XXXIX. STUDIES ON THE COLORIMETRIC DETERMINATION OF PHOSPHATE

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IN 1914 Taylor & Miller introduced a method for the colorimetric determination of phosphate, based on the reduction of phosphomolybdic acid. The imperfections of the original method led to many modifications by other workers, but, owing to inadequate knowledge of the chemical mechanism of the reaction, these modifications were based largely on methods of trial and error. The following investigations on the mechanism of the reaction were undertaken to serve as a more accurate basis for further improvements of the method.

I. *Reduction of molybdic acid in the absence of phosphate*

Previous modifications consisted largely of the use of new reducing agents, on the assumption that certain reducing agents act specifically on phosphomolybdic acid without reducing molybdic acid itself. (For literature see Tschopp & Tschopp [1932].) This assumption required further investigation.

This was carried out by comparing, over a wide acid range, the effects of different reducing agents on molybdic acid, using a "Spekker" Photoelectric

Fig. 1. Colour intensities at different acidities produced by the reduction of molybdic acid in the absence of phosphate. A, with the reagent of Fiske & Subbarow [1925]. Readings taken after 3 days. B, with SnCl_2 (reagent of Kuttner & Cohen [1927]). Readings taken after 5 min. The intensities of blue colour in Figs. 1-7 inclusive represent actual readings on the "Spekker" Photoelectric Absorptiometer without filter.

Absorptiometer without filter for estimating the intensities of blue colour production. Fig. 1 shows the results with two reducing agents (stannous chloride

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as used by Kuttner & Cohen [1927] and 1: amino-2: naphthol-4: sulphonic acid as used by Fiske & Subbarow [1925]). In these and other experiments with different reducing agents it was found that, given sufficient time for the reaction to be completed, most reducing agents will, under suitable conditions, reduce molybdc acid in the absence of phosphate.

The velocity of the reduction is greatest at about $0.2\text{ }N\text{ H}_2\text{SO}_4$, being low in alkaline and neutral solutions and rapidly diminishing to zero in strongly acid solutions. At $0.2\text{ }N$, all available reducing agent is used up provided that there is sufficient molybdate present.

In solution, molybdc acid exists in many forms, but these are not all reduced equally readily [Britton & German, 1930]. The equilibrium depends on the acidity and on the total amount of molybdate present, and therefore the reducibility of molybdc acid is also a function of the acidity and molybdate concentration. The velocity of the reduction is similarly dependent on these factors, and also on the concentration and nature of the reducing agent.

The supposed specificity of certain reducing agents for phosphomolybdc acid appears therefore only in high acid concentrations.

II. Acceleration of the reduction of molybdc acid by phosphate

The effect of phosphate on the rate of reduction of molybdc acid was investigated at different acidities with different reducing agents, for 0, 10, 40, 100 and 500γ of phosphorus per 10 ml. of total fluid, and the intensities of blue colour which developed were determined after different time intervals. The

Fig. 2.

Fig. 3.

Fig. 2. Colour production at different time intervals, using 0.4 ml. of Fiske & Subbarow's reagent per 10 ml. at $0.2\text{ }N\text{ H}_2\text{SO}_4$.

Fig. 3. As Fig. 2 at $0.5\text{ }N\text{ H}_2\text{SO}_4$.

results of these experiments are shown in Figs. 2, 3, 4 and 5, from which the following conclusions may be reached.

(a) The rate of reduction of molybdc acid is greatly accelerated by the presence of phosphate.

(b) The acceleration occurs over a very wide acid range, but diminishes as the acidity increases (*v.* also Fig. 7).

(c) The end points may not be reached till after 30 min. or more at 0.5 *N* (Fig. 3) or after several days at *N* (Figs. 4 and 5). (With strong reducing agents such as stannous chloride, the end points are reached too rapidly for the velocity to be determined with accuracy.)

Fig. 4. As Fig. 2 at 1 *N* H₂SO₄.

Fig. 5. As Fig. 4 using HI as reducing agent (1 ml. of 10% KI per 10 ml.).

(d) At acid ranges where molybdic acid is itself reduced, the apparent end point may be followed by a further increase in colour (Fig. 2). The latter is due to the "non-specific" reduction of molybdic acid.

(e) The nature and concentration of reducing agent greatly influence both the accelerating effect of phosphate and the time required to reach equilibrium.

Since the effects of changes in acidity and in molybdate concentration are closely related (see end of section III), it follows that both the acceleration and the time required to reach equilibrium are also influenced by the concentration of molybdate.

III. *End points in the reduction of molybdic acid in the presence of phosphate*

When working in an acid range in which "non-specific" reduction is negligible, the end points reached are, with any given reducing agent, approximately proportional to the amount of phosphate present. But with different reducing agents different end points are obtained for the same amount of phosphate. The stronger the reducing agent the deeper the blue colour produced.

In order to be able to calculate the actual number of molecules of molybdic acid reduced per atom of phosphorus, the following experiments were undertaken on the relation between the amount of reduction and the intensity of blue colour produced.

Two different methods were used for estimating the amount of reduction. The results obtained by the two methods were in close agreement.

