THE FURTHER PURIFICATION AND PROPERTIES OF A PHOSPHATASE FROM SPLEEN ABLE TO HYDROLYZE COMPLETELY THE PHOSPHORUS OF α-CASEIN

JOHN A. GLOMSET

Institute of Medical Chemistry, University of Uppsala, Uppsala (Sweden)

(Received July 26th, 1958)

SUMMARY

1. The spleen phosphatase preparation of SINGER AND FRUTON has been further purified using chromatography on triethylaminoethyl-cellulose columns and preparative electrophoresis.

2. The purified enzyme moves as a single peak on electrophoresis in acetate buffer, pH 5.6, and has a blue-violet color.

3. The enzyme is active toward phosphoramidate, *p*-nitrophenylphosphate, inorganic pyrophosphate and adenosinetriphosphate, but not towards glycerylphosphate or Ca(bis(*p*-nitrophenyl)phosphate)$_2$.

4. The enzyme is able to remove completely the phosphorus of α-casein and of unfractionated "Hammarsten casein".

INTRODUCTION

Since 1946, when HARRIS[1] first discovered the presence of phosphoprotein phosphatase activity in frog eggs, the occurrence of similar actvity has been demonstrated in a number of additional biological materials[2,3,4]. Of these, mammalian spleen has been shown to be a particularly rich source, and the preparation of partially purified "phosphoprotein phosphatase" from this organ has been described by several authors[5,6,7,8]. However, there are several points regarding the nature of the spleen enzyme that are not clear. The several preparations appear to differ with respect to both substrate specificity and physical properties. Furthermore, it is difficult to evaluate the extent to which they are contaminated with extraneous proteins. Nor is it entirely certain that the "phosphoprotein phosphatase" activity itself is due to a single enzyme.

The present study was undertaken in an attempt to resolve some of these questions. The specific plan of the investigation was to select the most convenient method of preparation of starting material from bovine spleen, attempt the further characterization and purification of the enzyme using chromatographic and electrophoretic methods, and finally investigate the activity of the purified enzyme toward α-casein and other substrates.

References p. 357.

MATERIALS AND METHODS

Starting material

For most experiments the starting material was prepared according to SINGER AND FRUTON[6], freeze-dried, and stored at —16° before use. Enzyme prepared according to SUNDARARAJAN AND SARMA[9] was employed in some preliminary experiments.

Chromatography

TEAE-cellulose* was prepared from DEAE-cellulose according to PORATH[10]. It was employed in the chloride form as in previous experiments[11,12]. Generally the columns contained 3–20 g of the exchanger. The exchanger bed was contained in glass tubes, plugged at the bottom with glass wool, and having an inner diameter of 1–2 cm. Stepwise elution was performed as before[11]. 1 ml of 0.1 *M* sodium acetate–acetic acid buffer, pH 5.6 was pipetted into each of the tubes of the fraction collector before beginning the chromatographic experiment. This was done in order to reduce the pH of the effluent as quickly as possible and thus diminish the risk of denaturation of the enzyme, which is partially inactivated on standing at pH 7.4.

In some experiments carboxymethyl-cellulose, prepared according to PETERSON AND SOBER[13] was employed. The columns contained 10 g of the exchanger (0.4 milliequiv. of acid groups/g dry weight).

Electrophoresis

Preparative electrophoresis was performed using the technique of MÜLLER-EBERHARD AND KUNKEL[14] on blocks of "Pevikon"[15], a copolymer of polyvinyl chloride and polyvinyl acetate containing 13 % of the latter (Superfosfatbolaget, Stockholm). The block size was generally 1 × 30 × 60 cm. The buffer used was 0.06 *M* sodium acetate–acetic acid, pH 5.6. Both electrophoresis and chromatography were carried out at +6°.

Concentration of the protein solutions

The volume of the protein solutions was reduced by ultrafiltration in the ARONSSON apparatus[16] and through collodion membranes (Membranengesellschaft, Göttingen).

