

An Improved Method for the Determination of Orthophosphate Suitable for Assay of Adenosine Triphosphatase Activity

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► In view of poor recovery and lack of reproducibility and stability frequently experienced in the assay of orthophosphate (Pi) by the method of Martin and Doty, the method has been modified in three respects: protein is precipitated with ClO_4^- at pH 1.5–1.8; the precipitate is removed prior to formation of phosphomolybdic acid; phosphomolybdic acid is measured, after extraction into isobutanol–benzene (1:1), in the unreduced (yellow) form. Optimal concentrations of H_2SO_4 and molybdate have also been determined. The method developed is superior in precision and accuracy, as well as sensitivity, to those presently available, and the product measured is stable for at least 48 hours. The procedure was applied, with satisfactory results, to the determination of the adenosine triphosphatase activity of extracts of muscle tissue and proteins isolated from such tissue.

ORTHOPHOSPHATE (Pi) is almost always determined as phosphomolybdic acid, usually after reduction (5, 18, 24). Duval (4) aptly remarks on the “very numerous modifications” of the method, and of “profound contradictions on the subject of the optimal concentration of molybdic acid, of acidity, and of the temperature of reduction.” He further states that if reduction is carried out at room temperature, as is commonly done, the blue color is unstable. Kondrashova *et al.* (14) likewise comment on the “existence of many modifications [and] the absence of a single one that is sufficiently satisfactory.” Our experience fully confirms these statements.

The object of the investigation presently described was to determine conditions which are optimal for the determination of Pi and compatible with the presence of adenosine-5'-triphosphate (ATP), proteins and various salts. It was found necessary to consider the following factors: the agent used for precipitation of the protein, the necessity of removing the (precipitated) protein, the form of the phosphomolybdate during extraction and measurement, the $[\text{H}^+]$, and the concentration (aqueous) of molybdate.

EXPERIMENTAL

All spectra were obtained with a Bausch and Lomb Spectronic 505 recording spectrophotometer. Other absorbance measurements were made with a Beckman DU spectrophotometer which was calibrated for wavelength and absorbance (12). The level of phosphorus is expressed as final concentration in mg. of P/liter. Except where otherwise indicated, the unit of absorptivity a is $(\text{cm})^{-1} (\text{mg. P/liter})^{-1}$. When phosphorus was extracted from an aqueous into an organic phase, the absorptivity figures refers to the concentration of phosphorus in the latter phase. In such cases the absorptivity is “apparent” in the sense that the extraction was assumed to be quantitative and the volume of the organic phase was assumed to be unaffected by equilibration with the aqueous phase.

The reduced and unreduced forms of the colored phosphomolybdate product(s) are indicated by the superscripts *B* (blue) and *Y* (yellow), respectively. Additions of inorganic phosphate were in the form of KH_2PO_4 , Fisher primary standard, dried at 110° C. for 24 hours. The levels of acids and salts are expressed as final concentrations in the aqueous phase.

Adenosine Triphosphatase Assay.

To 1 ml. of enzyme solution containing from 10 to 400 $\mu\text{g.}$ of myosin (6) in buffer A were added two ml. of substrate solution, containing ATP in buffer B (24). The samples were incubated in a water bath at 22° C. for five minutes. Some assays were carried out using the buffers suggested by Haga *et al.* (9).

RESULTS

I. Tests with Phosphomolybdate Blue. The procedure of Martin and Doty (18), as modified by Szent-Györgyi and Holtzer (24), was applied to a series of standard phosphate solutions. The absorbance spectrum of the blue product (Figure 1A) has a broad peak in the visible range, and peaks in the ultraviolet at 313 $\text{m}\mu$ and at 273 $\text{m}\mu$. An absorptivity $a_{273}^{B_{675}} = 0.512$ was found, agreeing with Szent-Györgyi and Holtzer (24). However, fading of the extracts was observed. The fading rate increased with increasing concentration of Pi, being a first-order function of phosphate concentration up to 1.3 mg. P/liter, and a higher order function above this level. Furthermore, the rate of fading was not reproducible in different experiments. This could not be attributed to variability in reagents. Chilling the reduced extract in an ice bath improved color stability considerably, but the results were still too erratic to be acceptable. The substitution of *p*-semidine for stannous chloride, as recommended by Dryer *et al.* (3) was tried, but very little reduction to the blue product was obtained as measured by the color yield. The use of isoamyl acetate (iAA) as the extracting solvent, and addition of stannous chloride in the dry form, as reported by

