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A New Technique for the Determination of Phosphorus by the Molybdenum Blue Method

By W. I. M. HOLMAN, *From the Rowett Institute, Aberdeen*

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Numerous methods are available for the colorimetric determination of phosphorus by molybdenum blue. A number of reducing agents have been applied, but no method can be regarded as entirely satisfactory. The methods using $SnCl_2$ have the advantage of high sensitivity, but in general do not obey Beer's Law over a wide range, and the variation in tint at different intensities is sufficient to cause difficulty in matching. Bodansky [1932] corrected for the deviation from Beer's Law in the method of Kuttner & Cohen [1927]. When absolute colour measurement is adopted difficulty is experienced in controlling the colour development and in eliminating variation caused by instability of the colour with time and with changes in room temperature. In methods using other reducing agents these disadvantages are reduced, but the sensitivity is also diminished. The method of Fiske & Subbarow [1925] is probably the most popular and, even though the colour is not quite stable, Allen [1940] has found that a Pulfrich photometer can be used for matching. Probably the only method which gives a stable colour under conditions which are easy to control is that of Zinzadze [1935] using metallic Mo as the reducing agent. A recent review of methods is given by Woods & Mellon [1941].

Arising from an observation that KI in acid solution is a suitable reducing agent for phosphomolybdic acid, a new method has been developed which has none of the above disadvantages and is simple to operate. The range is 1–100 μg. P and, where applicable, it gives a higher degree of accuracy than existing methods. The sensitivity is almost three times that of the Fiske-Subbarow method. Wu [1920] suggested the use of HI as a reducing agent for phosphomolybdic acid and devised a rough test. Berenblum & Chain [1938] used HI in a study of the mechanism of the reduction to Mo blue.

Suitable conditions for reduction to Mo blue and for removal of liberated I_2 were determined. A temperature of 100° was found to be suitable for the development of the colour. The colour intensity increases with the amount of molybdate used, but the 'blank' colour increases in the same manner. The 'blank' can be reduced by increasing the acidity or by using less reducing agent, but if the acidity is too high the colour fades after reaching its maximum intensity at 100°. If too little reducing agent is present the colour is not stable. Suitable concentrations of reagents were ascertained. $Na_2S_2O_3$ cannot be used to remove the liberated I_2 owing to precipitation of S, but Na_2SO_3 was satisfactory. Only a small excess is permissible, otherwise it imparts a yellow colour to the solution. The H_2SO_4 and $(NH_4)_2MoO_4$ must be added separately since a combined reagent gives a high and variable 'blank'. The solution of KI keeps satisfactorily if it contains a small amount of Na_2CO_3 to prevent liberation of free HI.

METHODS

Reagents

(1) *10N H_2SO_4.*

(2) *2·5% ammonium molybdate solution.* Dissolve 25 g. $(NH_4)_2MoO_4$ in distilled water by warming. Cool and dilute to 1 l. Preserve the solution under liquid paraffin to prevent diffusion into the air of the NH_3 liberated by salt hydrolysis. An aspirator bottle may be used, which has been fitted with a well-greased tap at the base and the inside of which has been coated with a thin film of liquid paraffin, to prevent the solution wetting the sides.

(3) *20% KI solution.* Dissolve 200 g. KI and 5 g. Na_2CO_3 in distilled water and dilute to 1 l. The solution is stable for at least 3 months and probably indefinitely.

(4) *0·5% Na_2SO_3 solution.* Dissolve 1 g. $Na_2SO_3.7H_2O$ in distilled water and dilute to 200 ml. Prepare the solution freshly each day.

(5) *Standard P solution.* Prepare a stock solution containing 1 g. P/l. by dissolving 4·3885 g. pure dry KH_2PO_4 in distilled water, adding 2 ml. concentrated H_2SO_4 as a preservative before diluting to 1 l. To prepare a working standard, dilute 5 ml. of the stock solution to 250 ml. with distilled water. This solution contains 20 μg. P/ml.