Enzyme-activity determinations

Incubation of the enzyme was performed in Michaelis' veronal acetate buffer, pH 6.0, containing 0.02 *M* mercaptoethanol as activator and Hammarsten casein (Merck) in a concentration of 10 μmoles P/ml medium as substrate. The total reaction mixture was 1.4 ml in volume. The reaction was started and stopped in an ice bath. The incubation was carried out for 15 min at 37°. Subsequently, 0.1 ml of 10 *N* H_2SO_4 was added and after centrifugation an aliquot was taken for determination of inorganic phosphate by the method of MARTIN AND DOTY[17] (using their silico-tungstate reagent). Enzyme-activity values were calculated on the basis of incubation experiments where the amount of enzyme present was such that the final concentration of liberated inorganic phosphate did not exceed 10^{-3} *M*. The values are presented as μg P liberated/min/ml incubation medium unless otherwise stated.

* The following abbreviations will be used: TEAE-cellulose = triethylaminoethyl cellulose. DEAE-cellulose = diethylaminoethyl-cellulose. Tris = tris(hydroxyamino)methane. CM-cellulose = carboxymethyl-cellulose. ATP = adenosine triphosphate.

References p. 357.

Specific activity refers to enzyme activity units/mg enzyme N/ml as determined by micro-Kjeldahl.

Other substrates were also used in concentrations corresponding to 10 μmoles P/ml except when specifically stated otherwise. The α-casein used was a gift from Dr. T. L. McMeekin (Eastern Regional Lab., U.S.A.). The calcium (bis(*p*-nitrophenyl)-phosphate)$_2$ was a gift from Dr. Hans Boman (Uppsala). Enzyme measurements using the latter substrate and also *p*-nitrophenylphosphate were made according to Boman and Kaletta[18] and Boman[19] with minor modifications to include veronal–acetate buffer and mercaptoethanol.

RESULTS

Preliminary experiments in which the preparative procedure of Singer and Fruton[6] and Sundararajan and Sarma[9] were compared indicated that the enzymes obtained by the two procedures behaved similarly toward casein, *p*-nitrophenylphosphate, and glycerylphosphate. However, the first procedure[6] was technically easier and afforded a better yield. It was therefore employed in subsequent experiments*.

Chromatography on TEAE-cellulose

In experiments with the anion exchanger, TEAE-cellulose it was found that the phosphoprotein phosphatase activity of the freeze-dried Singer and Fruton enzyme was not retained on the column under the experimental conditions employed, whereas a great deal of inactive protein was. A typical experiment is shown in Fig. 1.

Here, 1 g of the enzyme preparation was dissolved in 4 ml of distilled water, yielding a dark brown solution. This was then filtered through a column containing

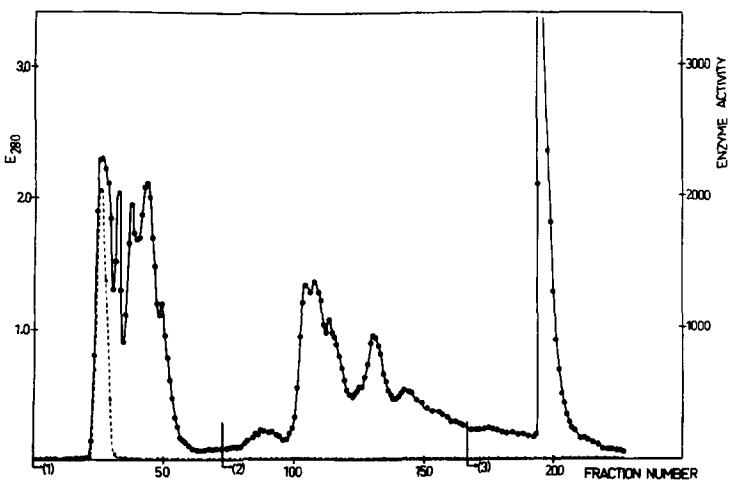

Fig. 1. Chromatogram of the starting material on TEAE. 1 g of the freeze-dried Singer and Fruton phosphatase chromatographed on 20 g TEAE-cellulose. ●—●— Protein (optical density at 280 mμ). ×--×-- Enzyme activity (units in complete incubation medium). Buffer-salt concentrations: (1) 0.001 *M* Tris–HCl, pH 7.4; (2) 0.01 *M* Tris–HCl, pH 7.4 + 0.1 *M* NaCl; (3) 0.01 *M* Tris–HCl, pH 7.4 + 1 *M* NaCl.

