THE DETERMINATION OF INORGANIC PHOSPHATE IN THE PRESENCE OF LABILE PHOSPHATE ESTERS

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Labile phosphate esters such as phosphocreatine, acetyl phosphate, and ribose-1-phosphate (1) have assumed great biological importance. These esters are so unstable that they are split with great rapidity by the reagents commonly used for inorganic phosphate determination. Therefore, the usual inorganic phosphate measurements in tissue extracts, etc., actually represent the sum of the inorganic phosphate and the phosphate of these labile esters. In order to determine the labile esters it has usually been necessary to perform a prior removal of inorganic phosphate by precipitation (2). By working very rapidly, Fiske and Subbarow (3) were able to measure inorganic phosphate in the presence of phosphocreatine, but, due to the speed of the reaction, this procedure has been difficult to use. The detection and determination of such esters would become very simple if it were possible to determine inorganic phosphate in their presence, since the labile phosphate could then be measured by the inorganic phosphate liberated as the result of very mild hydrolysis.

It has been found possible to establish conditions under which inorganic phosphate can readily be determined in the presence of labile esters. In addition to describing the procedure, data are presented as to the speed of hydrolysis of several of the labile esters under the prescribed conditions.

Method and Procedure

Phosphomolybdic acid is much more readily reduced to blue molybdous compounds than is molybdic acid itself. This fact is the basis of most colorimetric methods for inorganic phosphate. In the well known Fiske and Subbarow procedure (4) this reduction is effected in 0.5 N sulfuric acid by means of bisulfite with 1,4-aminonaphtholsulfonic acid as a catalyst. In the present determination the following changes from the original Fiske and Subbarow procedure are made. The pH is shifted from 0.65 to 4.0, the molybdate concentration is reduced from 0.25 per cent to 0.1 per cent, and ascorbic acid is substituted for the original mixture of bisulfite and 1,4-aminonaphtholsulfonic acid. Under these conditions the labile esters are much more stable than with the reagents prescribed by Fiske and Subbarow. The use of ascorbic acid as a reducing agent in the determination of phosphate, but at a strongly acid pH, has been previously described (5).

The sample to be analyzed is deproteinized under conditions which will not hydrolyze the particular ester; *e.g.*, ice-cold 0.3 N (5 per cent) trichloroacetic acid or 0.3 N (3 per cent) perchloric acid, or, particularly with very labile esters, saturated ammonium sulfate which is 0.1 N in acetic acid and 0.025 N in sodium acetate (pH 4). If either of the acid precipitants is used, the extracts are rapidly brought to pH 4 to 4.2 by adding 4 volumes of 0.1 N sodium acetate. Most of the labile esters are reasonably stable at this pH. The extracts are diluted with acetate buffer of pH 4 (0.1 N acetic acid, 0.025 N sodium acetate) until the inorganic phosphorus is 0.015 to 0.1 mM (0.05 to 0.3 mg. per cent of P). Ammonium sulfate extracts should be diluted at least 5-fold. To each volume of extract is added 0.1 volume of 1 per cent ascorbic acid and 0.1 volume of 1 per cent ammonium molybdate in 0.05 N sulfuric acid. Readings are made at 5 and again at 10 minutes after the molybdate addition at a wave-length of 700 mμ. (Any wave-length between 650 and 950 mμ is satisfactory.) Simultaneous readings are made on a 0.05 mM standard and a blank, both of the same composition, as far as possible, as the unknown. If a difference is observed in the unknown readings at 5 and at 10 minutes compared to the standard, the values are extrapolated to zero time. The ascorbic acid and molybdate may be combined before addition but must then be used within 15 minutes.

In the presence of certain tissue extracts, the reaction is delayed, in which case an internal standard must be used. A standard amount of inorganic phosphate is added to a duplicate tissue aliquot, and values of the unknown are calculated from the difference between the readings of the unknown and of the unknown with added phosphate. The inhibitory effect may be partially overcome by dilution. For example, in order to avoid undue inhibition, brain and muscle extracts should be diluted to a volume 150 to 250 times that of the tissue, and liver extracts to a volume 300 to 500 times that of the original liver. In addition, the molybdate concentration may be increased to 0.15 per cent; *i.e.*, 0.1 volume of 1.5 per cent ammonium molybdate in 0.05 N sulfuric acid is added in place of the 1 per cent solution. This results in an acceleration of color development. In studying isolated enzyme systems, this problem of inhibition is ordinarily not encountered.

