

Micromethod for Determination of Orthophosphate in Presence of Adenosine Triphosphate after Deproteinization with Trichloroacetate Buffer

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For the determination of orthophosphate following partial enzymatic hydrolysis of adenosine-5'-triphosphate, a method with the following characteristics and advantages has been developed: A sample containing a given concentration of orthophosphate yields an absorbance nearly three times the highest value hitherto attainable (1). Potassium ion, frequently present in samples subjected to analysis for orthophosphate enzymatically released from adenosine triphosphate, does not interfere, even in high concentration (0.6M). Background absorbance is low. The absorbance of the product, yellow (unreduced) phosphomolybdate in isobutanol-benzene-ethanol, decreases at a rate less than 0.1% per hour. Reproducibility is excellent. Operation is made relatively simple by performing the extraction in tubes especially designed for this purpose, which are mounted in batches of 10-15 on a shaking device.

IN A PREVIOUS COMMUNICATION from this laboratory, the status of existing methods for the determination of orthophosphate in solutions containing ATP (adenosine-5'-triphosphate) and protein was reviewed, the need for a reliable method was made evident, and such a method was described (2). In that procedure protein was precipitated with ClO_4^- . The method is applicable to solutions containing K^+ at concentrations less than 0.05M, levels which occur in ATPase (adenosine triphosphatase) measurements with myofibrils (3) and actomyosin (4), and occasionally with myosin. However, subsequent experience has shown that at higher levels of K^+ , frequently encountered in work with myosin (5), P_i (ortho-

phosphate) is lost by coprecipitation with KClO_4 . Because compatibility with K^+ is essential in many enzymatic studies, a method providing this characteristic has been developed. In addition to compatibility with higher levels of K^+ , the present method yields an absorbance six times that of the former, for a sample containing a given concentration of P_i . Such sensitivity is highly desirable in determination of the kinetic parameters of phosphohydrolases such as myosin with a K_m of the order of $10\mu\text{M}$, where one must deal with substrate concentrations of the order of $10\mu\text{M}$, and consequently with solutions containing P_i at levels of the order of $1\mu\text{M}$.

EXPERIMENTAL

Apparatus. The design of the extraction tube is shown in Figure 1. Up to 15 such tubes are clamped to a horizontal rod of 0.5-inch diameter, which can be rotated about its axis. The rod is held by a Castaloy Flexaframe foot (No. 14-666-25) near each end which serves as a bearing. The bearings are mounted on a simple wooden frame, 12 inches above the base of the frame. To one end of the rod is attached a crank to permit manual rotation of the rod for the purpose of shaking the contents of the tube or to change its position. A holding device or brake is used to keep the tubes in any desired position.

Reagents. Hydrolysis medium was KCl , 0.60M; CaCl_2 , 6.67mM; Tris [tris(hydroxymethyl)aminomethane] buffer, pH 7.5, 0.055M. Reagents for the P_i determination are as follows. To make the trichloroacetate buffer, trichloroacetic acid, 1.78M, and sodium trichloroacetate, 1.78M, are mixed in the proper proportions so that when 4.5 ml of the resulting buffer is added to 25 ml of sample (or sample solvent) a pH of 1.85 ± 0.05 results. (When, as usual, the sample solvent is hydrolysis medium, the pH of the trichloroacetate buffer is made 1.22, a value obtained with a salt to acid ratio of approximately 10:1.) For the diluent, 25 ml of sample solvent (no P_i or compounds which yield P_i on hydrolysis) are mixed

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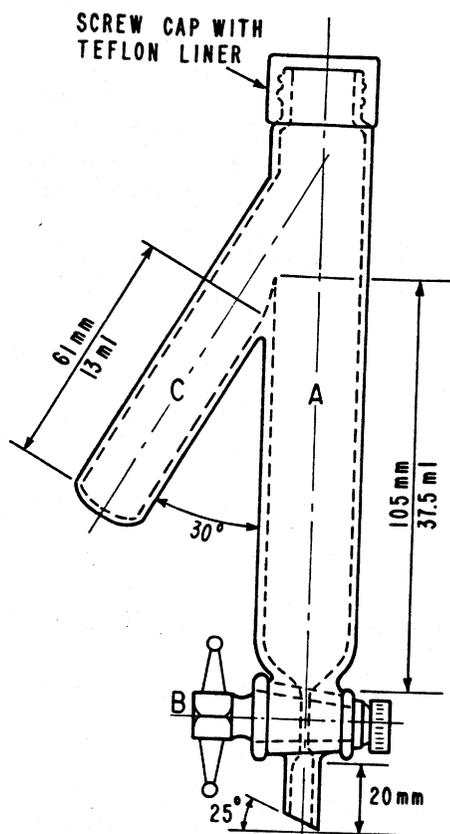