* The bulk of these experiments were performed before the paper of Hofman[8] appeared, in which the method of Sundararajan and Sarma was simplified.

References p. 357.

TABLE I

PURIFICATION OF THE SINGER AND FRUTON[6] SPLEEN PHOSPHATASE PREPARATION

Enzyme	*Total activity*	*Percent recovery*	*Specific activity**	*Purification*
1 g freeze-dried starting material	50,550 E.U.		566	
After filtration through TEAE	33,620 E.U.	66.5	2694	4.8
Final, single peak obtained after 3 consecutive electrophoresis experiments	5,236 E.U.	10.6**	9768	17.3

* Enzyme units/ml incubation medium/mg enzyme N.
** Corrected for aliquots taken for analysis.

20 g of TEAE–Cl in equilibrium with 0.01 *M* Tris–HCl, pH 7.4, and displaced with the same buffer solution. 5 ml fractions of the effluent were collected. The flow rate was about 60 ml per h. It will be seen from the figure that the enzyme emerges in the first protein peak, the succeeding peaks containing negligible activity. As indicated in Table I, a recovery of about 66 % of the enzyme activity was obtained, while the specific activity increased by a factor of 4.8.

When starting material of a particularly high enzyme content was chromatographed, a blue band could be seen moving down the column shortly after beginning the experiment. The progress of the band was easily followed and it could be seen to emerge from the column as a solution lightly tinged with violet into the tubes corresponding to the first peak of the chromatogram. In the experiment shown in Fig. 1 the band was seen with difficulty, but when the contents of the tubes corresponding to the first peak were ultrafiltered the violet color of the concentrate was unmistakable.

It was of interest to examine the behavior of the SUNDARARAJAN AND SARMA enzyme[9] on TEAE. Some difficulty was encountered in preparing this enzyme so that only a small amount of material was available. When this material was chromatographed protein peaks apparently corresponding to several of those in Fig. 1 could be detected. As in the case of the SINGER AND FRUTON enzyme, the enzyme activity was found in the first peak. This was taken as another indication of the similarity of the active principles of the two preparations.

Chromatography on carboxymethyl-cellulose

In an attempt to purify the enzyme further an experiment with CM-cellulose was performed. The protein corresponding to the first peak in Fig. 1 was concentrated and filtered through a 10 g column of the ion exchanger in Na^+ form, at pH 6.0 (sodium acetate–acetic acid, 0.01 *M*). All of the protein was retained on the column and most could not be displaced with 2.0 *M* sodium acetate–acetic acid, pH 6.0. The protein material of the first TEAE peak thus behaved similarly to the thymus histone preparation of CRAMPTON, MOORE AND STEIN[20] which was strongly adsorbed to the carboxylic acid ion exchanger, IRC 50. Although these authors achieved favorable results using alkaline earth buffers, the effect of this type of buffer was not tested in the present experiments.

Preparative electrophoresis

In preliminary filtration experiments made as a routine measure before attempting the electrophoretic separation of the TEAE-purified enzyme, the latter was filtered

References p. 357.

through different types of supporting media. It was found that varying amounts of the enzyme were irreversibly adsorbed to ethanolyzed cellulose[21]. Under the assumption that this adsorption was due to the presence of small amounts of carboxyl groups (see CM-cellulose experiment) the columns of cellulose were washed with 0.1 *M* barium acetate–acetic acid, pH 6.0 and filtration performed in this solution. This reduced the adsorption somewhat but did not eliminate it completely. However, when "Pevikon", a polyvinyl acetate–polyvinyl chloride copolymer was used as supporting medium no adsorption of the enzyme could be detected. Therefore this material was used throughout in the electrophoresis experiments.

Fig. 2 shows an electropherogram of enzyme purified in a TEAE experiment similar to that shown in Fig. 1. The tubes corresponding to the first peak were combined and concentrated to 3 ml by ultrafiltration. The violet solution was then applied to a 1 × 30 × 60 cm block of Pevikon in a narrow trough constructed 15 cm from the anode. The buffer used was 0.06 *M* sodium acetate–acetic acid, pH 5.6. For additional details, see the legend to the figure. During the experiment the blue-violet zone could be seen to migrate toward the cathode and at the end of the experiment it corresponded in position to the enzyme activity. It is evident that the enzyme is a highly basic substance, as could also be inferred from the TEAE and CMC experiments.