The ascorbic acid concentration may be increased, if necessary, to accelerate the reaction, but with final concentrations greater than 0.2 to 0.3 per cent the readings of the standard increase unduly with time. The final pH may be varied, if desired, between 3.5 and 4.2.

DISCUSSION

The following observations suggested the possibility that inorganic phosphate might be determined under conditions which would be less likely to

hydrolyze labile phosphate esters. As the acidity of a mixture of molybdic acid and reducing agent is progressively changed, an interesting phenomenon occurs (Fig. 1). Molybdic acid itself is reduced at intermediate pH levels, resulting in a large and progressive blank. With further increase in pH, molybdate is no longer reduced; yet phosphomolybdate may still undergo reduction. Thus, with aminonaphtholsulfonic acid and bisulfite as reducing agents, a large reagent blank is encountered between pH 0.8 and 1.8, and with the stronger ascorbic acid reducing agent, the reagent blank becomes very large between pH 0.9 and 2.8 (Fig. 1). Phosphomolybdate continues to be reduced at a useful rate as far as pH 3.2 with bisulfite, and as far as pH 4.6 with ascorbic acid. Therefore, with the Fiske and

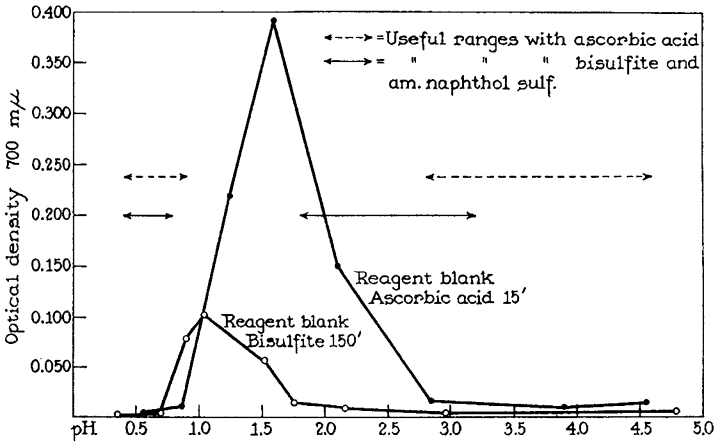

FIG. 1. Reagent blank values at different pH levels and useful pH ranges with (*a*) ascorbic acid and (*b*) bisulfite-aminonaphtholsulfonic acid as reducing agents.

Subbarow reagents, phosphate may be measured between pH 0.4 to 0.7 and pH 1.8 to 3.2, and with ascorbic acid between pH 0.4 to 0.9 and pH 2.8 to 4.6 but not at intermediate pH levels. Since acidity accelerates the splitting of labile phosphate esters, ascorbic acid with its more nearly neutral effective range was chosen. Actually, the less acid pH range with ascorbic acid is limited to pH 3.5 to 4.5, since between pH 2.8 and 3.5 color continues to develop unduly with time.

Effect of Ascorbic Acid and Molybdate Concentrations on Color Development—In general, it has been found that the higher the ascorbic acid concentration the more rapid is the reduction of phosphomolybdic acid. If the ascorbic acid concentration is too great, the standard continues to develop color with time. The concentration recommended, 0.1 per cent, is a satisfactory compromise. Decreasing the molybdate concentration

tends to slow the rate of color development. Reduction in the concentration of molybdate is desirable in order to decrease its catalytic effect in splitting phosphocreatine or acetyl phosphate; however, the concentration cannot be reduced much below 0.05 per cent without inordinate decrease in the rate of color development. Indeed, if the concentration is much below this figure full color is never developed. It is worth noting that if ascorbic acid stands with the weak molybdate reagent at pH 4.0 before phosphate is added the reagent becomes incapable of developing color rapidly and the reaction does not proceed to completion. The mechanism of this effect is obscure; it is not due to destruction of ascorbic acid. It is, therefore, essential that the ascorbic acid and molybdate are not mixed together until just before use.

The reaction is slower the higher the pH. With 0.25 per cent molybdate the reaction is sufficiently rapid up to pH 4.6. With 0.05 per cent molybdate pH 4.2 is about the practical pH limit. Since change of hydrogen ion concentration has less effect in this pH range on the splitting of phosphate esters than the molybdate concentration, it is desirable to work in the more acid region with the lowest permissible molybdate concentration.

The reaction is accelerated by raising the temperature; for example, the reaction is essentially complete in 2 minutes at 35° and 6 minutes at 25°. At 0° almost no color is produced.