Procedure

Transfer a sample containing 1–100 μg. P to a 10 ml. volumetric flask or to a 25 ml. test tube provided with a stopper and marked at levels corresponding to exactly 5 and 10 ml. If the solution is acid or alkaline add phenolphthalein and neutralize with *N*/10 NaOH or *N*/10 HCl; then add H_2O to give exactly 5 ml. Add 1 ml. 10*N* H_2SO_4, 1 ml. $(NH_4)_2MoO_4$ and 1 ml. KI solution, in the order named and mixing the contents after each addition. Transfer to a briskly boiling water-bath, using an inverted specimen tube to cover the neck of the flask or tube. After exactly 15 min. transfer at once to cold running water. Replace the stopper. After cooling, add Na_2SO_3 from a burette, 0·1 ml. at a time, mixing after each addition, until all the liberated I_2 has been removed and the solution is a pure blue. The end-point is very sharp in daylight or when a daylight lamp is used, but yellow light obscures it somewhat. Then add 0·2 ml. Na_2SO_3 in excess. Make the volume to 10 ml. with H_2O and mix. Match the colour by means of a photoelectric absorptiometer or a visual colorimeter.

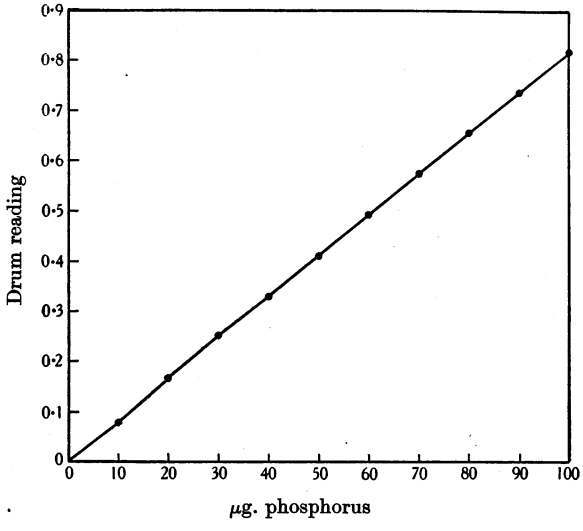

Fig. 1. Typical calibration curve, using the Spekker photoelectric absorptiometer.

If a Spekker photoelectric absorptiometer is used it is necessary to prepare a 'blank' from distilled water treating it in the same way as the test solution. Place red glass filters (no. 1) in front of the two photocells and a 1 cm. glass cell containing the test solution in front of the right photocell. Set the drum to zero and bring the galvanometer reading to zero by adjusting the shutter aperture in front of the left photocell. Then place a 1 cm. glass cell containing the 'blank' in front of the right photocell and again bring the galvanometer reading to zero by rotating the drum, while keeping the shutter aperture constant. Record the drum reading. Switch off the lamp in the interval between each of a series of matchings to prevent fatigue in the photocells, but not in the interval between the two operations described above which comprise a single matching. To obtain a calibration curve from which the amount of P in the test solution can be read match a series of standards against the 'blank' and plot the drum readings against the corresponding amounts of P. The curve should be prepared by each worker for his own instrument and checked periodically. Colours giving drum readings greater than about 1 are too intense for accurate results to be obtained and, if 1 cm. glass cells are used, only the range 1–30 μg. P can be covered.

To cover the range 30–100 μg. P the colour must be diluted to one-third of its intensity if 1 cm. glass cells are to be used. After developing the colour remove the liberated I_2 with 0·5% Na_2SO_3 and add 0·5 ml. in excess. Dilute to 30 ml. instead of 10 ml. and match against a 'blank' prepared in the same way. A calibration curve using this procedure is shown in Fig. 1.

If a visual colorimeter is used for matching dilution to 30 ml. is not required. It is necessary, however, to correct for the 'blank' and for this reason a 'blank' and a standard containing 1 μg. P should be prepared. Match the 'blank' against the standard and calculate the amount of P to which its colour is equivalent as follows:

$$c = \frac{b}{(a-b)},$$

where c = μg. P to which 'blank' is equivalent; a = length of column of 'blank'; b = length of column of 1 μg. standard which matches a.

Make the following correction for the 'blank' when calculating the result for the test:

$$x = \frac{b(y+c)}{a} - c,$$

where x = μg. P present in test solution; y = μg. P present in standard; c = μg. P to which 'blank' is equivalent; a = length of column of test solution; b = length of column of standard which matches a.

DISCUSSION

Order of accuracy of the method

The colour is stable, no difference in intensity being detectable when standards were kept up to 24 hr.

in stoppered test tubes or when the temperature was varied from 12 to 32°. After keeping for some days the excess of Na_2SO_3 gradually becomes oxidized and further I_2 is liberated thus altering the colour. Small variations in the time of development of the colour at 100° have little effect on the intensity. The variations when the time was increased or decreased by 5 min. are shown in Table 1. The average error per min. of deviation from the standard time of 15 min. is 0·3 %.