Fig. 3 is an electropherogram of enzyme purified first by filtration through TEAE and then by three successive electrophoretic experiments of the type shown in Fig. 2 (the segments corresponding to the blue enzyme band were eluted, ultrafiltered, and subjected to electrophoresis three consecutive times).

Although the manipulations involved in the three consecutive electrophoretic runs (elution from the Pevikon using suction through sinter glass filters plus concentration by ultrafiltration through collodion membrane) apparently caused a considerable loss of the enzyme (Table I), the use of TEAE coupled with electrophoresis can be seen to have brought about a 17.3-fold increase in the specific activity of the enzyme

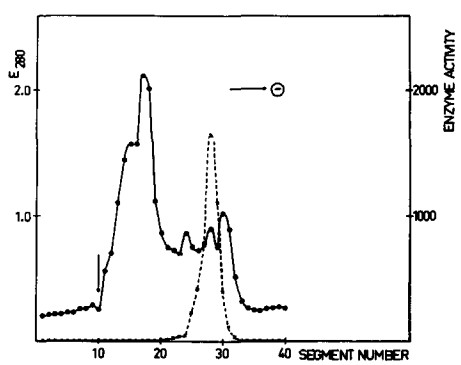

Fig. 2. Electropherogram of TEAE-purified enzyme. The first (enzyme-containing) peak from the TEAE chromatogram ultrafiltered and applied to a Pevikon block in the region indicated by the arrow in the figure. Block size: 1 × 30 × 60 cm. Buffer: sodium acetate–acetic acid, 0.06 *M*, pH 5.6. Voltage: 5.5–6.0 V/cm. Current strength: 120 mA. Duration: 15 h. Segment width: 1.25 cm. Segments eluted with 5 ml buffer. Position of the blue band at the end of the experiment corresponded to segments 27 and 28.

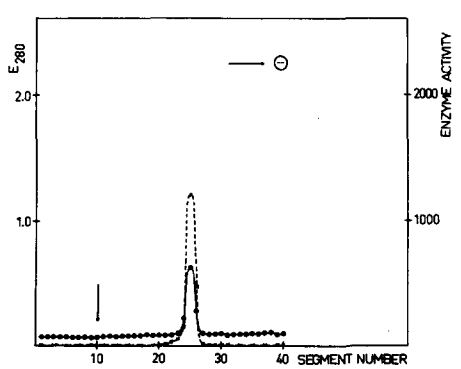

Fig. 3. Electropherogram of the final enzyme preparation. Enzyme previously purified by TEAE chromatography + 2 previous electrophoretic experiments. Conditions as in the experiment illustrated in Fig. 2 except that the voltage was 5.0–5.5 V/cm. The position of the blue band corresponded to segments 23 and 24.

References p. 357.

resulting in an enzyme preparation which moves as one peak on electrophoresis and appears to have a higher specific activity than any of the spleen phosphatase preparations hitherto reported in the literature (in spite of the use of lyophilized starting material—which according to SINGER AND FRUTON has a reduced specific activity—preparations with as high a specific activity as 13,200 enzyme units/mg protein N have been obtained).

A solution of the purified enzyme in acetate buffer, pH 5.6, is stable for at least 3 weeks at +6°. On lyophilization a violet powder is obtained, which when dissolved in a few drops of water yields an intense blue-violet solution.

The action of the present enzyme on casein

It was of interest to study the action of the purified enzyme obtained in the present study on casein. It was found that in the presence of suitable enzyme and substrate concentrations the phosphorus of both α-casein and unfractionated Hammarsten casein was completely hydrolyzed. See Fig. 4 for experimental details. The effect of using different amounts of enzyme on the course of the hydrolysis is also shown in the figure. The importance of using an adequate amount of enzyme is evident.

