Some Observations on the Role of Copper Ions in the Reduction of Phosphomolybdate by Ascorbic Acid and their Application in the Determination of Inorganic Orthophosphate

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The reduction of molybdate in the presence of phosphate gives rise to a blue compound, and under suitable conditions the colour intensity is proportional to the amount of orthophosphate present. This principle forms the basis of several methods which have been described for the determination of phosphate and a variety of reducing agents has been used for this purpose. In the method of Lowry & Lopez (1946) ascorbic acid is used at pH 4, in contrast to the strongly acid conditions used in most other methods, and in addition a relatively low molybdate concentration is employed. This reduces the breakdown of labile phosphates such as creatine phosphate, acetyl phosphate and ribose 1-phosphate, which is otherwise very rapid (Fiske & Subbarow, 1929; Lipmann & Tuttle, 1944; Lowry & Lopez, 1946), to such an extent that it can be corrected for. This method thus permits the determination of inorganic phosphate in the presence of labile phosphate esters.

Difficulty was experienced in applying the method as described by Lowry & Lopez to the determination of phosphate in reaction mixtures containing extracts of a rumen micro-organism. Similar observations were made in the presence of heat-coagulated potato extracts, and Lowry & Lopez (1946) observed a slight interference with concentrated preparations of liver and brain extracts. The results presented in this paper indicate that the reduction of phosphomolybdate by ascorbic acid requires copper ions and show that the process is powerfully inhibited by metal-binding agents; a suitable modification to the method as described by Lowry & Lopez is suggested. A preliminary account of part of this work has already been given (Peel, Fox & Elsden, 1955).

Since this work was completed, Bruemmer & O'Dell (1956) have reported that phosphomolybdate reduction is inhibited by the reduced, but not the oxidized, forms of glutathione and cysteine, and that copper sulphate reverses these inhibitions and also that due to liver extract. Their results, which are in agreement with our own, are referred to in more detail below.

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MATERIALS AND METHODS

Bacterial extract. This was prepared from the rumen organism LC1 described by Elsden, Volcani, Gilchrist & Lewis (1956). The organism was grown on a medium containing 1·4% (w/v) of sodium lactate and 1% (v/v) of corn-steep liquor; growth conditions and the preparation of extracts of vacuum-dried cells were essentially the same as those described by Stadtman & Barker (1949) for *Clostridium kluyveri*. The full details will be given in a separate paper and need only be considered briefly. The main factor of interest to the present investigation is that the dried cells were extracted with 0·01 M potassium phosphate buffer, pH 6·8, with the addition of 0·03% $Na_2S,9H_2O$ to maintain anaerobic conditions. The clear supernatant after centrifuging will be referred to as 'bacterial extract'. For certain experiments the extract was deproteinized by the addition of 7 ml. of the ammonium sulphate–acetate reagent of Lowry & Lopez (1946) for each millilitre of bacterial extract. The supernatant obtained after centrifuging will be referred to as 'deproteinized bacterial extract'.

Liver and brain extracts. The two tissues were provided by Dr R. Whittam from a freshly killed guinea pig and samples were homogenized with 0·125 M sodium acetate buffer, pH 4, in the apparatus of Potter & Elvehjem (1936), made of stainless steel. With liver, 10 g. of tissue was homogenized with 40 ml. of buffer for 4 min.; with brain, 4 g. of tissue was homogenized with 20 ml. of buffer for 1 min. After removal of the coarser debris by centrifuging at 1800 *g* for 10 min., the supernatant was deproteinized by saturating with $(NH_4)_2SO_4$ and recentrifuging at 9000 *g* for 20 min. The supernatants, which will be referred to as 'deproteinized extracts', were stored at 2° and used within a few days.

Chemical reagents. All inorganic chemicals except Cu_2O were of A.R. grade. Cuprous oxide and ascorbic acid were obtained from J. Preston Ltd. (Sheffield), cysteine hydrochloride from Light and Co. Ltd. and the ethylenediaminetetraacetic acid and sodium diethyldithiocarbamate were of laboratory reagent grade obtained from British Drug Houses Ltd. and Hopkin and Williams Ltd. respectively. Lithium acetyl phosphate was prepared with the help of

Dr G. C. N. Jayasuriya from *iso*propenyl acetate by the method of Stadtman & Lipmann (1950). This preparation had been stored for a considerable time and was contaminated with inorganic phosphate. Solutions were assayed by the method of Lipmann & Tuttle (1945).

Cuprous chloride reagent. Cuprous oxide (0·5 g.) was dissolved in approx. 10 ml. of hot N-HCl and the solution cooled to precipitate the Cu_2Cl_2. The precipitate was filtered off, washed with a little water and then used to saturate a sample of 0·125 M sodium acetate buffer, pH 4.