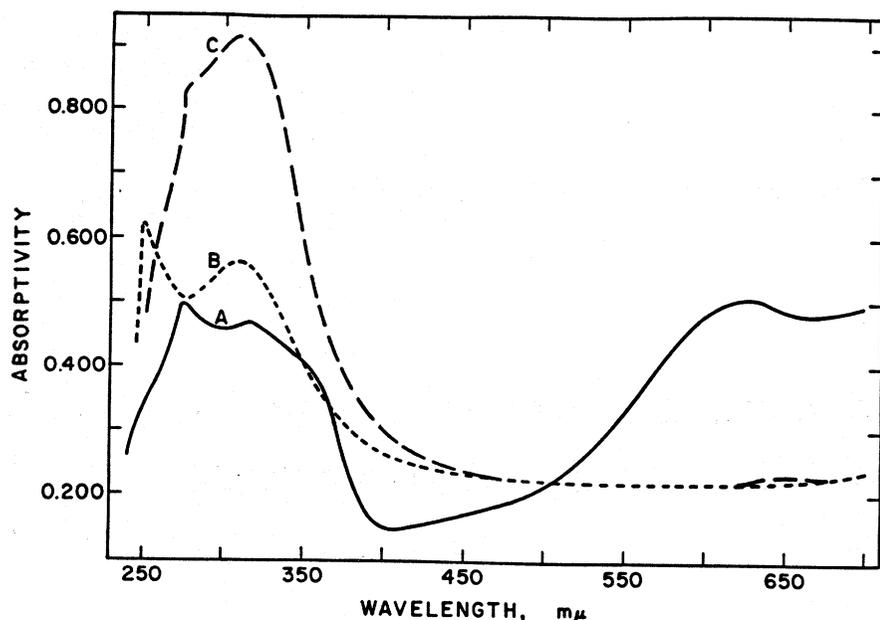


Figure 1. Absorbance spectra of phosphomolybdic acid

(A) Reduced acid in iBB, 1:1 (18)

(B) Unreduced acid in iAA

The aqueous phase contained 45.2 $\mu\text{g.}$ of P (KH_2PO_4), 40 meq. H_2SO_4 , and 405 μmoles $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ in a volume of 50 ml.

Ten ml. of the aqueous phase was extracted with 10 ml. iAA

(C) Unreduced acid in iBB

As for (B) above, except that the aqueous phase was extracted with iBB

Kirsten and Carlsson (13) was also attempted. Color stability at 720 $\text{m}\mu$ was improved but the fading rate was still unacceptably high. Moreover, the sensitivity found, $a_{720}^{\beta} = 0.471$, was much lower than expected (13).

II. Tests with Yellow Phosphomolybdic Acid: Conditions for Its Formation and Extraction.

A. PRELIMINARY EVALUATION. Figure 1 also shows the absorbance spectra of yellow phosphomolybdic acid in iAA and iBB (isobutanol-benzene, 1:1, v/v). Stability was satisfactory in both solvents, but higher sensitivity was obtained with the latter. It is also evident that greater sensitivity was obtained by measuring the heteropoly acid without reduction ($a_{313}^{\gamma} > a_{675}^{\beta}$). Furthermore, the spectrum of the yellow, unreduced acid was completely stable for at least two hours, in contrast to the poor stability of the blue reduction product. Adherence to Beer's law was demonstrated for the iBB extract.

In an attempt to prevent hydrolysis of organic phosphate (ATP) prior to extraction, the reagents used to obtain the yellow phosphomolybdate product were made up in dimethyl formamide and dimethyl sulfoxide (20). However, it was found that both of these solvents inhibit formation of the heteropolyacid and its extraction into iBB.

B. OPTIMAL MOLYBDATE CONCENTRATION. Samples containing a constant amount of KH_2PO_4 were reacted with varying amounts of molybdate [$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$]. The prod-

ucts were extracted into iBB according to the procedure of Szent-Györgyi and Holtzer, and the absorbance of a portion of each organic phase was measured at 313 $\text{m}\mu$ against the corresponding reagent blank. The aqueous phase contained 2.26 $\mu\text{g.}$ P, 3.84 meq. H_2SO_4 , and 1-100 μmoles $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ in a volume of 6 ml., and was extracted with 5 ml. iBB. The data (Figure 2) indicate that optimal color development for this set of conditions occurs at a concentration of 4 to 5 mM molybdate. A molybdate concentration of 4.17 mM was selected for further experiments.