(1) The blue compound (produced by stannous chloride at *N*) was extracted with butyl alcohol, washed free from reducing agent, and titrated with *M*/100 potassium permanganate, the end point being the disappearance of blue colour in the butyl alcohol layer and the persistence of a violet colour in the aqueous layer.

(2) Krumholz's method. The reducing agent used was hydriodic acid, the iodine liberated being titrated in carbon tetrachloride with *M*/200 sodium thiosulphate.

It has been established [Jakób & Koslowski, 1929; Hein *et al.* 1936] that the blue compound formed is a combination of 6-valent and 5-valent molybdenum. Using therefore the schematic equation

the titration results could be expressed in terms of molecules of MoO_3 reduced. Fig. 6 shows the titration values plotted against the intensity of blue colour.

Fig. 6. Relation between titration values (represent amounts of reduction) and colour production.

From this it is possible to calculate the number of molecules of Mo^{VI} reduced to Mo^{V} under different conditions. This is summarized in Table I.

Table I

Reducing agent	Acidity	Time	No. molecules MoO ₃ reduced per atom of P
Potassium iodide	1 N H ₂ SO ₄	10 min.	0
	1 N H ₂ SO ₄	24 hr.*	2
Reagent of Fiske & Subbarow	0.5 N H ₂ SO ₄	30 min.*	2
	1 N H ₂ SO ₄	30 min.	0.7 (approx.)
	1 N H ₂ SO ₄	3 days*	4
Reagent of Kuttner & Cohen	1 N H ₂ SO ₄	10 min.*	9

* Equilibrium reached.

With HI as reducing agent, 2 mol. of MoO₃ are reduced by one of phosphorus. This is in agreement with the results of Krumholz [1933]. With Fiske & Subbarow's reagent at 0.5 N (at which the test is normally carried out), 2 mol. are also reduced, while at N twice as many molecules are reduced, but at this acidity the velocity of the reaction is too slow for practical application (over 3 days). With SnCl₂ as used by Kuttner & Cohen, as many as 9 mol. are reduced. (The value of 6 mol. obtained by Hein *et al.*, was due to the reduction being carried out on 12-phosphomolybdic acid without any excess of molybdic acid. Under such conditions, the theoretical maximum cannot be more than 6.)

Since stannous chloride is one of the strongest reducing agents available, and since higher concentrations than those used above tend to cause reduction beyond the 5-valent state, it would appear that the limit of sensitivity is reached in the method of Kuttner & Cohen. (Stannous chloride was originally used by Denigès [1920], but the method was greatly improved by Kuttner & Cohen [1927].)

Fig. 7. Colour-acidity curves with stannous chloride.

The relation between end points and acidity was studied mainly with SnCl₂, as equilibrium is reached very rapidly with this reagent. In Fig. 7 curves are

given showing the colour intensities produced with different amounts of phosphate at different acidities. Two points of interest arise out of this:

(1) The existence of a very narrow acid zone (around 1.1 *N*) within which the reaction may be used for the quantitative determination of phosphate. A greater acidity causes marked diminution in colour; less acidity causes colour to appear in the control. (With weaker reducing agents, the margin of safety is greater.)

(2) At 0.2 *N*, at which intense colour develops in the control, the presence of phosphate causes a diminution in colour intensity, due evidently to the fact that some of the reducible molybdic acid, combined with phosphoric acid, is no longer available for reduction.

The curves in Fig. 7 were obtained from experiments in which the concentration of ammonium molybdate was 0.5%. With lower concentrations, the curves remain similar in type but are all moved to the left. Thus changes in acidity and in molybdate concentration have opposite effects on the equilibria which exist in solution between the different forms of molybdic and phosphomolybdic acids.

Fig. 8. Intensities of yellow colour (production of yellow phosphomolybdic acids) at different acidities. The intensities of yellow colour represent readings on the instrument, using a purple filter.

IV. *The reducible phosphomolybdic acids*

The compounds between phosphoric acid and molybdic acid have been investigated by several workers. (For literature see Gmelin [1935].) Many such compounds are known to exist, two main types being described, the colourless and the yellow phosphomolybdic acids. Little is known of the former except that they exist both in neutral and in strongly acid solutions. Of the yellow compounds, two have been isolated, the 12 (24) and the 9 (18) phosphomolybdic acids. In alkaline solutions, all phosphomolybdic acid complexes are almost completely dissociated.

Wu [1920] has studied the reducibility of these compounds, by isolating them in a pure state, bringing them into solution and adding reducing agents. It is known, however [Krumholz, 1933], that, in solution, the different phosphomolybdic acids all exist in a state of equilibrium, which is a function of several factors (acidity, concentration, temperature etc.). So that, on theoretical grounds, Wu's conclusion, that one of the yellow phosphomolybdic acids is more reducible than the others, is untenable.