Figure 1. Extraction tube

- A. Culture tube with screw cap, 25 × 150 mm, Corning No. 9826
- B. Teflon plug, straight stopcock, 2-mm plug bore, Corning No. 9288-B10
- C. Medium wall tubing, 3/4-inch o.d., 1/16-inch wall, Corning No. 237,320

with 4.5 ml of trichloroacetate buffer. Other reagents include H_2SO_4 , 19.32*N*; isobutyl alcohol-benzene (iBB)—isobutyl alcohol (iB), 1.77 volume, benzene (B), 1 volume; molybdate— $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, 30*mM*; H_2SO_4 , 0.6*N*; isobutyl alcohol; ethanol, absolute.

Procedure. To 4.5 ml of trichloroacetate buffer in an 85-ml capacity polycarbonate centrifuge tube, 25 ml of sample containing at least 1 μg of P as P_i are added. The contents are mixed by swirling for 45 seconds, avoiding foam formation, and centrifuged at $40,000 \times g$ (20,000 rpm in Spinco rotor No. 21) for 20 min (including acceleration time) at room temperature.

Into the side arm of the extraction tube, 7 ml of iBB and 5 ml of molybdate are placed, and into the main body 29.5 - V ml diluent, where V is the sample volume (see below). The tube is mounted on the shaking rack, and a volume, V (ml), of deproteinized sample containing 1–14 μg of P is added to the diluent, followed by 1.5 ml of 19.32*N* H_2SO_4 . The tube is capped and tilted so that the contents of the side arm empties into the main body, the mixture is shaken vigorously 20–30 times over a period of 20–30 sec, and the phases are allowed to separate with the tube in a horizontal position. The tube is tilted to a vertical position, the “bubble” of organic phase near the stopcock dislodged by rapping the tube with a rubber stopper, and the aqueous phase is drained and discarded.

Twenty milliliters of 0.6*N* H_2SO_4 and 2 ml of isobutyl alcohol are added; the tube is capped, shaken, and the aqueous phase discarded as above. A 4-ml aliquot of the organic phase is transferred into a tube containing 0.3 ml of ethanol and, after mixing, the absorbance (A) at 310 μm is measured with a suitable spectrophotometer calibrated

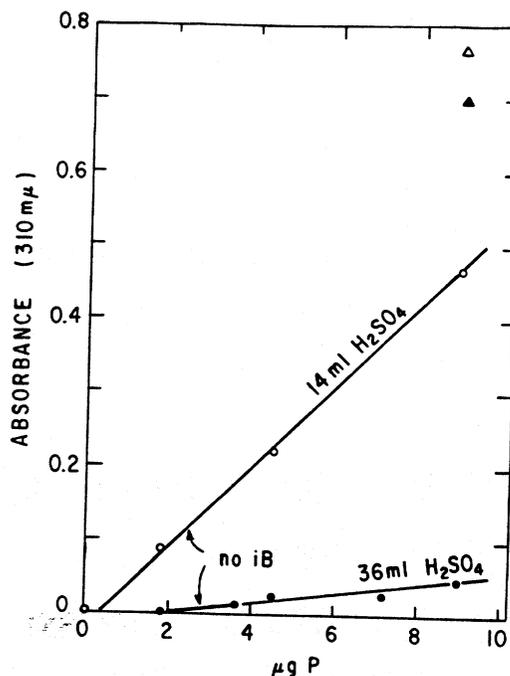


Figure 2. Absorbance as a function of P_i content for samples washed with different volumes of H_2SO_4 and isobutyl alcohol

- 14 ml of H_2SO_4 , no iB
- △ 14 ml of H_2SO_4 + 1.4 ml of iB
- 36 ml of H_2SO_4 , no iB
- ▲ 36 ml of H_2SO_4 + 3.6 ml of iB

for wavelength and absorbance (6). In the experiments described a Beckman DU was used.

Orthophosphate content is calculated from a standard curve prepared with samples containing varying amounts of P_i in a medium simulating that of the unknowns, especially with regard to salt concentrations. The level of phosphorus is expressed as final concentration in mg P/liter, and absorptivity as $(\text{cm})^{-1} (\text{mg P/liter})^{-1}$.

The iBB, molybdate, and diluent may be placed in the extraction tube the day before the determination. The extraction tube should be capped tightly until ready for use.