Since the heteropolyacid forms in, and is extracted from, an acidic medium which frequently contains salts, the

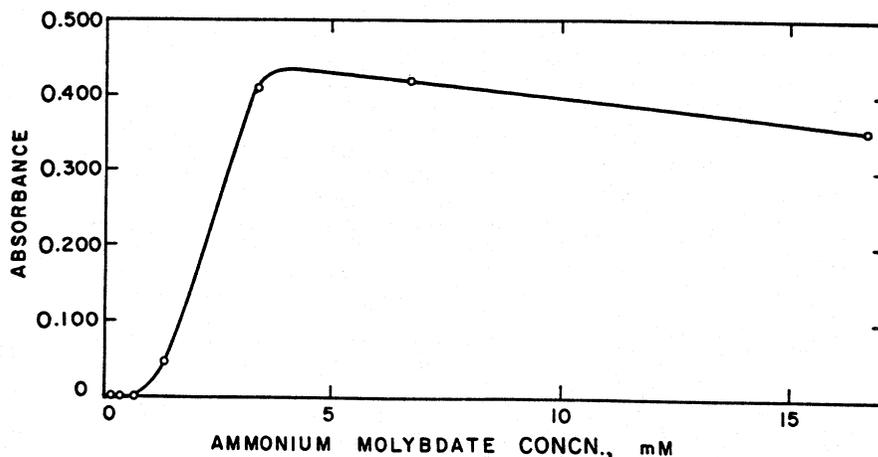


Figure 2. Absorbance of extracted unreduced phosphomolybdic acid as a function of concentration of molybdate

effect of acidity, ionic strength, and species of ion needed careful scrutiny.

C. OPTIMAL ACID STRENGTH. The effect of varying acid concentration (H_2SO_4) from 0 to 3.5 N on the yield of heteropolyacid extracted into the organic phase was measured. The aqueous phase contained 2.26 $\mu\text{g.}$ phosphorous, 25 μmoles molybdate, and 0-21 meq. H_2SO_4 in a volume of 6 ml., and was extracted with 5 ml. iBB. Absorbance was nearly independent of $[\text{H}^+]$ over the range 0.5-0.9 N (Figure 3), where $a_{313}^{\gamma} = 0.93$. The drop in absorbance was very steep beyond an acid strength of 1 N . For some of the experiments to be described the $[\text{H}^+]$ was set at 0.6 N . In later work a concentration of 0.7 N was used. The interplay of hydrogen ion concentration, ionic strength, and ionic species is discussed further in the text.

D. INTERFERING SUBSTANCES. Preparatory to running adenosine triphosphatase (ATPase) determinations according to Szent-Györgyi and Holtzer (24) the buffer and salts involved were individually checked for possible interference in the measurement of phosphate as yellow phosphomolybdate. It was found that sodium chloride, imidazole, and calcium chloride, at the levels indicated, made no contribution to the absorbance at 313 $\text{m}\mu$. Furthermore, $a_{313}^{\gamma} = 0.923$ was found for Pi when two μmoles of ATP were present in the sample. This value is in good agreement with our previously found a_{313}^{γ} values, where ATP was absent.

The following salts were also investigated for possible interference at levels likely to be encountered in practice: EDTA, 0.67 mM ; MgSO_4 , 0.67 mM ; KCl, 0.4 M . These were tested singly in the presence of calcium chloride, sodium chloride, and imidazole. There was no reduction in precision or absorbance. In the presence of calcium chloride, sodium chloride, and imidazole, an absorbance curve was run in the range 0-1.36 mg. P/liter of solvent phase.

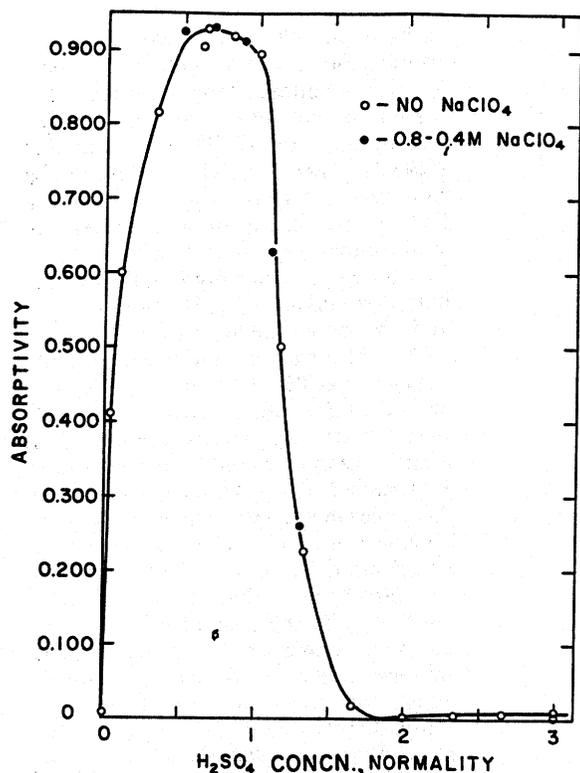


Figure 3. Absorbance of extracted unreduced phosphomolybdic acid at $313\text{ m}\mu$ as a function of concentration of H^+

The standard deviation of absorbance was ± 0.013 , corresponding to 1.4% at the 1 mg. P/liter level. Glycerol at a level of 2.28 mM in the aqueous phase gave no interference.