Reduction of phosphomolybdate. Formation of the blue chromogen by the reduction of phosphomolybdate was studied under the conditions described by Lowry & Lopez (1946). Phosphate, as KH_2PO_4, was contained in 0·5 ml. or less, and to this were added other substances as indicated, followed by 0·125 M sodium acetate buffer, pH 4, to a total volume of 5 ml., and 0·5 ml. of 1% (w/v) ascorbic acid. The reaction was started by adding 0·5 ml. of 1% (w/v) ammonium molybdate $[(NH_4)_6Mo_7O_{24}, 4H_2O]$ in 0·05 N-H_2SO_4. The course of the development of the blue colour was followed with the EEL photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, Essex) with the red filter (no. 205) supplied with the instrument. Colour intensities were measured relative to a blank cell containing water and are expressed in terms of the scale readings of this instrument (range 0–100 scale units) which are directly proportional to optical density.

When phosphate determinations were carried out on incubation mixtures, these were first deproteinized with ammonium sulphate–acetate reagent in the same way as the bacterial extract described above. Where acetyl phosphate was present, colour intensity was measured 10 and 20 min. after addition of the molybdate reagent and the intensity at zero time, obtained by extrapolation, taken as the measure of true inorganic phosphate (cf. Lowry & Lopez, 1946). The rate of colour development was found to vary with different batches of reagents and even by as much as 25% with the same reagents on different days (cf. the various experiments of Table 1). All operations were carried out at uncontrolled room temperature (approx. 20°) and temperature variations may therefore account for this latter effect, since Lowry & Lopez (1946) observed that at 35° colour development was complete in one-third of the time required at 25°, and we have observed an approximate doubling of the rate of colour development between 15° and 25°.

RESULTS

Inhibition of phosphomolybdate reduction by bacterial extract. The present detailed investigations arose after attempts to determine inorganic phosphate in an incubation mixture resulting from the action of an extract of the rumen organism LC 1 on pyruvate (Peel, 1956). It was known that a considerable amount of inorganic phosphate was present, and colour development during the assay was expected to be completed within 5 min. After 10 min., however, no measurable colour had developed, though a marked colour did appear after 30 min. It was concluded that some component of the incubation mixture was interfering with colour development and the trouble was traced to the bacterial extract

The effect of a quantity of deproteinized bacterial extract, equivalent to that present in the assay on the incubation mixture, is shown in Fig. 1. In the absence of interfering substances (curve *A*), colour development was linear with time and with 20 μg. of P reached a maximum value in 4–8 min. Thereafter only a very slow change, an increase of approx. 1 scale unit/hr., was observed. In the presence of bacterial extract the rate of colour development remained linear with time but was considerably depressed, and the reaction was not complete after 10 min. (curve *B*, Fig. 1). Similar effects have been observed with heat-coagulated extracts of potato, and by Lowry & Lopez (1946) in the presence of relatively high concentrations of liver and brain extracts, though the bacterial extract was a far more potent inhibitor than these other preparations.

Inhibition by sulphide. By comparison with other extracts, the most unusual feature of the bacterial extract appeared to be the presence of 0·03% of sodium sulphide. The effect of a quantity of sulphide equal to that originally present in the bacterial extract tested was therefore examined and complete inhibition of colour development observed (curve *C*, Fig. 1). Further experiments showed that the inhibition was complete with a sulphide concentration as low as 5×10^{-7} M (Expt. 1, Table 1),

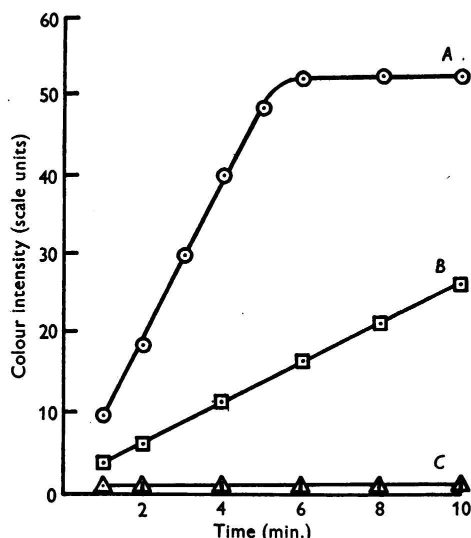


Fig. 1. Effect of deproteinized bacterial extract and of sodium sulphide upon phosphomolybdate reduction. Tubes contained: 0·5 ml. of KH_2PO_4 containing 20 μg. of P, 0·5 ml. of deproteinized bacterial extract or $Na_2S,9H_2O$ to give the concentrations indicated, 0·5 ml. of 1% ascorbic acid; 0·5 ml. of 1% ammonium molybdate in 0·05 N-H_2SO_4 was added at zero time and 0·125 M sodium acetate buffer, pH 4, to make total vol. 6 ml. Curve *A*, no additions; curve *B*, 0·83% of deproteinized bacterial extract present; curve *C*, $1{\cdot}3 \times 10^{-6}$ M-Na_2S present.

