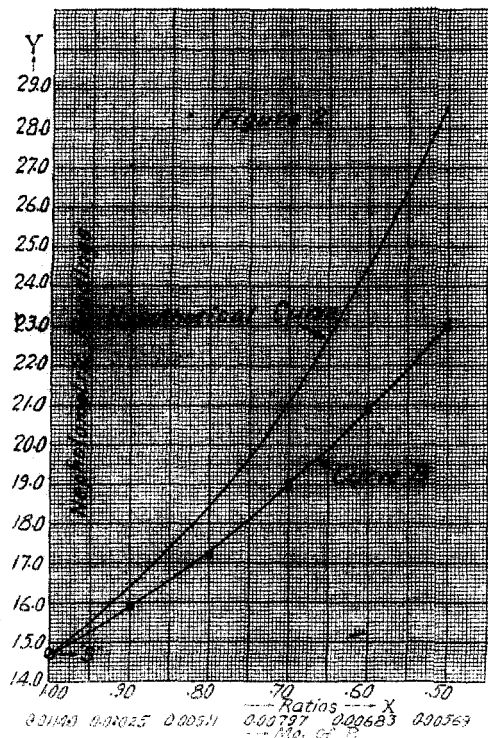


on being matched with the standard remaining on the right side, gave a reading of 14.95 (this and similar readings are denoted by "y" in the formula and curve). Then solutions of 0.800, 0.700, 0.600, and 0.500 standard strength were put on the left side and matched successively, thus giving, respectively, 16.40, 18.10, 19.90, and 22.00 mm. A line drawn through these points gave us Curve A, and similarly Curve B was obtained.

If the instrument and light remained constant so that the readings of the standard solution always remained the same, in this case, 13.92 mm., the curve would be sufficient for practical work. The readings of unknown solutions, by reference to the curve, could be expressed directly in milligrams of substance. But most instruments do not remain constant (not even a good balance) and therefore the readings of standard

<sup>1</sup> By using "s" as the potential height of the standard liquid, any inequality in light, tubes, and plungers of the two sides is eliminated. It is similar to weighing by substitution.

on the left side (the readings of the right side being the same, usually 15.0 mm.) changes, from time to time, similarly to the zero point of the balance, and the value of the curve itself is thereby destroyed. Therefore,



we always check up the "zero-point" of the instrument, before beginning a series of analyses, by putting standard solution on both sides of the instrument and making readings.

To circumvent this difficulty of changing instrument, without using a nephelometric formula, two methods are available: (1) to redilute either the standard or the unknown solutions until they give the same readings. Unless the two readings are identical, which is difficult to obtain, an assumption or hypothesis must be introduced to finish the calculation, *i. e.*, that the readings are inversely proportional to the concentration of the substance, as in the curve marked "hypothetical." How

erroneous this assumption is and how near the readings of the "unknown" must be to those of the standard solutions to avoid appreciable error, can be seen at a glance by the difference between the hypothetical and the actual curve (A). Therefore, this way is not only tedious, but not very accurate. (2) The second way to obviate the inconstancy of the instrument, is, of course, to make a fresh curve each time, which again is unnecessarily tedious.

If a nephelometric curve could be expressed by an equation, any change in any one of the variables (*i. e.*, the readings) would be of no consequence, and in general, the behavior of the reagents could then be followed and expressed with ease. This has been attempted<sup>1</sup> in the formula

$$y = \frac{s}{x} - \frac{(1-x)K}{x^2}$$

where  $y$  = height of "unknown" solution, on the left side of the instrument, when standard solution is kept on the right side at a definite height,  $s$  = height of standard solution on the left side and  $x$  = the ratio of the concentrations of the two solutions.  $K$  = a

<sup>1</sup> Kober, *J. Biol. Chem.*, 13, 485 (1913).

constant, obtained by substitution of standardization values of  $s$ ,  $y$ , and  $x$ .

When  $K$  obtained with one height of standard solution is compared with that of another height, it is found that  $K$  is proportional to the height of the standard solution, and the equation then becomes for any heights of liquid within moderate limits

$$y = \frac{s}{x} - \frac{(1-x)sk}{x^2} \text{ where } k = \frac{K}{s}.$$

When solved for  $x$  the equation becomes

$$x = \frac{s + sk + \sqrt{(s + sk)^2 - 4sky}}{2y}.$$

This equation, then, actually expresses the mathematical relationships between the ratios of the concentrations of the substances and the readings, and  $k$ , which is constant for a given substance and its reagent or precipitant, is an index of the amount of deviation of the readings from the colorimetric or hypothetical curve. Its value in the colorimetric curve is zero and the maximum thus far obtained for suspensions is 0.29.

One of us (K.) conjunctively with Sara S. Graves has found it convenient to estimate the maximum deviation or constant possible from a given suspension. This can be obtained by precipitating a standard solution, then diluting an aliquot portion with more reagent to a definite volume, and comparing the two suspensions in the nephelometer. The readings of " $y$ " in this case will be slightly lower, and the constant slightly greater than the one used in actual analysis, or that obtained by diluting the solution before adding the reagent. This is due to the fact that when substances are precipitated in very dilute solutions, a small and often an appreciable amount remains unprecipitated, through its solubility or hydrolysis, but by precipitating before diluting, this solubility of the substance is eliminated, *as both standard and "unknown" or weaker solution then contain, as under ideal conditions, the same proportion of precipitated and dissolved substance.*<sup>1</sup> We shall hereafter designate it as the *theoretical constant of a substance*, and the difference between it and the actual constant may then be taken as representing the amount of substance not precipitated, under the conditions of analysis.