If the sample has little or no salt, 15 mmoles of KCl may be incorporated into the diluent to facilitate phase separation and to reduce solubility of iB in the aqueous phase.

For a sample containing a high concentration ($>0.025M$) of base, the concentration of H_2SO_4 should be increased (from 19.32*N*) to provide for the extra H^+ requirement, the final $[\text{H}^+]$ in the 36-ml aqueous phase being thus maintained at 0.6*N*.

Reagents and solutions are allowed to contact only glass or plastic resistant to them.

RESULTS

Extraction Apparatus. Because the optimal concentration of hydrogen ion for extraction is 0.6*N*, and because ATP in 0.3*N* (trichloroacetic) acid was found to hydrolyze to P_i at the rate of 1%/hour, it became important to reduce extraction time to a minimum, and to maintain this interval uniform for all samples. The extraction tube and the shaking rack described were designed for this purpose. For 10–15 samples, the interval from addition of H_2SO_4 to completion of extraction is less than 5 min; within another 10–15 min the organic phase is isolated from the aqueous phase.

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Table I. Absorptivity of P_i in iBB-Ethanol in the Presence and Absence of ATP and Myosin

	Myosin, μg	ATP, μmoles	a^a	s_a^b	100 s_a/a , %
A	0	0	0.656	0.0068	1.03
B	1.25	0	0.645	0.0056	0.86
C	1.25	25	0.640	0.0109	1.70

^a $\text{cm}^{-1} (\text{mg P/liter})^{-1}$.

^b Standard error of a .

As originally conceived, the side arm was to be used not only for simultaneous addition of reagents to all of the samples, but also to isolate a major portion of the stable organic phase from the unstable aqueous phase immediately after completion of the extraction. We have found it unnecessary to resort to the latter in our experiments with ATP. However, with more labile phosphates this application may be highly useful.

Deproteinization, Extraction, and Washing. Recognizing the limitations of ClO_4^- for deproteinization, the possibility of using trichloroacetic acid for this purpose was reinvestigated. Preliminary experiments confirmed the efficacy of washing the organic extract obtained from samples containing trichloroacetic acid in reducing background absorbance (7). However, with samples containing ATP variable results were obtained. These were shown to be attributable, at least in part, to the hydrolysis of ATP in the acidic medium. Trichloroacetate buffer was therefore substituted for the acid, the composition of the buffer being chosen to yield a final pH of 1.85 ± 0.05 (2). This modification reduced the rate of hydrolysis of ATP from 1.1 to 0.1% per hour.

In initial efforts to extract the P_i from 25-ml aqueous samples, extraction resulted in a marked decrease in the volume of the organic phase (iBB, 1:1 v/v) (2). To offset this loss of iB to the aqueous phase the extracting solvent was supplemented with additional iB. A ratio of iB:B of 1.77:1 was found suitable.

The effect of washing with H_2SO_4 (alone) on the recovery of P_i in the organic extract is illustrated in Figure 2. In this experiment, extracts containing varying amounts of P_i as phosphomolybdate in iBB were washed with 14 or 36 ml 0.7N H_2SO_4 , and the absorbances read after addition of ethanol (7) to clarify the extract. Extracts washed with the larger volume of H_2SO_4 contained one tenth or less of the amount of phosphomolybdate present in those washed with the smaller volume. Assuming that the loss resulted from the extraction of isobutyl alcohol, and with it phosphomolybdate, from the organic phase into the aqueous phase, the wash reagent was supplemented with isobutyl alcohol. Addition of 10% by volume of iB to the H_2SO_4 increased the absorbance for the smaller wash volume by a factor of 1.6, showing that even the smaller volume of H_2SO_4 washes out phosphomolybdate (in the absence of iB). The lower absorbance after washing with 36 ml of $\text{H}_2\text{SO}_4 + 3.6$ ml of iB (0.703 vs. 0.767) is due to the larger volume of the organic phase in this case, and was taken into account in calculating recoveries (below).