Table 1. *Inhibition of phosphomolybdate reduction by metal-binding agents*

Tubes contained 20 μg. of P and 1 ml. of inhibitor; ascorbic acid, ammonium molybdate and acetate buffer were present as in Fig. 1; total vol. 6 ml. Rates were measured over the linear phase of colour development. The four experiments were done on different days and two different batches of reagents used, one for Expts. 1 and 4 and the other for Expts. 2 and 3.

Expt.	Inhibitor	Concn. of inhibitor (M)	Rate of colour development (scale units/min.)	Percentage inhibition
1	Sodium sulphide	0	9·4	—
		5×10^{-9}	9·3	1
		5×10^{-8}	5·5	41
		5×10^{-7}	0	100
		5×10^{-6}	0·3	97
2	Diethyldithiocarbamate	0	11·0	—
		1×10^{-7}	9·3	15
		2×10^{-7}	8·0	27
		5×10^{-7}	1·3	88
		5×10^{-6}	0·45	96
3	Ethylenediaminetetraacetic acid	0	13·0	—
		5×10^{-7}	9·2	29
		2×10^{-6}	7·3	44
		5×10^{-6}	1·4	89
		5×10^{-5}	0·6	95
4	Cysteine	0	7·3	—
		$1{\cdot}7 \times 10^{-5}$	7·3	0
		$1{\cdot}7 \times 10^{-4}$	2·3	68
		$1{\cdot}7 \times 10^{-3}$	0·95	87

corresponding to 3 μm-moles of sulphide in the mixture. This amount was far less than the amount of phosphate present (645 μm-moles) and therefore suggested that the sulphide interferes with some catalytic process involved in phosphomolybdate reduction.

Inhibition by other metal-binding agents. It was suspected that sulphide might inhibit by binding a metal, and this was confirmed when other metal-binding agents were tested (Table 1). Diethyldithiocarbamate inhibited the rate of colour development almost as effectively as sodium sulphide, and ethylenediaminetetraacetic acid was only slightly less inhibitory, 5×10^{-6} M giving an almost complete inhibition. Cysteine, although a much less powerful complexing agent, nevertheless gave a strong inhibition at concentrations of the order of 10^{-3} M.

Bruemmer & O'Dell (1956) observed a marked inhibition with 8×10^{-5} M cysteine, in agreement with our own results. These authors also noted a comparable effect with glutathione and showed that the oxidized forms of these two compounds did not inhibit. This latter observation is consistent with the idea of inhibition by metal-binding since the free sulphydryl group is known to be important in metal-binding (Albert, 1952).

Effect of added cupric ions on colour development during phosphomolybdate reduction. The oxidation of ascorbic acid by air, both enzymically and non-enzymically, is stimulated by copper ions (see Meiklejohn & Stewart, 1941) and ascorbic acid oxidase is in fact a copper protein (Dawson & Mallette, 1945). It therefore appeared possible that copper ions might be involved in the reduction of phosphomolybdate by ascorbic acid, and their effect upon the system was investigated. The findings were rather complex, three distinct effects being observed as the concentration of cupric ions was increased.

Table 2. *Effect of low concentrations of added copper ions on colour development during phosphomolybdate reduction*

Tubes contained 20 μg. of P and 1 ml. of $CuSO_4$ to give the concentrations indicated; ascorbic acid, ammonium molybdate and acetate buffer were present as in Fig. 1; total vol. 6 ml. Rates were measured as in Table 1.

Concn. of added $CuSO_4$ (M)	Rate of colour development (scale units/min.)	Colour intensity after 10 min. (scale units)
0	10	50
2×10^{-8}	14	50
2×10^{-7}	19	50
2×10^{-6}	35	50
2×10^{-5}	170	55

Copper sulphate up to 2×10^{-5} M caused a marked increase in the initial linear rate of colour development (Table 2). Otherwise the general course of the reaction was unaffected (cf. curve *A*, Fig. 1 and

curve A, Fig. 2). After the initial rapid increase there was no further change with time and the final colour intensity attained after 10 min. was not affected by $CuSO_4$ up to 2×10^{-6} M. With 2×10^{-5} M-$CuSO_4$, the first signs of a second effect began to appear, namely an enhancement of the final colour.