III. Investigation of Agents for Deproteinization Prior to Formation and Extraction of Unreduced Phosphomolybdic Acid. The following protein precipitants were examined:

Boiled silicotungstic acid (18, 19) was evaluated at three levels: 1.5, 5.0, and 8.0 mM in the aqueous phase. The pH of the STA reagents that were used was adjusted to 1.5–1.8. It was found that the absorbances of the extracts were 0.90, 1.11, and 1.39, respectively, when read against iBB at $313\text{ m}\mu$; blanks having this order of absorbance would diminish photometric sensitivity. Purification of STA by recrystallization (22) did not improve the results.

Sulfosalicylic acid (27) was tried at the 0.39M level in the aqueous phase, but it adversely affected extraction of the yellow product by the solvent (iBB). Furthermore, it was preferentially extracted by the solvent, giving an extremely high blank ($A > 2$ vs. iBB).

Trichloroacetic acid (TCA) (17) at the 0.20M level in the aqueous phase gave a reasonable blank, 0.28; a_{313}^Y was found to be 0.680.

Early in this investigation, perchloric acid (26) was used in the deproteinizing step with the intention of having it serve both as the protein precipitant and as the source of H^+ during color

development. It was satisfactory insofar as sensitivity is concerned, both with samples devoid of protein and with samples containing protein (which were deproteinized). However, it was found that background color was directly correlated with the concentration of perchloric acid in the aqueous phase subjected to extraction. Furthermore, poor reproducibility and instability of the color yield were found. Also, it became apparent that perchloric acid from different sources gave very different background colors. These disadvantages led us to consider a substitute for perchloric acid.

Owing to the possibility that the high background color obtained with HClO_4 was produced by an impurity such as Pi, arsenate, or silicate (25), and in order to maintain a low $[\text{H}^+]$, the use of NaClO_4 (0.6M) at acid pH values was investigated. The recovery of protein was found to increase with decreasing pH and was complete at 1.85. This pH thus represents the minimal acid strength adequate for removal of myosin. NaClO_4 in an acidic medium was therefore chosen as the precipitating agent.

Figure 4 shows the results of determining phosphate in the presence and absence of protein. The aqueous phase contained the following amounts of the solutes given in a volume of 6 ml.: phosphorus, 1.13, 2.26, or 4.16 μg .; myosin

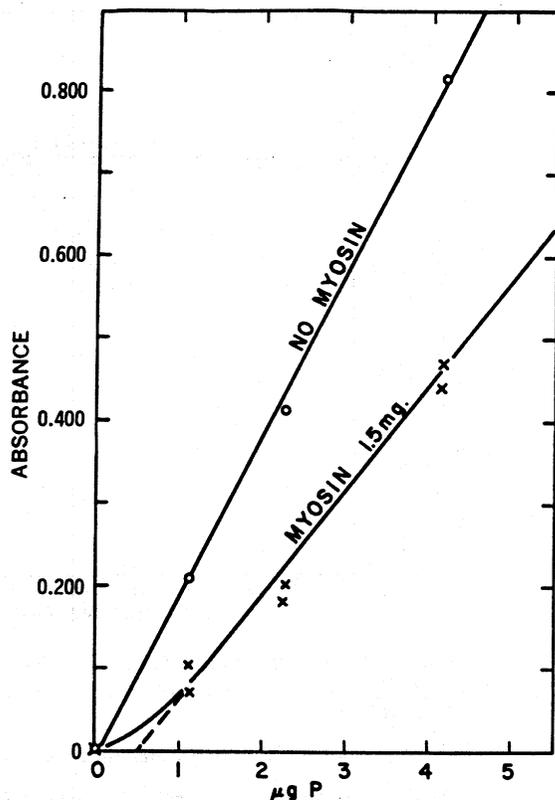


Figure 4. Absorbance at $313\text{ m}\mu$ with and without myosin present during formation and extraction of phosphomolybdic acid

(when present), 1 mg.; NaClO_4 , 3.6 mmoles; glycine, 0.16 mmole; HCl , 0.40 mmole; H_2SO_4 , 4.2 mmoles; molybdate, 25 μmoles . This was extracted with 5 ml. iBB. It is evident that the presence of protein [beef myosin (6)] during extraction of the heteropolyacid, as in the procedure of Martin and Doty (18), results in substantial loss of phosphomolybdate, in spite of the fact that the protein is in the form of a precipitate.