With the Graves reagent for ammonia it was found that the theoretical constant was 0.26<sup>2</sup> and the actual 0.14.<sup>3</sup> For this new reagent for phos-

<sup>1</sup> It is, of course, probable that the suspension in the weaker solution will redissolve or hydrolyse slightly, until the same equilibrium is reached as in the one precipitated after dilution, but the speed of solution, near the saturation point, as is well known, is extremely slow, and any error due to this must be negligible.

<sup>2</sup> From the note book of S. S. Graves, dated February 10, 1915.

<sup>3</sup> Even if the amount not precipitated is appreciable, no error in analysis results, as it is eliminated through standardization.

phorus, the theoretical constant was found to be 0.23, and the actual as shown above is near 0.22 (the average, including two high values, was 0.23), which demonstrates that the precipitation of phosphorus in this extreme dilution is remarkably complete. (The half standard solution contained 1 part of phosphorus in 2,000,000, while the final suspension in the nephelometer only contained one part in 10,000,000.)

Another point to be considered in connection with the completeness of precipitation is that of stability of the suspension. Experience has shown that the more complete a precipitation the less stable the suspension, *i. e.*, the quicker it agglutinates. Thus it is sometimes necessary to sacrifice somewhat on the completeness of the precipitation to gain stability of suspension (*i. e.*, time for making readings). These and other properties of opalescent solutions will be taken up in detail in the near future.

#### IV. Applications.

Our first intention was to apply the reagent to the estimation of phosphorus in various organic and inorganic substances, but lack of opportunity compels us to content ourselves with a few *preliminary trials*. As Pouget and Chouchak have already suggested and tried the nitric acid reagent for metals, ores and biological material, and Greenwald has shown that the same is useful for blood work, it will not be necessary to give many details and data for the purpose of showing its usefulness. The accuracy obtainable with the new reagent is shown by the nephelometric curves.

As the dilution of the solution for most phosphorus estimations is so large, very few substances are likely to interfere with the reaction. In an extreme case re-solution of a phosphomolybdate might be necessary. As a rule, any solution containing phosphates, but no organic matter, may be used at once, provided the solution is neutral or slightly acid and free from turbidity.

Ten cc. of urine were digested, as in a Kjeldahl nitrogen estimation, with 20 cc. of concentrated sulfuric acid and 10 g. of potassium sulfate, until the solution was clear. After the melt had cooled it was dissolved in water and made up to 500 cc. Twenty five cc. of the solution, neutralized roughly with ammonia and filtered, made up to 200 cc. gave a solution of suitable dilution for nephelometric work. As this gave a suspension slightly stronger than the standard used, it was put on the right side of the instrument and the standard on the left was used as an "unknown." The ratio of the diluted urine to the standard is therefore equal to the reciprocal of  $x$ , in this case.

Titration<sup>3</sup> with uranium acetate gave 0.053 and 0.054 g. of phosphorus

<sup>3</sup> For making these estimations, I am indebted to Dr. Thomas LeClair of Fraser & Co. Laboratory, New York City.



in 100 cc. of urine. Further work is of course necessary to determine which of these values is the more accurate, and if the conditions for the application of the nephelometric method are satisfactory. It is possible that the ammonia used for neutralization has a slight inhibitory effect, and that sodium or lithium hydroxide will give better results.

TABLE II.

s.	y.	Ratio of solution to standard = $1/x$ .	Mg. P. in 1 cc. solution (1 cc. of standard 0.001034 mg. P).	G. of P in 100 cc. urine (total vol. dilution for 100 cc. = 40.000 cc.).
14.8	(a) {	17.2		
15.0		17.5		
		17.35	Av., 1.218	0.001259
14.9	(b) {	17.4		
14.9		17.2		
		17.30	Av., 1.215	0.001256
	(c) {	17.4		
		17.5		
14.90		17.45	Av., 1.226	0.001267
				0.0504
				0.0502
				0.0507
				0.0504

### VI. Summary.

1. The reagent of Pouget and Chouchak has been modified, so as to be: (a) stable, (b) colorless, (c) quantitatively and (d) nephelometrically applicable.

2. It is shown that 0.005 mg. of phosphorus in 10 cc. of solution, or one part of phosphorus in 2 million parts of water, is easily determined quantitatively with the nephelometer.

NEW YORK, N. Y.

### NOTE.

**Alternating Current Thermoregulators.**—Davis<sup>1</sup> has recently described an alternating current thermoregulator, designed for operation on the usual 110 v., 60 cycle lighting current. In my description of a large incubator<sup>2</sup> a very similar system was described, differing mainly from Davis' in that a 200 ohm, main line, telegraph relay was used to break the circuit, this relay operating in series with a 10 watt lamp and being controlled by a "fire-alarm" thermometer. This system operated very well for several months, the only trouble it gave being that incident to the sparking at the mercury-platinum contact of the thermometer; replacing the thermometer with a thermoregulator of larger bore improved

<sup>1</sup> THIS JOURNAL, 37, 1520 (1915).

<sup>2</sup> J. Ind. Eng. Chem., 6, 939 (1914).