Table 1. *Effect of time of heating on the development of the colour*

Time of heating min.	P present μg.	P found μg.	Error %
10	10	9·7	−3·0
10	50	49·2	−1·6
10	100	98·0	−2·0
15	10	10·0	Nil
15	50	50·0	Nil
15	100	100·0	Nil
20	10	9·8	−2·0
20	50	50·0	Nil
20	100	101·0	+1·0

When a Spekker photoelectric absorptiometer is used for matching, results accurate to within 2 % can be obtained for the range 3–100 μg. P. With special care the error may be reduced still further. Fig. 1 shows that the relationship between drum reading and amount of P is very nearly linear. Deviations from strict linearity are attributable to the photocells themselves as well as to the colour.

When a visual colorimeter is used and a correction is made for the 'blank', Beer's Law is obeyed closely enough for reasonable accuracy to be obtained even when the test solution is widely different from the standard. A high degree of accuracy can be assured if each test solution is matched against a standard which does not differ from it by more than about 30 %. With less than 5 μg. P the colours are too weak for accurate matching, and the deviation from Beer's Law is greatest in this region. Results for a series of standards containing widely different amounts of P are shown in Table 2. The colours were matched by means of a Klett colorimeter.

Table 2. *Order of accuracy with a visual colorimeter*

P present μg.		Relative lengths of columns		P found μg.	Error %
Test	Standard	Test	Standard	Test	
'Blank'	1	40·0	25·0	1·7*	—
10	50	30·0	6·7	9·84	−1·6
10	100	30·0	3·5	10·16	+1·6
50	10	6·7	30·0	50·7	+1·4
50	100	10·0	5·1	50·2	+0·4
100	10	3·5	30·0	98·6	−1·4
100	50	5·1	10·0	99·7	−0·3

* This value was used to correct for the 'blank'.

Application of the method

Salts likely to be present in solutions to be tested were included in standards in various concentrations before development of the colour. The maximum amount which may be present in the sample without affecting the colour was determined in each case (Table 3).

Table 3. *Maximum amounts of various salts which may be present without affecting the result*

Salt	Maximum amount permissible (mg.)	
$FeCl_3$	0·1	Fe^{+++}
$CaCl_2$	2	Ca^{++}
$MgCl_2$	40	Mg^{++}
NaCl	100	Cl^-
Na_2SO_4	10	$SO_4^=$
Na acetate	5	CH_3COO^-
Na trichloracetate	100	CCl_3COO^-
Na citrate	5	$C_6H_5O_7^{\equiv}$

These results were obtained when a single salt was present and do not necessarily apply to mixtures of salts. Thus, a mixture consisting of the maximum amounts of all these salts was found to reduce the colour intensity by approximately 4 %. It is therefore advisable, before applying the method to any particular type of solution, to make certain that the recovery of added P is complete, even though the amounts of salts present do not exceed the limits given in Table 3.

Not more than a trace of Cu^{++} may be present, owing to precipitation of CuI with liberation of I_2. The maximum amount which may be present was found to be 200 μg. Cu^{++}.

Since As and Si produce a blue colour in the same way as P, the maximum amounts which may be present without affecting the test were determined and are for Na_3AsO_4 1 μg. As, and for waterglass 10 μg. SiO_2.

The method is suitable for the analysis of HCl extracts of residues from dry ashing or Na_2CO_3 fusion. It cannot be used to distinguish between inorganic and organic P compounds. Several organic P compounds were found to be hydrolysed under the conditions of the test.

SUMMARY

A new technique has been developed for the determination of phosphorus by molybdenum blue, using potassium iodide as reducing agent. The range of the method is 1–100 μg. P. The colour is stable and obeys Beer's Law closely if a correction is made for the faint colour present in the 'blank'.

