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THE ESTIMATION OF INORGANIC PHOSPHATE IN THE PRESENCE OF ADENOSINE TRIPHOSPHATE

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SUMMARY

The estimation of inorganic phosphate in the presence of ATP by the formation of "molybdenum blue" may be accompanied by appreciable molybdate-catalysed hydrolysis of ATP. A method to overcome this is described; excess molybdate is removed as a citrate complex after the extraction of phosphomolybdate by butanol, and phosphomolybdate absorbance is determined at 310 mμ.

INTRODUCTION

The phosphate content of biological material is usually determined by measurement of the depth of colour of "molybdenum blue" produced by the phosphate-activated reduction of molybdic acid. One such method, that of ALLEN[1], which has been employed successfully for many years, was used recently to estimate the adenosine tri-

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phosphatase (ATPase) activity of muscle fibres in the presence of a large excess of substrate. The results were most unsatisfactory. As an example of the difficulties encountered, Table I shows the results of an attempt to determine the inorganic phosphate content of a freshly-prepared, neutralised solution of crystalline disodium ATP (Schwarz) following storage in dry form at —5° for 2 years. Inorganic P (P_I) was later found to account for only 1.05 % of the 10′ P (P present after 10 min in *N* HCl at 100°). Even quite small errors in estimating the P_I content of ATP may cause appreciable errors in ATPase determinations. Thus in one experiment in this laboratory, the ATPase activity of a muscle preparation appeared to decline rapidly with increasing substrate concentration. This false result was due to an over-estimate, amounting to only 0.5 % of the acid-labile P, of the P_I content of the ATP solution.

TABLE I

ESTIMATION OF INORGANIC P IN PRESENCE OF ATP BY ALLEN'S METHOD

Phosphate solution ml	*After molybdate addition min*				
	5	*10*	*15*	*20*	*30*
	Apparent P_I in per cent of 10′ P				
0.25	2.32	2.43	2.53	2.64	2.83
0.50	1.45	1.55	1.64	1.75	1.96
1.00	0.99	1.08	1.18	1.27	1.46

Certain phosphate esters, notably creatine phosphate, have long been known to undergo rapid acid hydrolysis in the presence of molybdate (FISKE AND SUBBAROW[2]), and WEIL-MALHERBE AND GREEN[3] have drawn attention to the very appreciable molybdate catalysis of ATP dephosphorylation in acid solution. This molybdate effect probably accounts for the quite unsatisfactory results mentioned above, and a method was sought in which it could be overcome. The methods of FISKE AND SUBBAROW[4] and KING[5] are liable to the same errors as that of ALLEN[1], while the procedure of LOWRY AND LOPEZ[6], originally devised to prevent the hydrolysis of labile phosphate, is peculiarly ill-suited for use with ATP[3]. The method of BERENBLUM AND CHAIN[7], in which phosphomolybdic acid is extracted into isobutanol before reduction to molybdenum blue, offered promise, but is unwieldy and time-consuming if 20–30 estimations are to be made daily.

WADELIN AND MELLON[8] introduced a sensitive method which, unlike those mentioned above, depends on the colour of phosphomolybdic acid rather than that of molybdenum blue. Phosphomolybdic acid is extracted with 20 % (v/v) butanol in chloroform, this being used in preference to butanol alone in order to reduce the large blank absorbance due to molybdate in the solvent phase. The solvent mixture, however, extracts phosphomolybdic acid rather poorly, and two extractions, each of a minute, are required. As a result, the method is relatively slow and, when applied to the present problem, allows a dangerously long period of molybdate-catalysed hydrolysis of ATP before the final separation of the two phases. In the present modification of the method, the two extractions with butanol–chloroform have been replaced by one with butanol alone in order to greatly accelerate extraction and to permit the operation to be performed in one flask. The ability of citrate to form

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a stable complex with molybdate (DAVIES AND DAVIES[9]) is utilised to reduce blank absorbance by removing molybdate from the solvent phase, and to prevent molybdate catalysis of ATP hydrolysis in the water phase.