IV. Optimal Conditions for Formation and Extraction of Unreduced Phosphomolybdic Acid after Deproteinization.

A. CONCENTRATION OF SULFURIC ACID AND OF NaClO_4 .

To determine the optimal concentration of H_2SO_4 in the presence of NaClO_4 , experiments were carried out in which the former component was varied from 0.5 to 1.3N; NaClO_4 , in combination, was varied from 0.8 to 0.4M. This component was introduced since it was to be present in protein filtrates to be analyzed. When compared with absorbances measured in the absence of ClO_4^- , the results (Figure 3) show that absorbance was unaffected by ClO_4^- . At $[\text{H}^+] = 0.7N$ $a_{313}^Y = 0.932$; background absorbance was 0.20.

In a further experiment H_2SO_4 was kept constant at 0.7N and NaClO_4 was varied from 0.35 to 1.62M (Figure 5). It is seen that absorptivity, due to Pi,

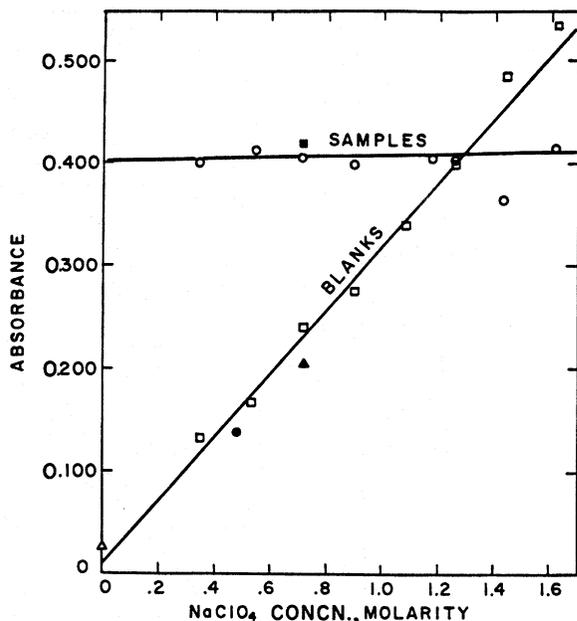


Figure 5. Absorbance of experimental samples (vs. blanks) and blanks (vs. iBB) at 313 μ as a function of $[\text{NaClO}_4]$

The different symbols refer to experiments run at different times under the same conditions
 Details as for Figure 3 except that H_2SO_4 was fixed at 4.2 meq. and NaClO_4 varied from 2.10 to 9.72 mmoles

is virtually independent of the sodium perchlorate concentration through the entire range, whereas background color is strongly dependent on NaClO_4 concentration.

Samples from which protein has been removed contain ClO_4^- at a concentration of 0.6M. Since a 4-ml. aliquot of this is taken into an aqueous volume of 6 ml., the perchlorate ion concentration cannot in practice be reduced below 0.4M. We chose to maintain the perchlorate ion concentration at 0.6M by adding NaClO_4 to the H_2SO_4 reagent; the increase in background color is small, 0.05 absorbance unit, and provision is thereby made for samples containing higher concentrations of ClO_4^- . In the latter case, the NaClO_4 content of the H_2SO_4 reagent would be reduced. For an inorganic phosphate test in a sample containing no protein, perchlorate can be completely eliminated.

Having selected the conditions of making the aqueous phase 0.7N H_2SO_4 -0.6M NaClO_4 , a Beer's law test was run. A straight line relationship of absorbance to Pi concentration was found, the slope yielding $a_{313}^{\text{P}} = 0.906 \pm 0.003$ (standard deviation); background color was 0.18. The data are part of Figure 6, which will be discussed below. One of the solvent extracts was monitored at room temperature (water-cooled lamp housing) in a Gilford absorbance recording system for a 40-hour period; perfect color stability was found. Similar stability was found for deproteinized samples discussed below.

B. EFFECT OF PROTEIN. Solutions containing varying amounts of KH_2PO_4 and a constant amount of myosin were deproteinized and analyzed as set forth in the procedure recommended below. The data, plotted as part of Figure 6, yield a slope $a_{313}^{\text{P}} = 0.905 \pm 0.021$;