Disturbing Substances—The following concentrations of added substances were found not to cause disturbing change in color development: sodium acetate 0.5 M, sodium sulfate 0.3 M, potassium nitrate 0.3 M, ammonium sulfate 0.2 M, sodium chloride 0.15 M, sodium perchlorate 0.15 M, sodium trichloroacetate 0.15 M, sodium fluoride 0.13 N, glycine 0.02 M, sodium oxalate or citrate 0.05 M, sodium nitrite 0.004 M, sodium tungstate 0.004 M, and ferric chloride 0.003 M. With larger amounts of many of these substances the color development is delayed. This delay can often be overcome by increasing the ascorbic acid concentration to 0.2 per cent. Unfortunately, color develops with both arsenate and silicate. Not over 2 mg. per cent of arsenate or 0.5 mg. per cent of silicate is tolerable. It is, therefore, necessary to avoid reagents such as sodium hydroxide, which may contain silicate. In neutralizing acids, sodium acetate is desirable, since it is not likely to contain silicate and it buffers the reaction at an appropriate pH.

Proportionality and Reproducibility—The optical density of solutions with different amounts of phosphate is linear from 10 to 75 $\times 10^{-6}$ mole per liter (0.03 to 0.25 mg. per cent of P). This is equivalent to extinctions (optical densities with a 1 cm. light path) from 0.05 to 0.35 at 700 mμ. The reproducibility in the Beckman spectrophotometer is ± 1 or 2 per cent over this range, depending on the concentration. The absorption is essentially the same from 700 to 900 mμ, with a maximum at 860 mμ.

Effect on Labile Esters—Figs. 2 to 4 illustrate the hydrolytic rates for various phosphate esters under different conditions of pH and molybdate concentration. The rates were measured at room temperature without any attempt at exact temperature control. All of the rates are, of course, influenced somewhat by the temperature. The accelerating effect of molybdate on the splitting of phosphocreatine and acetyl phosphate has been previously observed (2, 4). Phosphocreatine (Fig. 2) is split under the conditions described by Fiske and Subbarow for inorganic phosphate determination with a half time of about 4 minutes. At pH 4, with 0.25

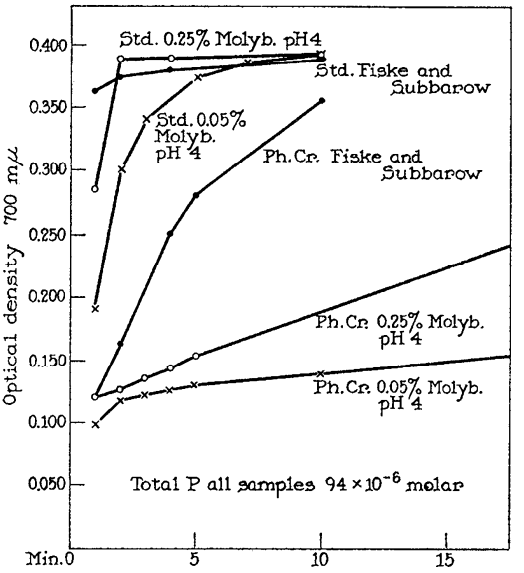

FIG. 2. Splitting of phosphocreatine

per cent molybdate, the half time is nearly 20 minutes, and with 0.05 per cent molybdate at pH 4.0 the half time is about 70 minutes. With acetyl phosphate (Fig. 3) in the strong acid reagent the half time of hydrolysis is 30 or 40 seconds, at pH 4.5 with 0.25 per cent molybdate it is 12 minutes, and at pH 4 with 0.05 per cent molybdate it is approximately 90 minutes. It would appear that the change in pH affects both esters to about the same degree but that molybdate has a greater relative effect on acetyl phosphate than on phosphocreatine.

Ribose-1-phosphate (Fig. 4) has a half time of about 2.5 minutes in the Fiske and Subbarow reagents. The hydrolysis is much slower at pH 4.0. The half time with 0.25 per cent molybdate appears to be over 3 hours. Thus, the change in stability with pH is relatively greater for ribose-1-