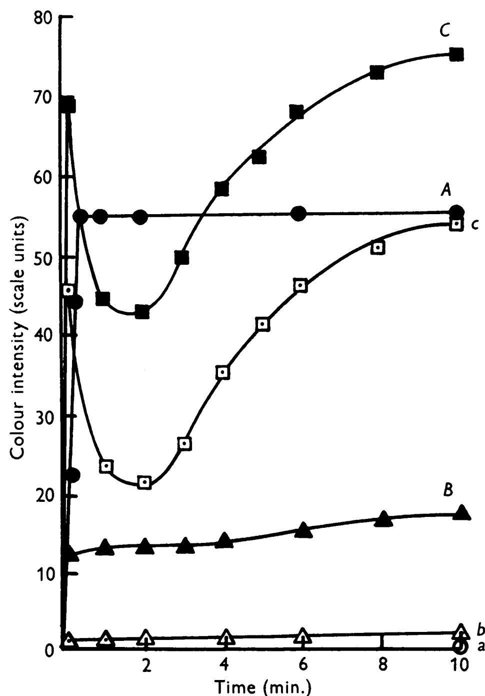


Fig. 2. Effect of added copper ions on the progress of colour development during phosphomolybdate reduction. Tubes contained: 0·5 ml. of KH_2PO_4 and 1 ml. of $CuSO_4$ to give the amounts indicated; ascorbic acid, ammonium molybdate and acetate buffer were present as in Fig. 1, total vol. 6 ml. Curve A, 2×10^{-5} M-$CuSO_4$ and 20 μg. of P; curve B, $1{\cdot}7 \times 10^{-4}$ M-$CuSO_4$ and 4 μg. of P; curve C, $1{\cdot}7 \times 10^{-3}$ M-$CuSO_4$ and 4 μg. of P; curves a, b, c, as for A, B, C respectively but no P present.

In studying the effect of higher concentrations of $CuSO_4$, the amount of phosphate present was reduced to one-fifth of that used in the preceding experiments in order to keep the colour intensities produced within the range of the colorimeter. With concentrations of $1{\cdot}7 \times 10^{-4}$ M and above, there is a marked increase in the colour intensity after 10 min. (Table 3; also compare slopes, Table 4). With $1{\cdot}7 \times 10^{-4}$ M-$CuSO_4$, the initial rapid appearance of colour was followed by a slow increase which still persisted after 10 min. (curve B, Fig. 2). At this concentration of $CuSO_4$ the enhanced colour obtained after 10 min. was directly proportional to the amount of phosphate present over the range 0–20 μg. of P.

With still higher concentrations of $CuSO_4$ a third effect became apparent, namely, the development of a pronounced colour in the absence of phosphate. Without phosphate, the colour after 10 min. was

Table 3. *Effect of higher concentrations of added copper ions on colour development during phosphomolybdate reduction*

Tubes contained 4 μg. of P and 0·01 M-$CuSO_4$ to give the concentrations indicated; ascorbic acid, ammonium molybdate and acetate buffer were present as in Fig. 1; total vol. 6 ml.

Concn. of added $CuSO_4$ (M)	Colour intensity after 10 min. (scale units): 4 μg. of P present	No P present
0	11	0
$1{\cdot}7 \times 10^{-4}$	17	2
$3{\cdot}3 \times 10^{-4}$	23	3
$6{\cdot}7 \times 10^{-4}$	42	14
$1{\cdot}0 \times 10^{-3}$	55	—
$1{\cdot}2 \times 10^{-3}$	—	46
$1{\cdot}7 \times 10^{-3}$	90	66

Table 4. *Slopes of calibration curves*

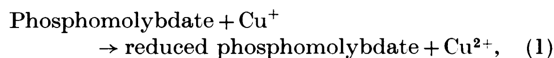
The slopes with bacterial extract and liver extract are derived from the data of Fig. 3. For the others data were used from similar experiments without inhibitor or with 0·25 ml. of $Na_2S,9H_2O$ or 0·5 ml. of deproteinized brain extract to give the concentrations indicated.

Inhibitor	Concn. of added $CuSO_4$ (M)	Slope of calibration curve (scale units/μg. of P)	Range of added P (μg.)
None	0	2·68	0–24
	$1{\cdot}7 \times 10^{-4}$	3·95	0–20
Sodium sulphide ($1{\cdot}3 \times 10^{-6}$ M)	0	0·03	0–24
	$8{\cdot}4 \times 10^{-7}$	2·46	0–12
	$4{\cdot}2 \times 10^{-6}$	2·65	0–24
Deproteinized bacterial extract (0·83%; containing 5 μg. of P)	$4{\cdot}2 \times 10^{-6}$	2·72	0–24
Deproteinized brain extract [1·7% (v/v); containing 5 μg. of P]	$4{\cdot}2 \times 10^{-6}$	2·72	0–16
Deproteinized liver extract [1·7% (v/v); containing 12 μg. of P]	$4{\cdot}2 \times 10^{-6}$	2·34	0–16
	$8{\cdot}4 \times 10^{-6}$	2·72	0–16