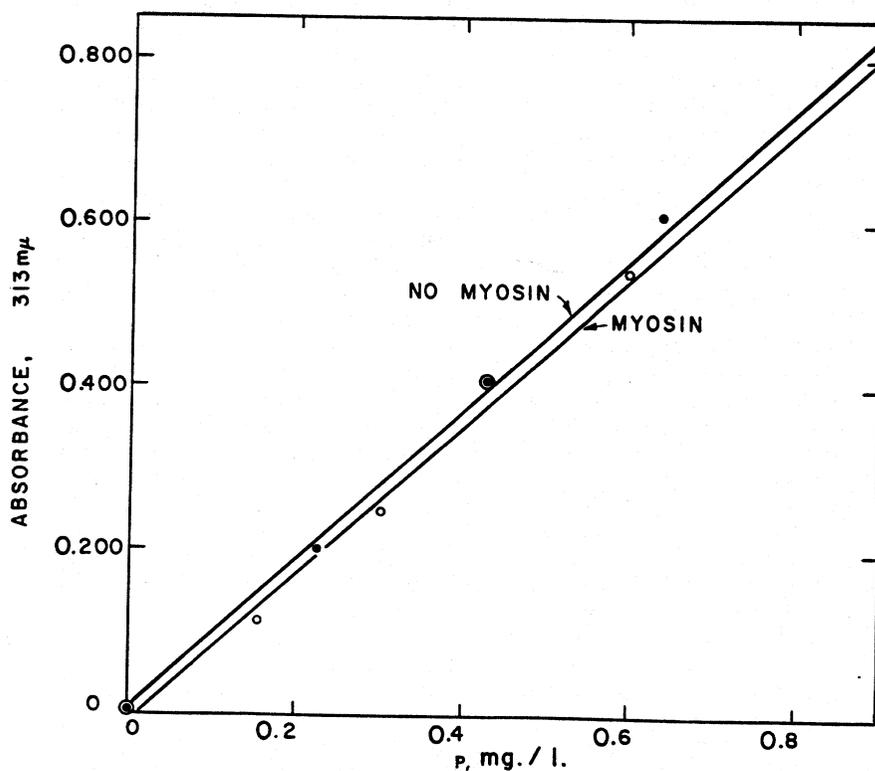


Figure 6. Effect of deproteinization with ClO_4^- on the recovery of Pi

background color was 0.208. For the samples not containing protein, the aqueous phase contained the following components in a volume of 6 ml.: phosphorus, 1.13 to 4.52 μg ; NaClO_4 , 3.6 mmoles; H_2SO_4 , 4.2 meq.; molybdate, 25 μmoles . This was extracted with 5 ml. iBB. For the samples containing protein, a 3-ml sample containing 1.13 to 4.52 μg . Phosphorus, 1.16 mg. myosin, 100 μmoles imidazole buffer, pH 7, 80 μmoles NaCl , and 15 μmoles CaCl_2 was deproteinized with 3 ml. of a solution containing 3.6 mmoles NaClO_4 , 0.24 mmole glycine, and 0.60 mmole HCl . After removal of the precipitate by centrifugation, a 4-ml. aliquot was taken and to it was added 1.2 mmole NaClO_4 , 4.2 meq. H_2SO_4 , and 25 μmoles molybdate; the final aqueous volume was 6 ml. This was extracted with 5 ml. iBB. A statistical analysis of the data showed that, at a confidence level of 95%, the absorptivity after removal of myosin was identical to that in samples originally devoid of protein. Thus, there was no loss of Pi from samples containing the protein. An absorptivity of 0.906 therefore applies to both cases. The standard deviation is higher for the samples containing protein (2.3%) than for those that do not (0.3%).

V. Application of the Phosphate Determination to the Assay of Adenosine Triphosphatase Activity. Portions of a myosin preparation (6) were diluted with buffer to yield a range of protein concentrations and incubated with substrate. For most samples a 4-ml. aliquot of the incubation mixture

was taken for analysis. In a number of cases, where large amounts of Pi were produced, a 1-ml. aliquot was taken and diluted to a volume of 4 ml.; NaClO₄ was incorporated into the diluent to maintain its concentration constant (0.6M) in all samples. In several tests the solvent extract was diluted with the organic phase from blank samples to reduce absorbance to a more accurate measuring range. Satisfactory results were obtained with both methods of dilution, but the first is preferred.

The yield of Pi for the five minute hydrolysis of this experiment is shown in Figure 7 as A/V , absorbance per unit volume of deproteinized aliquot, plotted against enzyme concentration. The yield of Pi is linear for the myosin range 0.04 to 0.4 mg. of protein/3 ml. of incubation mixture. The straight line portion of this curve is drawn on the basis of a least-squares fit, discarding data obtained with concentrations of myosin below the 0.04 level. Also plotted are data from a separate experiment where both extremes of the range were investigated. After hydrolysis of 50 per cent (1 μ mole) of the ATP, the rate of hydrolysis decreases appreciably (the data are not shown in Figure 7). A lower rate of hydrolysis is also evident at very low concentrations of myosin. The latter effect may be attributed to denaturation, and could possibly be overcome by a stabilizer such as hydrolyzed gelatin. From the linear section of the curve the ATPase activity of the myosin preparation was 0.58 μ mole of Pi per mg. protein per minute.

IMPROVED METHOD

Reagents are as follows: protein precipitant: NaClO₄, 1.2M; glycine, 0.080M; HCl, 0.2M; diluent: NaClO₄, 0.6M; acid-perchlorate reagent: H₂SO₄, 4.2N; NaClO₄, 1.2M; isobutanol-benzene (iBB): isobutanol, 1 volume; benzene, 1 volume; molybdate: (NH₄)₆Mo₇O₂₄, 25mM.