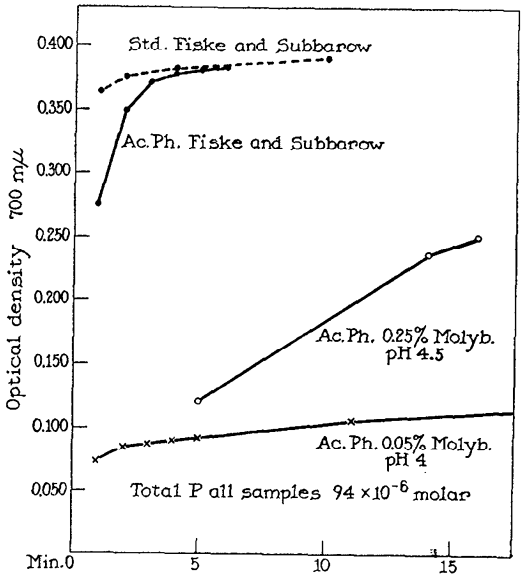

FIG. 3. Acetyl phosphate splitting

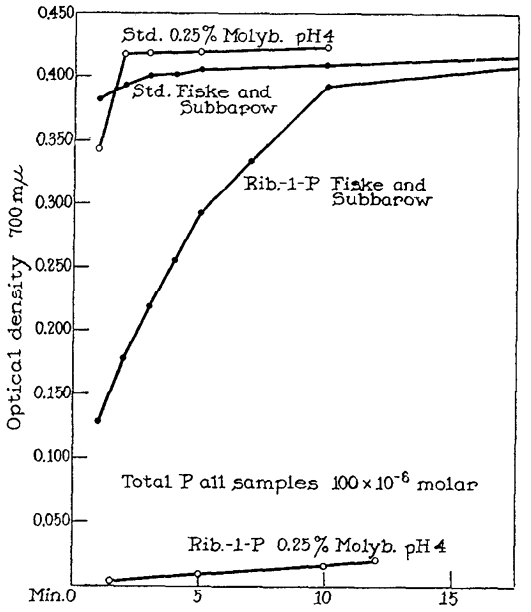

FIG. 4. Ribose-1-phosphate splitting

phosphate than for phosphocreatine or acetyl phosphate. Dr. H. M. Kalckar, in this laboratory, has observed that molybdic acid does not accelerate the splitting of ribose-1-phosphate.

Representative Tissue Analyses—Rat skeletal muscle and brain were analyzed for inorganic phosphorus with both the Fiske and Subbarow reagent and the ascorbic acid reagent at pH 4.0. The extracts used were prepared with ice-cold ammonium sulfate at pH 4.0 from tissues frozen *in situ* with petroleum ether chilled with solid carbon dioxide. The final

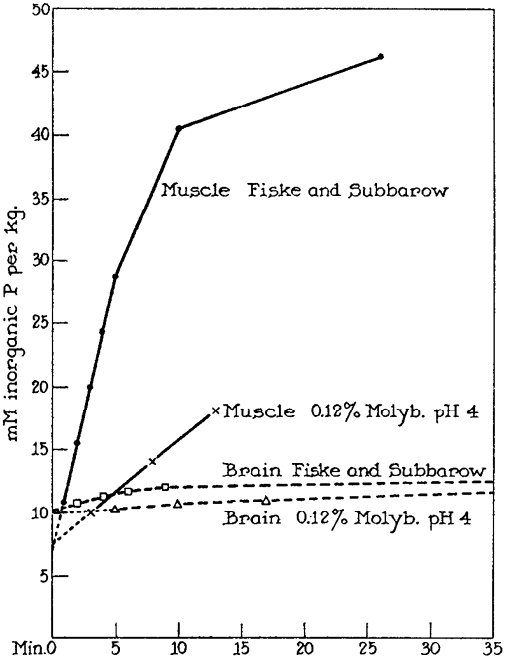

FIG. 5. Inorganic phosphate in muscle and brain

reagent was 0.125 per cent in ammonium molybdate and the final tissue dilution was about 500-fold. With both reagents approximately the same extrapolated values are obtained for inorganic phosphate. This indicates the absence of significant amounts of acetyl phosphate (Fig. 5). With the ascorbic acid reagent the change in the inorganic phosphate concentration is slow enough to make it easy to obtain the initial inorganic phosphate value.

SUMMARY

1. A procedure is described for the measurement of inorganic phosphorus in the presence of labile phosphate esters. The reaction is based on the reduction of phosphomolybdate by ascorbic acid at pH 4.0.

2. Under the reaction conditions the splitting of phosphocreatine, acetyl phosphate, and ribose-1-phosphate is decreased to 5 per cent or less of the rate observed in the presence of the Fiske and Subbarow reagents. This makes possible a simple determination of the concentration of such labile esters.

3. There do not appear to be significant amounts of acetyl phosphate in skeletal muscle or brain.

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