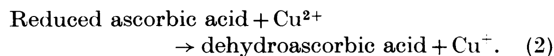
small with $CuSO_4$ concentrations of $3{\cdot}3 \times 10^{-4}$ M and below (Table 3; curves *a*, *b*, Fig. 2), but with $6{\cdot}7 \times 10^{-4}$ M and above it became very large, accounting for a large proportion of the colour intensity developing in the presence of phosphate. In addition, the time course of colour development became more complex at these higher concentrations (curves *C*, *c*, Fig. 2), an initial rapid development of colour being followed by a sharp drop in intensity and then a prolonged rise, both in the presence and the absence of phosphate. The actual readings obtained were not readily reproducible (cf. the 10 min. readings in the presence of $1{\cdot}7 \times 10^{-3}$ M-$CuSO_4$ and 4 μg. of P in Fig. 2 and Table 3). The colour developing in the absence of phosphate was blue, but of a somewhat more greenish hue than in the presence of phosphate and a low concentration of copper ions; the colour of $CuSO_4$ itself at these concentrations was negligible. No adequate explanation for the above effects can be offered.

Effect of other metal ions on phosphomolybdate reduction. Colour development was unaffected by $MgSO_4$, $CoSO_4$, $NiSO_4$, $FeSO_4$ and $FeCl_3$ at $1{\cdot}7 \times 10^{-3}$ M, apart from slight inhibitions in some cases. At $1{\cdot}7 \times 10^{-6}$ M and $1{\cdot}7 \times 10^{-5}$ M, $SnCl_2$ had no effect upon the rate of phosphomolybdate reduction, though at higher concentrations the phosphomolybdate was reduced by the $SnCl_2$ itself. These experiments confirmed that the effects observed with $CuSO_4$ were indeed due to cupric rather than sulphate ions.

Reduction of phosphomolybdate by cuprous ions. It seems probable that copper ions catalyse the reduction of phosphomolybdate according to reactions 1 and 2:



$$\text{Phosphomolybdate} + Cu^{+} \rightarrow \text{reduced phosphomolybdate} + Cu^{2+}, \quad (1)$$



$$\text{Reduced ascorbic acid} + Cu^{2+} \rightarrow \text{dehydroascorbic acid} + Cu^{+}. \quad (2)$$

Reaction 1 has been known for a considerable time and has in fact been used by Folin & Wu (1920) in the determination of reducing sugars. We have established that it occurs under the conditions used for phosphate assay by treating phosphate plus molybdate with cuprous chloride reagent (see Methods). A marked blue colour developed almost instantaneously. The chromogen could be extracted