To determine the optimal wash volume, a solution of phosphomolybdate in iBB was prepared in the usual way. Samples containing 9.088 μg of P, in a volume of 6.40 ml, taken from

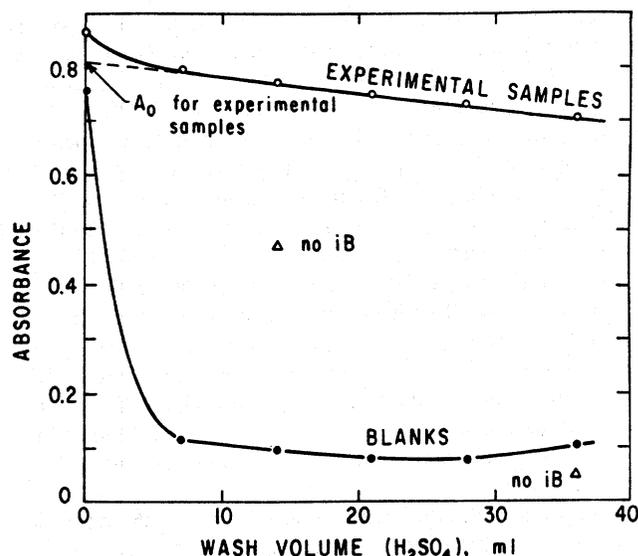


Figure 3. Absorbance as a function of wash volume

The absorbance of each experimental sample was measured at 307.5 $m\mu$ against its corresponding blank as optical reference. For the blanks iBB-ethanol served as optical reference.

- Samples washed with $\text{H}_2\text{SO}_4 + 10\%$ by volume of iB
- △ Samples washed with H_2SO_4 , no iB
- Blanks washed with $\text{H}_2\text{SO}_4 + 10\%$ by volume of iB

the organic extract were then washed with varying volumes of 0.7N H_2SO_4 , together with 10% by volume of isobutyl alcohol. To a 4-ml aliquot from each washed extract, 0.3 ml of ethanol was added and the absorbance measured. The results are shown in Figure 3. The recovery of P_i from the experimental samples was calculated as $R_s = AV/A_0V_0$ where A is the absorbance of the sample measured against the corresponding blank as optical reference, V is its volume, A_0 is the absorbance extrapolated by linear least-squares analysis to zero wash volume, and V_0 is the volume of extract taken for washing (6.40 ml). R_B was calculated in the same way for the blanks, using for A (including A_0 , the absorbance of the unwashed blank) the absorbance measured against iBB-ethanol as optical reference. R_B represents the recovery of undesired absorbing material, relative to the unwashed blank. A P_i recovery of $101 \pm 0.5\%$ was obtained. The ratio R_B/R_s has a minimum at a wash volume of 21–28 ml (H_2SO_4). Twenty milliliters of H_2SO_4 , with 2 ml of iB, was therefore chosen as convenient and optimal for the washing operation. The volume of the extract resulting (6.9 ml) is virtually equal to that of the extracting solvent (7 ml).

Incorporation of ammonium molybdate at a level of 0.4mM into the wash reagent has no effect.

A reinvestigation of the effect of varying the sulfuric acid concentration during extraction and washing under the new conditions showed the optimum concentration to be 0.6N, with rapid loss of absorbance below 0.5N and above 0.9N. These results are similar to those obtained previously (2) except that the optimum is ca. 0.1 pH unit less than in the earlier procedure.

Spectra of washed extracts in iBB-ethanol prepared in the usual way had a maximum at 310 $m\mu$. The absorbances at 308 and 312 $m\mu$ differed from those at 310 $m\mu$ by less than 0.2%. The absorbance at 310 $m\mu$ decreased with time to a new level ca. 7% lower than the original, the decay being exponential, with a rate of ca. 1% per hour. The overall rate of change of absorbance was thus less than 0.1% per hour.

(7) I. Berenblum and E. Chain, *Biochem. J.*, **32**, 286, 295 (1938).

Effect of Protein and ATP. To evaluate the possible effects of myosin and ATP, a determination of P_i was carried out as set forth in the Procedure section with three groups of samples containing varying amounts of KH_2PO_4 in hydrolysis medium. Group A (see Table I) contained neither myosin nor ATP; group B contained myosin at a level frequently used; and group C contained both myosin and ATP, the latter at the highest level encountered in our experiments on the kinetic parameters of the enzyme. The ATP was added after the trichloroacetate buffer, simulating the "zero-time control" in the determination of rate of enzymatic hydrolysis. The slope of each of the three curves of A vs. P_i was calculated by a least-squares analysis, which yielded the absorptivities and standard errors shown in Table I. Statistical comparison showed that no significant differences in absorptivity could be attributed to the presence or absence of ATP or protein.