Procedure. Pipet a sample containing 0.7 to 30 μ g. phosphorus as Pi into a 10-12 ml. screw-cap test tube, dilute with water to 3 ml., and add 3 ml. of protein precipitant. It is preferable to add this and all other reagents with automatic syringes of all-glass construction. Cover the tube tightly with a screw-cap lined with Teflon and mix. After ten minutes, centrifuge at 6200 \times g. for twenty minutes. (We centrifuge at 10° C., but this operation can usually be done at room temperature.) An aliquot of the supernatant containing 0.5 to 5 μ g. phosphorus is taken and diluted, if necessary, to 4 ml. with 0.6M NaClO₄. Add 1 ml. of acid-perchlorate reagent and mix. Add 5 ml. of iBB and 1 ml. of molybdate. Mix the phases completely by inverting 15-20 times during a period of 15-20 seconds. Centrifuge for 10-15 minutes at 3300 \times g. to provide complete separation of the phases. Place a portion of the organic phase in a cuvet with a ground glass joint, stopper, and read the absorbance

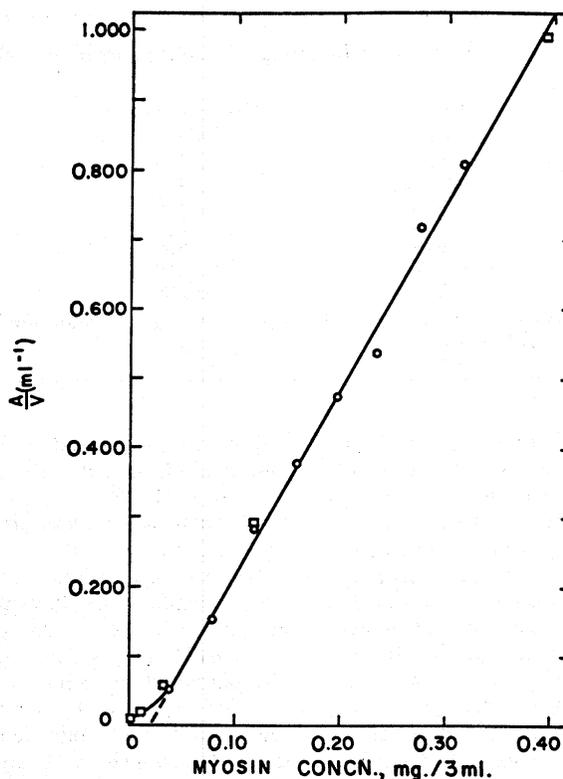


Figure 7. Application of the determination of Pi to the measurement of ATPase activity

ATP was incubated with myosin as described (Experimental) Deproteinization and subsequent steps were as for Figure 6, except that in some cases the aliquot taken for extraction was 1 ml. (see text)

at 313 $m\mu$ against a suitable blank treated identically.

Comments. The inorganic phosphate should be extracted and isolated from the aqueous phase without undue delay, since residual nucleotide phosphate does slowly hydrolyze in the acidified medium. Under the conditions described for "Adenosine Triphosphatase Assay" (Experimental section), this hydrolysis occurs at the rate of 0.1 micromole ATP (5% of the amount of ATP initially present) per hour. When isolated, the absorbance of the organic phase is constant for at least 48 hours.

It should be noted that once the incubated samples have been deproteinized, the remaining steps of the procedure should be run at room temperature. In an experiment where 0 to 3° C. was maintained from the protein precipitation step until measurement of absorbance, the absorbance was low and nonreproducible.

To maintain an adequate excess of molybdate (14), the aliquot taken for reaction with this reagent should contain not more than the recommended limit of 5 μ g. of phosphorus.

DISCUSSION

This investigation was initiated by our requirement for a reliable means of measuring the ATPase activity of muscle extracts and proteins isolated therefrom. Existing methods gave surprisingly erratic results, in agreement

with the findings of other investigators (4, 14). It was established that the difficulty was not associated with the enzymatic incubation but rather with the determination of Pi itself.

Isolation of Pi. We chose the solvent extraction of Berenblum and Chain (1), as modified by Martin and Doty (18), since it appeared that it would be applicable to large numbers of samples with relative ease. To avoid the troublesome reduction step, we investigated the possibility of measuring the yellow phosphomolybdate directly. The results were highly rewarding in that it was shown to be possible to eliminate the reduction without the concomitant loss of sensitivity, which has generally been assumed unavoidable (8); a gain in sensitivity was in fact obtained. In addition, the results indicate that ATP is not measurably hydrolyzed during the formation and extraction of the heteropolyacid. Whether ATP is itself extracted into the organic phase as a molybdate complex (17) is open to question. In any case, no Pi is formed in the organic phase subsequent to the extraction.