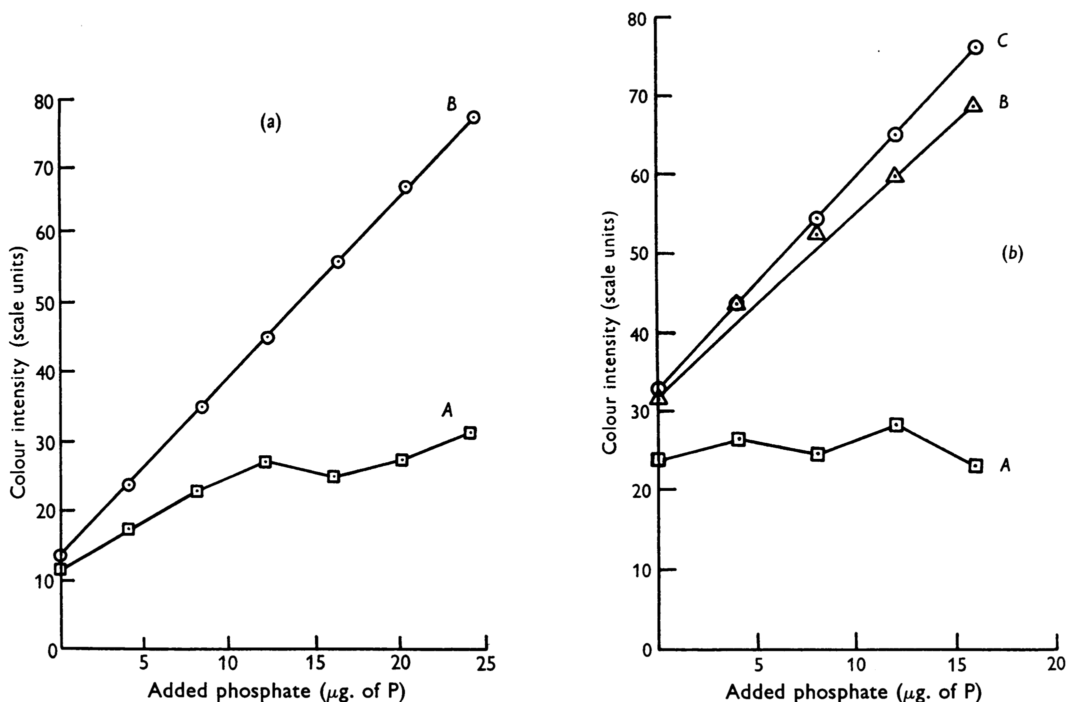


Fig. 3. Effect of copper ions on colour development in the presence of bacterial and liver extracts. Tubes contained: KH_2PO_4 (40 μg. of P/ml.), 0·5 ml. of $CuSO_4$ and 0·5 ml. of soln. of deproteinized bacterial extract or deproteinized liver extract to give the amounts indicated; ascorbic acid, ammonium molybdate and acetate buffer were present as in Fig. 1. Readings were taken after 10 min. (*a*) 0·83% of deproteinized bacterial extract present. Curve *A*, no $CuSO_4$; curve *B*, $4{\cdot}2 \times 10^{-6}$ M-$CuSO_4$. (*b*) 1·7% (v/v) of deproteinized liver extract present. Curve *A*, no $CuSO_4$; curve *B*, $4{\cdot}2 \times 10^{-6}$ M-$CuSO_4$; curve *C*, $8{\cdot}4 \times 10^{-6}$ M-$CuSO_4$.

with *iso*butanol after acidifying to pH 1 with HCl (cf. Berenblum & Chain, 1938) and was not formed when phosphate was absent, confirming that it was reduced phosphomolybdate. A slow reduction of phosphomolybdate was also observed when a few milligrams of cuprous oxide powder were used as the reducing agent.

Effect of added cupric ions in the presence of inhibitors of phosphomolybdate reduction. If copper ions are essential for the reduction of phosphomolybdate by ascorbic acid it would be expected that the inhibition by sulphide, presumed to be due to metal-binding, might be overcome by adding sufficient copper ions. This proved to be the case. With $1{\cdot}3 \times 10^{-6}$ M-Na_2S, the inhibition could be overcome by adding $4{\cdot}2 \times 10^{-6}$ M-$CuSO_4$, in the presence of which the calibration curve was linear over the range 0–24 μg. of P and the slope was restored to the value observed in the absence of inhibitor and added $CuSO_4$ (Table 4). With one-fifth of the same concentration of copper ions the curve was only linear over the range 0–12 μg. of P.

We have not studied the effect of copper ions on the inhibitions due to other metal-binding agents, but Bruemmer & O'Dell (1956) have shown that $6{\cdot}9 \times 10^{-5}$ M-$CuSO_4$ counteracts the inhibitory effect of $8{\cdot}3 \times 10^{-5}$ M glutathione.

The possibility of reversing the inhibitions observed with the biological extracts was next explored. The experiments were complicated by the presence of inorganic phosphate in the extracts themselves, but the slopes of the calibration curves (Fig. 3, Table 4) provide a convenient criterion of effective reversal. The inhibition caused by deproteinized bacterial extract (Fig. 3*a*) was completely overcome by $4{\cdot}2 \times 10^{-6}$ M-$CuSO_4$ and qualitatively similar results were obtained with deproteinized brain extract. With the latter the inhibition was relatively slight, the maximum inhibition being 39 % with 16 μg. of added P. This same concentration of $CuSO_4$ was not quite adequate with the liver extract, but $8{\cdot}4 \times 10^{-6}$ M was completely effective (Fig. 3*b* and Table 4). Bruemmer & O'Dell (1956), using $6{\cdot}9 \times 10^{-5}$ M $CuSO_4$ and 5 μg. of P in 7·2 ml., observed a reversal of the inhibition due to a concentration of liver extract similar to our own.

The inhibition with bacterial extract was less than that observed with the amount of sulphide known to be originally present in the extract. This discrepancy may be due to traces of copper ions in the deproteinized extract, or to losses of sulphide by oxidation during its preparation.

DISCUSSION

The principal finding reported here is that copper ions counteract the inhibitory effect of sulphide and biological extracts upon the reduction of phosphomolybdate by ascorbic acid under the conditions of Lowry & Lopez (1946). A similar reversal of inhibitions due to glutathione and liver extract has been reported independently by Bruemmer & O'Dell (1956). Both investigations demonstrate the desirability of adding copper ions to the assay system for phosphate devised by Lowry & Lopez.

The strong inhibitions which we have observed with metal-binding agents indicate that a metal is required for phosphomolybdate reduction, probably as an intermediate electron carrier, and suggest that the inhibitions with sulphydryl agents, including cysteine and glutathione, are due to binding of the metal. The inhibitions with tissue extracts may be attributed to the same cause, since not only cysteine and glutathione, but also many other tissue constituents, are known to form complexes with metals (see Albert, 1950, 1952, 1953). Our results suggest that the metal concerned is copper and that the reduction observed in the absence of added copper ions is due to traces present as impurity in the reagents. The evidence, however, is entirely circumstantial: attempts to demonstrate an absolute requirement for copper ions were abandoned owing to difficulties encountered in trying to free the reagents of copper.