REAGENTS

(1) *Acid molybdate.* 50 ml concentrated hydrochloric acid and a solution of 20 g sodium molybdate dihydrate in water are mixed and diluted to 500 ml.

(2) *Citrate.* A solution of 100 g sodium citrate dihydrate is diluted to 500 ml after adjustment of pH to 7 with hydrochloric acid.

(3) *Butanol.*

(4) *Methanol.*

PROCEDURE

The sample, containing up to 25 μg (preferably 1–10 μg) of P_i, is transferred to a 25 ml volumetric flask and diluted with water to 10–12 ml. 10.0 ml butanol is added, followed by 1 ml acid molybdate. The flask is shaken vigorously for 5–10 sec, after which 2 ml citrate is added. The volume is increased to 25 ml with water and the flask is again shaken for 5–10 sec. About 3 ml of the upper phase is transferred by pipette to a 1 cm silica cell, 2 drops of methanol are added to remove a turbidity which occasionally develops, and the cell is inverted to mix the contents. Absorbance is measured at 310 mμ (hydrogen lamp) against a blank prepared as above but containing no phosphate.

In a simplified procedure, the volumes of added reagents are reduced to 0.6 of those recommended above, and a centrifuge tube calibrated at 15 ml is used in place of the 25 ml flask. Following the second shaking, the tube is centrifuged for a few seconds to remove the last droplets of water from the butanol layer, and part of the upper phase is then decanted to the cell. Sensitivity is increased by 60 %, and the desirable P_i content of the sample is reduced to about ½–6 μg.

It is important that the citrate should be added *after* the extraction of the phosphomolybdate to the butanol phase. If the first shaking is omitted, citrate causes a quite rapid diminution of absorbance, presumably by decomposing the phosphomolybdate complex in the water phase. If the method above is followed, however, the absorbance is constant for at least 10 min, and decreases by only about 1 % of its value in the next 15 min.

The method permits quite wide variations in the volumes of added reagents. Thus in estimating 5 μg of P by the first procedure no significant error was introduced by adding either 0.5 or 1.5 ml of acid molybdate, the blank containing the recommended 1.0 ml in each case. Similarly no change in absorbance was observed when the volume of citrate was varied between 1.8 and 2.3 ml.

The second procedure was employed to determine the P_i content (relative to 10′ P) of a dilute solution of the ATP previously examined by ALLEN's method. The results are shown in Table II. Two reproducibility studies were also performed. In the first, the mean ($\pm$ standard deviation) of the P_i content of an ATP solution was found to be 2.55 $\pm$ 0.028 μg/ml (eight replicates; extremes 2.51, 2.59). In the second, single P_i and 10′ P estimations were made whenever fresh solutions were

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prepared from the same solid ATP; the mean P_i content, relative to 10′ P, was 1.05 ± 0.03 % (six analyses in five months; extremes 1.01, 1.10).

TABLE II

ESTIMATION OF INORGANIC P IN PRESENCE OF ATP BY NEW METHOD

Phosphate solution ml	*After molybdate addition min*				
	5	*10*	*15*	*20*	*30*
	Apparent P_i in per cent of 10′ P				
0.25	1.05	1.05	1.05	1.05	1.04
0.50	1.05	1.05	1.05	1.05	1.04
1.00	1.05	1.05	1.05	1.04	1.03

EFFECT OF INTERFERING SUBSTANCES

Up to 2.4 ml *M* KCl is tolerated by the first procedure without change in absorbance, but readings increase if this amount is exceeded. Interference by higher concentrations, up to at least 6 ml 2 *M* KCl, is completely suppressed by adding an equivalent amount of KCl to the blank.