It is usually assumed that one of the yellow phosphomolybdic acids is involved as an intermediate product in the colour reaction for phosphates. However, in Fig. 8, showing the intensities of yellow colour which develop at different

acidities, it is seen that no yellow colour is detectable at *N* (at which reduction easily occurs in the presence of phosphate by the method of Kuttner & Cohen), or on the alkaline side (at which reduction was carried out originally by Taylor & Miller). However, this evidence is not conclusive, and the problem can only be solved by a quantitative study of the complicated equilibria between the different forms of molybdic and phosphomolybdic acids.

The formation of a blue colour by the reduction of phosphomolybdic acid, has been used by Folin and others as a method for the quantitative determination of reducing agents (sugars, uric acids etc.). It follows from the above experiments that, at the correct acidity, molybdic acid without phosphate can be used for such determinations.

V. *Interferences in the reaction*

Many substances have been described which interfere with the colorimetric tests for phosphates. These can now be classified as follows.

A. Substances which alter the acidity of the medium, thereby altering the amount of reducible molybdic acid: i.e. acids, alkalis and buffers.

B. Substances which form molybdenum complexes which are difficult to reduce, thereby diminishing the amount of molybdic acid available for reduction: e.g. fluorides, citrates, oxalates etc.

C. Substances which change the concentration of reducing agent: e.g. nitrites, hypochlorites etc.

Additional evidence in support of this scheme of classification was obtained from experiments in which different inhibitors were added while the acidity, concentration of molybdate or of reducing agent were varied. It was found, for instance, that small amounts of inhibitors from group B caused the acid curve (Fig. 7) to be moved to the left, indicating that the available amount of reducible molybdic acid was diminished.

It has been shown [v. Gmelin, 1935] that complexes of the type of group B are also formed with chlorides and sulphates. It follows, therefore, that the decrease in the reduction velocity of molybdic acid at high acidities is not merely an effect of H^+ ions but is also due partly to the formation of hetero-polyacids with the particular acid present.

VI. *Mechanism of the reaction*

Since phosphates accelerate the velocity of reduction of molybdic acid, the process may be considered as catalytic. This view, originally suggested by Feigl [1922] (v. also Feigl & Krumholz [1929]), is confirmed by the present results.

The catalytic theory is incomplete, however, because, with a given amount of phosphate, a definite limit to the amount of colour production is reached, even in the presence of excess of reducing agent. No such limit should exist unless, as suggested by Krumholz, the catalyst is made ineffective during the course of the reaction. That this occurs, is confirmed by the following experiment. The blue compound, produced with stannous chloride in the presence of phosphate, was destroyed by titration with potassium permanganate; on further addition of stannous chloride, the original intensity of blue colour reappeared. Thus, by breaking up the blue compound, the phosphate can activate once again the reduction of Mo^{VI} .

The formation of a phosphomolybdic acid as an intermediate product naturally suggests itself. No such compound with more than 12 mol. of molybdic

acid per atom of phosphorus has yet been described. Since it is shown above that 9 mol. can be activated by one of phosphorus, one must assume either the existence of a phosphomolybdic acid with at least 18 mol. of molybdic acid in the complex, or else that excess of molybdic acid from the solution is taken into the complex molecule to replace the reduced molybdic acid.

The catalytic theory is interesting from the biochemical standpoint, in view of the importance of phosphoric acid in oxidation-reduction systems. Further investigation of the mechanism of this reaction may therefore prove of importance.

VII. *Practical applications*

The belief in the existence of certain reducing agents which act specifically on phosphomolybdic acid, is shown to be untenable. From the point of view of sensitivity, the ideal reducing agent is one which will reduce the maximum number of MoO_3 mol. per atom of phosphorus. This appears to be achieved by dilute stannous chloride. However, in addition to sensitivity, the method must have a wide margin of safety as regards acidity, concentration of reagents and potential inhibitors.

Of the two most frequently used methods for the colorimetric determination of phosphates, that of Kuttner & Cohen is very sensitive, but it loses in safety what it gains in sensitivity; that of Fiske & Subbarow allows a wider variation in acidity and concentration of reagents, but it is less sensitive and is affected by changes of temperature. Interfering substances affect both methods, but to different degrees [Berenblum & Chain, 1938].

From the present investigations, it is possible to devise conditions for a method of high sensitivity which would avoid most of the disadvantages of the previous tests. A method based on these considerations is described in the following communication [Berenblum & Chain, 1938].

SUMMARY

Investigations were carried out on the reduction of molybdic acid, on which the colorimetric determination of phosphate depends. All the reducing agents tested reduce molybdic acid in the absence of phosphate. The reduction velocity is greatest at about 0.2 *N*, and diminishes rapidly to zero with increasing acidity. Phosphates accelerate the rate of reduction of molybdic acid. This acceleration occurs over a wide acid range, but decreases with increasing acidity.

The rate of reduction of molybdic acid, the accelerating effect of phosphate and the end points reached in these reactions are all dependent on the concentration of molybdate and on the concentration and nature of the reducing agent. The stronger the reducing agent, the greater the number of molecules of MoO_3 reduced per atom of phosphorus.

The theory that the reduction of molybdic acid is catalytically activated by phosphate, has been confirmed. The reaction does not go to completion, however, but reaches definite end points, because the product of the reduction inactivates the catalyst.

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