The reversal by copper ions of the inhibitions caused by cysteine and glutathione is interpreted by Bruemmer & O'Dell as being due to a stimulation of the oxidation of these sulphydryl compounds in air, to give the inactive disulphide derivatives. Although such an effect may contribute to the reversal, it would seem likely from the present study that the saturation of the metal-binding agent by the added copper ions is also an important, if not the major, factor involved.

Our results show that in applying the method, the concentration of free copper ions must not be too high; above $3{\cdot}3 \times 10^{-4}$ M the reagent blank is very large and the course of colour development complex. Somewhat lower concentrations may have a useful application because of the increased sensitivity (50 % at $1{\cdot}7 \times 10^{-4}$ M). Under these conditions, however, the reagent blank must be corrected for and a rigid timing schedule is obligatory. In general it is preferable to avoid these disadvantages by keeping the concentration of free copper ions below about 2×10^{-5} M. A concentration of $4{\cdot}2 \times 10^{-6}$ M was sufficient to counteract the inhibitions observed with bacterial and brain extracts, though not quite sufficient with liver extract. Bearing in mind that in most applications such high concentrations of tissue extracts are not likely to be met, $4{\cdot}2 \times 10^{-6}$ M cupric sulphate is suggested as adequate for most purposes. This concentration was found to have no effect upon the hydrolysis of acetyl phosphate under the assay conditions, the liberation of inorganic phosphate being used as a measure of the

rate of hydrolysis, and Bruemmer & O'Dell (1956) found that $6 \cdot 9 \times 10^{-5}$ M cupric sulphate had no effect upon the hydrolysis of creatine phosphate.

With a little experience it is possible to judge visually whether sufficient copper ions are present, since if this is so there is a rapid development of colour within the first minute in the presence of inorganic phosphate. If not, more copper must be used, but with high concentrations of metal-binding substances it may prove difficult to ensure that the optimum concentration range of free copper ions is not exceeded. Where doubt exists, the setting up of an internal standard as suggested by Lowry & Lopez (1946) is the only true criterion as to whether the method is working satisfactorily. These points seem worth emphasizing in view of the widespread use of metal-binding agents in enzyme studies and of inorganic phosphate measurements as an index of enzymic activity.

The fact that copper is also necessary for the oxidation of ascorbic acid suggests that the requirement for copper ions in phosphomolybdate reduction may be peculiar to the use of ascorbic acid as the reducing agent. As far as we are aware, there has been no demonstration of a comparable metal requirement in the reduction of phosphomolybdate by other organic reducing agents, and we have not carried out any experiments specifically directed towards testing this point. We have, however, observed that $1 \cdot 3 \times 10^{-6}$ M sodium sulphide, which completely inhibits phosphomolybdate reduction by ascorbic acid, has no effect upon reduction by 1-amino-2-naphtholsulphonic acid under the conditions of Fiske & Subbarow (1925). In view of the extremely low solubility products of cupric and cuprous sulphides ($8 \cdot 5 \times 10^{-45}$ and 2×10^{-47} at 18°) the concentration of free copper ions remaining in solution in the presence of $1 \cdot 3 \times 10^{-6}$ M sodium sulphide is negligible, even at the lower pH used by Fiske & Subbarow. Copper ions do not therefore appear to be involved in the reduction of phosphomolybdate by 1-amino-2-naphtholsulphonic acid. In any case the absence of any effect with sulphide in the Fiske & Subbarow estimation indicates that the method is not susceptible to interference by metal-binding agents.

Table 5. *Use of the modified method to determine inorganic phosphate in the presence of bacterial extract and acetyl phosphate*

Mixtures for analysis were contained in a total volume of 1 ml.: 0·1 M triethanolamine–HCl buffer, pH 6·8, 0·2 ml. of untreated extract of organism LC1, KH_2PO_4 and 32 μ-moles of lithium acetylphosphate were present as indicated. Bacterial extract, where present, was added last and the mixture immediately deproteinized; 0·25 ml. samples of supernatant were taken for assay. In each experiment the experimental values due to added KH_2PO_4 were obtained by subtracting from the corresponding total values the total value found in the absence of added KH_2PO_4.