The presence of up to 2.5 ml *N* HCl (first procedure) does not affect absorbance. Appreciable errors are introduced, however, by alkali addition. With 0.1–0.9 ml *N* NaOH, the water phase becomes intensely yellow, a trace of this colour being extracted to the solvent layer on shaking; further addition of alkali causes a complete discharge of phosphomolybdate absorption from the butanol phase. For our purposes this raises no difficulty, estimations always being performed on neutral or acidified extracts. The acid tolerance of the method is useful in the estimation of labile phosphate in ATP by acid hydrolysis; a volume of up to 1.25 ml is heated with an equal volume of 2 *N* HCl and after cooling the colour is developed in the usual way. Similarly, in the determination of total phosphate after digestion in perchloric acid, the surplus acid is almost neutralised before molybdate addition, or alternatively the final colour development is performed on only a portion of the digest containing not more than the equivalent of 2.5 ml *N* acid. If, however, a certain tolerance to alkali is desirable, it can be achieved (with a corresponding decrease in acid tolerance) by making the molybdate reagent more strongly acid.

As little as 0.4 ml 5 % trichloroacetic acid (TCA) causes a significant change in absorbance in the first procedure. With 5 μg P present the error amounts to −2 %, or to +1 % if the acid is neutralised, the water phase in the latter case becoming very yellow. If an equivalent amount of TCA is added to the blank, however, no error is introduced by up to at least 10 ml 5 % TCA (not neutralised). The effect is due to a decrease in the absorbance of both the blank and the sample containing phosphate, the maximum lowering of about 0.06 being caused by 4 ml or more of 5 % TCA. This decrease, which diminishes the absorbance to that of butanol alone, appears to be due to the ability of TCA to expel final traces of molybdate or citrate–molybdate complex from the butanol phase. TCA does not interfere, therefore, provided an equivalent amount is added to the blank.

Using the FISKE-SUBBAROW method, BLUM AND CHAMBERS[10] observed that P_i

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estimations were too low in the presence of much ATP. The same effect has been observed in the present study, using the first procedure. In the absence of ATP the response to P_i is linear over the whole scale, but in the presence of 2000 μg acid-labile P of ATP, a departure from linearity occurs at an absorbance of about 1.6 (21.3 μg P). Further addition of ATP, even though accompanied by trace amounts of P_i, causes a decrease in absorbance; with 3600 μg acid-labile P, an absorbance of only 0.25 was observed. This effect, suggesting the formation of an ATP–molybdate complex, is being further studied.

The limitation imposed by the presence of ATP is not at all restrictive in view of the sensitivity of the method, and it is most improbable that errors due to this effect could be introduced during the estimation of ATPase activity. If it is suspected that the limit has been exceeded, a simple and rapid check may be carried out by estimating P_i on two aliquots of the sample, one of twice the volume of the other. A ratio of absorbances of less than 2:1 indicates that the permissible limit has been passed in at least the stronger, and possibly in both, of the aliquots.

DISCUSSION

In view of the wide-spread use of the methods of FISKE AND SUBBAROW, LOWRY AND LOPEZ, and ALLEN in ATP-ase determinations, it is disconcerting to observe appreciable errors due to the molybdate-catalysed hydrolysis of ATP during colour development; the warning issued earlier by WEIL-MALHERBE AND GREEN[3] appears to have gone unheeded. Furthermore, the effects of KCl and ATP on P_i estimations by the FISKE-SUBBAROW method might also lead to spurious results, as indicated by BLUM AND CHAMBERS[10]. In the method described in this paper, these two serious defects are satisfactorily eliminated: the first by butanol extraction of phosphomolybdate and removal of excess molybdate as a citrate complex, the second by a greatly enhanced sensitivity to inorganic phosphate. In view of the magnitude of the error which may be introduced in determinations of ATPase activity by a small error made in estimating the low P_i content of the ATP, the continued use of the molybdenum-blue type of reaction in homogeneous solution cannot be justified.

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