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Oxidizing Enzymes and Vitamin C in Tomatoes

By F. WOKES and JOAN G. ORGAN, *From Ovaltine Research Laboratories, King's Langley, Herts*

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Sixteen years ago Willimott & Wokes [1926 *a*, *b*, *c*] studied the occurrence of oxidizing enzymes and vitamin C in the peel and juice of citrus fruits. The enzymes were found to be present in highest concentration in the rind. When lemon rind was being assayed for vitamin C, in order to avoid destruction of the vitamin by these enzymes, the rind was cut into small pieces after removal, weighed, and mixed with 90% ethanol to prepare a tincture for administration to guinea-pigs. This tincture failed to prevent scurvy when administered as the sole source of vitamin C in daily doses equivalent to 0·5–1 g. of the rind, although the onset of symptoms was slower than in negative controls, so lemon rind was assumed to contain only slight amounts of the vitamin as compared with the juice, which has a considerable amount.

Later, when a satisfactory chemical test for vitamin C had become available, this was applied by Bacharach, Cook & Smith [1934] to orange and lemon rinds. Orange rind, which Willimott & Wokes [1926*c*] had reported to contain only moderate amounts of the vitamin, was found to contain a concentration of vitamin C several times that of the juice, whilst lemon rind also contained more than lemon juice. Bacharach *et al.* [1934] suggested that the discrepancy between their results and those of Willimott & Wokes might be due to loss of vitamin during preparation of the tincture, but made no reference to the oxidizing enzymes. Willimott & Wokes had drawn attention to these enzymes, but assumed that the use of 90% ethanol, in which the enzymes would not be soluble, would prevent their destroying the vitamin. Only within the last few months has it been realized that when these enzymes are set free by mincing the tissues a considerable amount of the vitamin may be destroyed in a few minutes.

In the interval between the appearance of the papers of Willimott & Wokes [1926 *a*, *b*, *c*] and that of Bacharach *et al.* [1934] Szent-Gyorgyi [1931] showed that his 'hexuronic acid' was rapidly destroyed by an oxidizing enzyme when the inner leaves of cabbage were minced. Qualitative tests indicated that most of the 'hexuronic' acid had disappeared within 5 min. of completion of mincing. The enzyme was named hexoxidase because it attacked hexuronic acid, but since the latter is now known as ascorbic acid the enzyme is now known as ascorbic acid oxidase.

Kohman, Eddy & Gurin [1931] reported that minced carrots lost much of their antiscorbutic activity on standing in air for 1 hr., but biological assays did not prove suitable for investigating this problem. Zilva [1934], using both biological and chemical methods, showed that apple juice contains an enzyme which destroys the vitamin C in lemon juice, approximately 84% being lost at *p*H 4·4 and 57% at *p*H 3·0, both in 6 hr. at room temperature, and that, as might have been deduced from the findings of Willimott & Wokes, the juice is a less potent source of the enzyme than the peel. However, during the following 8 years most workers on this enzyme neglected fruit and concentrated on vegetables. Thus Ahmad [1935] showed by chemical assays that 11% of the vitamin C in karela was lost in 10 min. and 17% in 20 min. when this common Indian vegetable was shredded. McHenry & Graham [1935] obtained similar results with shredded turnips, parsnips and cauliflower. Tauber, Kleiner & Mishkind [1935] found the enzyme in *Cucurbita maxima* and named it ascorbic acid oxidase. Hopkins & Morgan [1936] extracted the enzyme from cauliflower florets and showed that the enzyme extract would destroy about 70% of added ascorbic acid in 5 min. at *p*H 6 and 18°. It was apparent from their results that if the ratio of enzyme to ascorbic acid had been similar to that which occurs naturally even more rapid destruction of the vitamin would have occurred. Kertesz, Dearborn & Mack [1936] showed that when cabbage was ground with an equal weight of water it lost about 97% of its ascorbic acid content in 1 hr. and reported similar results with other vegetables. Johnson & Zilva [1937] expressed the juice from frozen cabbage, cauliflower, cucumber and marrow but found that the destruction in 1 hr. of ascorbic acid added to cauliflower juice was only 33% at *p*H 5 and 35% at *p*H 7, much slower than the rate found by Hopkins & Morgan [1936]. They confirmed Hopkins & Morgan's findings, however, that the optimum *p*H for the enzyme is about 6, and showed that at *p*H 3·0 the activity was very slight, but at *p*H 9·0 still fairly pronounced. Stone [1937] minced vegetables containing vitamin C, and showed that in cabbage juice a loss of 98% of free ascorbic acid and about 63% of total ascorbic acid occurred in 5 min. Like Kertesz *et al.* [1936] he found that the rate of destruction in spinach was much slower. Lampitt & Baker [1942] observed that when orange