	Inorganic phosphate (μmoles)		
		Experimental values	
	Added as KH_2PO_4	Total	Due to added KH_2PO_4
With bacterial extract	0	5·2	—
	6·5	12·0	6·8
	12·9	18·4	13·2
With acetyl phosphate	0	7·7	—
	6·5	14·1	6·4
	12·9	21·1	13·4
With bacterial extract plus acetyl phosphate	0	12·0	—
	6·5	18·7	6·7
	12·9	24·9	12·9

Modified method

The following modified procedure is suggested. The sample is first deproteinized with the ammonium sulphate–acetate reagent as described by Lowry & Lopez (1946), and to a portion of the resulting solution are added 0·5 ml. of 5×10^{-5} M $CuSO_4$ and 0·125 M sodium acetate buffer, pH 4, to a total volume of 5 ml., followed by 0·5 ml. of 1% ascorbic acid. The reduction is then started by the addition of 0·5 ml. of 1% ammonium molybdate in 0·05 N-H_2SO_4, and the colour intensity is measured after 10 min. When labile phosphates are present, readings are taken at 10 and 20 min., and the colour intensity corresponding to the inorganic phosphate present in the original sample is obtained by extrapolation to zero time. The taking of readings at 10 and 20 min. was found to be more suitable than at the times of 5 and 10 min. suggested by Lowry & Lopez (1946). A longer time is thereby allowed for completion of the reaction with the free phosphate.

The application of this modified procedure in the presence of bacterial extract and acetyl phosphate is illustrated in Table 5. Both the bacterial extract and the acetyl phosphate preparation contained free phosphate, but after correcting for these by subtraction, the recovery of inorganic phosphate was considered satisfactory, bearing in mind that four experimental readings were involved in the determination of each figure for recovered inorganic phosphate.

SUMMARY

1. The reduction of phosphomolybdate to a blue compound by ascorbic acid under the conditions of Lowry & Lopez (1946) is inhibited by extracts of a rumen micro-organism and by liver and brain extracts.

2. Strong inhibitions are observed in the presence of micromolar concentrations of sodium sulphide, diethyldithiocarbamate and ethylenediaminetetraacetic acid and in the presence of millimolar concentrations of cysteine.

3. At low concentrations (e.g. 2×10^{-6} M) cupric ions cause a marked acceleration of the rate of colour development. At higher concentrations

($1{\cdot}7 \times 10^{-4}$ M) the final colour attained is also increased, and above this concentration a marked colour appears in the absence of phosphate. No comparable effects are observed with Mg^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+} or Sn^{2+} ions.

4. Cuprous ions reduce phosphomolybdate to the blue complex under the conditions for phosphate assay. It is suggested that copper ions act as electron carriers in the reduction of phosphomolybdate by ascorbic acid and that normally sufficient copper is present as impurity in the reagents. In the presence of metal-binding agents, including those occurring in tissues, phosphomolybdate reduction is inhibited as a result of metal-binding action.

5. The addition of $4{\cdot}2 \times 10^{-6}$ M copper ions to the system overcomes the effect of such concentrations of metal-binding agents as are likely to be met with in normal practice. A suitable modification to the procedure of Lowry & Lopez is described.

6. By using a somewhat higher concentration ($1{\cdot}7 \times 10^{-4}$ M) of copper ions, the sensitivity of the method can be increased by 50 %, though the timing of operations becomes more critical under these conditions. Concentrations above $3{\cdot}3 \times 10^{-4}$ M interfere with the method.

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Hydrolysis of Amides by Extracts from Mycobacteria

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Amidases of bacterial origin have received less attention than amidases of animal origin. Amidase systems in micro-organisms which catalyse the hydrolysis of glutamine (Hughes & Williamson, 1952; McIlwain, 1948), asparagine (Altenbern & Housewright, 1954) and nicotinamide (Hughes & Williamson, 1953; Oka, 1954) have been described. Altenbern & Housewright (1954) were able to show the existence of two stereospecific asparaginases in extracts from *Brucella abortus*. Kirchheimer & Whittaker (1954) reported the presence of asparaginase activity in various species of Mycobacteriaceae.

This study deals with amidase activities of *Mycobacterium phlei* and *Mycobacterium tuberculosis* var. *bovis* B.C.G. and with the substrate specificities of the enzymes studied.

EXPERIMENTAL

Micro-organisms. The organisms used were *Mycobacterium phlei* 271 from the culture collection of this Department and *Mycobacterium tuberculosis* var. *bovis* B.C.G. 5692 (B.C.G.) obtained from the American Type Culture Collection (ATCC).

Cultivation of bacteria. The *Myco. phlei* strain was grown for 4 days at 35° in Roux bottles on nutrient agar supplemented with 0·1 % Tween 80 (polyoxyethylene sorbitan mono-oleate). The bacteria were taken up in water, washed 3 times by centrifuging and then resuspended in water. The B.C.G. strain was grown in Sauton's medium (Sauton, 1912) for about a month at 35°. The pellicles were filtered off, washed three times with water and a thick suspension of washed cells was prepared.

Bacterial extracts. Cell-free extracts were prepared by disintegrating the washed bacteria in a 9 kcyc./sec. Raytheon sonic vibrator for 25 min. The cell debris was removed in the