Application of the Phosphate Determination to the Assay of Adenosine Triphosphatase Activity. PRODUCTION OF P_i AS A FUNCTION OF ENZYME CONCENTRATION. ATP at a concentration of 0.998mM was incubated at 22.0° C for 5 min in 25 ml of hydrolysis medium with myosin at various concentrations ranging from 0 to 69 mg/liter. Enzyme action was terminated and P_i determined as in Procedure, the aliquot after deproteinization being 20 ml. The plot of absorbance vs. concentration of protein was linear, with an intercept at -0.005 ± 0.020 absorbance unit and a slope of 0.0183 absorbance unit/[5 min] (mg of myosin/liter)]. The intercept is thus zero, in contrast to the negative intercept obtained when ClO_4^- was used for deproteinization (2). A specific activity of $1.36 \pm 0.03 \mu\text{mole } P_i/(\text{sec} \cdot \text{gram})$ was calculated from the slope. The myosin was from beef *longissimus dorsi* muscle and had been stored at 0° C for 6 days prior to the assay.

PRODUCTION OF P_i AS A FUNCTION OF TIME. Myosin prepared from rabbit *longissimus dorsi* muscle was incubated with ATP at 22.0° C in hydrolysis medium at two levels of the substrate, 106.5 and 31.56 μ M. At varying time intervals a 25-ml sample was removed and, after deproteinization, a 10-ml aliquot was analyzed for P_i . For complete hydrolysis at the lower substrate concentration, it was calculated, from the absorptivity for P_i , that a 10-ml aliquot of the hydrolyzed solution would give an absorbance of 0.718. The predicted absorbance agrees well with the experimental values obtained after hydrolysis was virtually complete, viz., 0.719, 0.725, and 0.720. The initial velocities were 0.164 ± 0.001 and $0.156 \pm 0.000\mu\text{M}$ of P_i/sec .

DISCUSSION

The advantage of the present method, from the standpoint of sensitivity, is the high value of absorbance (A) obtained per unit concentration (c_0) of P (as P_i) in the aqueous sample subjected to analysis (Table II). This ratio A/c_0 we term a_0 , in keeping with the designation for absorptivity, a , which is defined as A/c , the absorbance obtained per unit concentration (c) of P in the organic extract. Clearly, for a case such as that considered here, where the volume of the reaction medium to be sampled may be chosen at will, a_0 , rather than a , should be the criterion for evaluating the sensitivity of a method. The two are simply related: $a_0 = \left(\frac{m}{m_0}\right) \left(\frac{v_0}{v}\right) a$, where m_0 is the mass of P (as P_i) in the aqueous sample, v_0 its volume, and m and v the corresponding quantities for the

Table II. A Comparison of Various Methods of Orthophosphate Determination

a^a	a_0^b	Reference
ca. 0.5	ca. 0.25	(7)
0.75	0.6	(8)
0.12	0.012	(9)
3.06	0.42	(10)
0.64	0.77	(1)
0.546	0.066	(11)
0.906	0.362	(2)
0.800	0.032	(12)
0.640	2.16	This paper

^a Absorptivity, cm^{-1} (mg of P/liter)⁻¹.

^b Absorbance obtained per unit concentration of P in the aqueous sample, cm^{-1} (mg of P/liter)⁻¹.

extract. a_0 is thus proportional to the absorptivity, to the ratio of the volumes, and to m/m_0 , the fraction of the amount of P (as P_i) in the aqueous sample which appears in the extract. (The latter is, in turn, a function of the size of the aliquots taken at various stages of the analysis. It is assumed that at every stage of the procedure, the volume of sample or aliquot taken is the maximum which the method permits.)

For the present method $a_0 = \left(\frac{4}{6.89}\right) \left(\frac{25}{4.3}\right) (0.640) = 2.16$

(Table II). This high value was achieved by choosing the composition of the extracting solvent so as to permit concentration of the phosphate from a large (aqueous) sample volume ($v_0 = 25$ ml) by extracting it, as phosphomolybdate, into a small volume of organic solvent, 6.9 ml, and (when desirable) by extracting the whole of the deproteinized supernatant.

Optimal conditions of acidity, molybdate concentration, etc., were determined for the formation and extraction of phosphomolybdate into isobutyl alcohol-benzene and for removal from the organic phase of interfering substance(s), possibly trichloroacetic acid itself, introduced by the protein precipitant. Removal of interfering substances is accomplished by washing the extract with dilute H_2SO_4 (7) containing isobutyl alcohol. The addition of iB was shown to be necessary to prevent loss of phosphomolybdate from the organic phase.

Extraction and washing of samples is carried out in specially designed tubes which serve both as extraction vessels and as separatory funnels. These are mounted on a rack in batches of 10-15 tubes. The reduction in time required to process the deproteinized samples, and the identical treatment which the samples receive, together with the mild conditions of deproteinization, virtually eliminate any error resulting from non-enzymatic hydrolysis of ATP.

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