Having chosen the method for isolating Pi in a stable and measureable form—i.e., as yellow (unreduced) phosphomolybdic acid in iBB, the effect of protein in the original samples on the yield of the product measured was investigated. The results indicated

Table I. Key Features of Various Methods of Orthophosphate Determination

| Organic solvent ^a | λ (m μ) | Absorptivity | Ref. |
|------------------------------|----------------------|--------------|------------|
| iB | White light | ca. 0.5 | (1) |
| None | 825 | 0.75 | (4) |
| None | 660-720 | 0.12 | (5) |
| iAA | 720 | 0.731 | (13) |
| B | 310 | 0.64 | (17) |
| BC | 310 | 0.800 | (25) |
| iBB | 625-725 | 0.546 | (18) |
| iBB | 313 | 0.906 | This paper |

^a iAA, isoamyl acetate; B, butanol; iB, isobutanol; iBB, isobutanol-benzene, 1:1 (v/v); BC, butanol-chloroform.

(Figure 4) that if protein (myosin) is present during extraction, the yield of phosphomolybdic acid in the organic phase is seriously reduced. Three alternatives are thus open for separating Pi from protein: extraction without prior removal of protein (18), with consequent loss of sensitivity and reproducibility; deproteinization, leaving Pi in solution; and precipitation of Pi, as in the method of Sugino (23), leaving the protein in solution. The first alternative being undesirable we chose deproteinization, since it is then unnecessary to redissolve the phosphate.

Deproteinization. Various methods of deproteinization were tried, as described in section III. Boiled STA (18) was found unsatisfactory. This is not surprising when one considers that silicate forms a heteropoly acid with molybdate (2, 25). TCA was not extensively investigated but is considered less desirable than a perchlorate medium, since extraction of phosphomolybdic acid from the former is more difficult (10), and since its use can be a source of error in the presence of small amounts of silicate (2).

HClO₄ was suggested as a protein precipitant by Neuberger *et al.* (21) and has been used for deproteinization prior to determination of Pi by Lowry and Lopez (16), Hagihara and Lardy (10), Hashimoto *et al.* (11), and Fukazawa *et al.* (7). We found this reagent, as commercially available, unsuitable, for reasons already given (Results).

It was found that myosin precipitated completely with 0.6M ClO₄⁻ at pH 1.85, but not at higher pH's (Section III), and Pi was quantitatively recovered in the supernatant (Figure 6). The ClO₄⁻ concentration used was suggested by the level of HClO₄ effective for precipitation of serum proteins (26, 27). The effect of pH on precipitation of proteins by anions such as ClO₄⁻ can be attributed to a requirement for protonation of carboxylate groups. In this connection it is interesting to note that Lipmann and Tuttle (15), working with

different proteins and a different precipitant, TCA, found that "an acidity of below pH 1.8 . . . was necessary to bring about a complete precipitation of protein . . ."

Optimal Conditions for Formation and Extraction of Unreduced Phosphomolybdic Acid. As pointed out by Duval (4), color yield due to phosphomolybdic acid is a complex function of both hydrogen ion concentration and molybdate concentration; the [H⁺] which is optimal at one molybdate concentration is not optimal at another, and vice versa. Furthermore, the optimal [H⁺] varies with the anion, being different for example, for HClO₄ than for H₂SO₄. The thorough analysis by Duval was unfortunately limited to the blue reduction product, but our experience indicates that even the yield of the unreduced heteropolyacid is a complex function of the above kind. Thus, multiple experiments were required to choose the conditions necessary to maximize the yield of this acid. The results of some of the experiments of this series relative to optimal concentrations of molybdate and H₂SO₄ are shown in Figures 2 and 3. In our method NaClO₄ is also present, at a concentration of 0.60M, together with a small amount of glycine and HCl, these components having been used during deproteinization.

Comparison of Methods. A comparison of the method proposed with representative methods taken from the literature is given in Table I.

Conclusion. The method of Martin and Doty (18) was found to be unsatisfactory. The difficulty was traced to the use of silicotungstic acid, the presence of protein during extraction of phosphomolybdic acid, and reduction of the latter to the blue form.

An improved method was developed which eliminates these sources of error. Protein (myosin) is removed after precipitation with ClO₄⁻ at pH 1.5-1.8, and the heteropoly acid is measured, after extraction into isobutanol benzene,

in the unreduced form. The extracted product has an absorptivity at 313 m μ of 0.906 (cm.)⁻¹ (mg. P/liter)⁻¹.

The improved method is superior to its predecessors with respect to reproducibility, stability, sensitivity, and ease of operation. The assay is suitable for application to the measurement of adenosine triphosphatase activity.

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