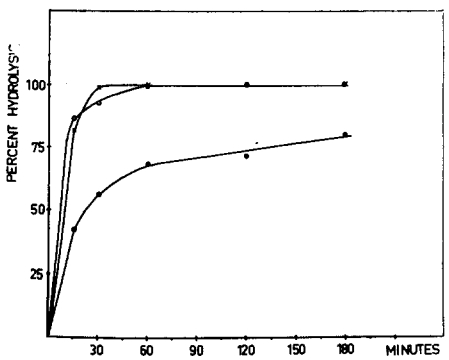

Fig. 4. The hydrolysis of unfractionated "Hammarsten" casein and α-casein by the purified enzyme. The α-casein and unfractionated casein media contained 4 and 5 mg protein/ml, respectively. Otherwise the incubation conditions were as usual. ×—×— α-casein hydrolysis curve. O—O— unfractionated casein curve. The amount of enzyme used in the α-casein experiment and that used in the unfractionated casein experiment designated by the open circles was 3.1 units/ml. In the unfractionated casein experiment denoted by the closed circles half this amount was used.

These results should be compared with those of SUNDARARAJAN AND SARMA[22], who—using rat spleen enzyme—obtained only about 80 % hydrolysis of unfractionated casein with almost similar substrate concentrations. In another experiment with 3.1 enzyme units and 4 mg α-casein/ml incubation medium, hydrolysis was allowed to proceed to completion (2 h) after which the amount of TCA-soluble amino nitrogen liberated during the course of the experiment was measured using the ninhydrin technique of V. HOFSTEN[23]. This value amounted to less than 2 % of the total amino nitrogen, in good agreement with the results of SUNDARARAJAN AND SARMA[22]. These results suggest that the purified enzyme contains little or no proteolytic activity. Additional experiments, however, with different substrates will be required before this can be considered proved.

The action of the purified enzyme on some low molecular weight phosphorus compounds

Some preliminary studies of the specificity of the purified enzyme were also performed. The enzyme was found to be active at pH 6.0 toward phosphoramidate, *p*-nitrophenylphosphate, inorganic pyrophosphate and ATP. On the other hand its activity toward glycerylphosphate and calcium(bis(*p*-nitrophenyl)phosphate)$_2$ was insignificant.

References p. 357.

DISCUSSION

The present study was performed in connection with an investigation of liver phosphoprotein metabolism now in progress at this institute[24, 25, 11, 12, 26, 27]. A well-characterized, highly purified phosphatase was required, active toward phosphoproteins and suitable for a number of different types of experiments with these proteins. Of special interest was the use of the enzyme in studying the effect of dephosphorylation on the structure and function of the phosphoproteins. Thus, it was not only necessary that the enzyme be as free as possible from contaminating enzymes and proteins, but also that methods be available which would allow the identification or removal of the enzyme from the reaction mixture on completion of the dephosphorylation. For this reason both TEAE chromatography and preparative electrophoresis were employed in the present study, since both techniques have been shown to be useful for the purification of phosphoproteins[11, 12].

The use of these methods has now been found to permit a considerable purification of the enzyme. In addition, the enzyme, which appears to be a strongly basic protein, behaves quite differently on chromatography and electrophoresis from the more acidic phosphoproteins[11]. It is possible that this difference in behavior may prove to be of value in connection with the second point mentioned above.

The cause of the loss of activity in the course of the present purification procedure is not known with certainty. The TEAE experiment was performed at pH 7.4 and at $+6°$. Although a solution of the enzyme is partially inactivated when kept at this pH and temperature for longer periods of time, no loss of activity could be detected during a 3 h period in preliminary experiments. Since filtration through the TEAE column required less than 3 h, it is possible that the loss of activity is due to interaction between the enzyme and the basic groups of the column. The loss of activity during 3 successive electrophoretic experiments is chiefly accounted for by the fact that only the peak tubes were re-run. However, it is possible that some additional factor may be involved.

Bovine spleen "phosphoprotein phosphatase" preparation have been made by SINGER AND FRUTON[7], SUNDARARAJAN AND SARMA[7, 9], HOFMAN[8], and ROCHE, THOAI AND PIN[5]. Some of the characteristics of these different enzyme preparations are compared in Table II. It will be seen that with the exception of the preparation of ROCHE *et al.*[5] the various enzymes have many properties in common, and in fact appear to differ mainly in their solubility properties. This suggests that the active principles of the various preparations may be identical, but that the enzymes of HOFMAN and SUNDARARAJAN AND SARMA are present in the form of a complex. Since the active principle seems to be strongly basic a nucleic acid–enzyme complex might be involved. It is of interest that the purified enzyme is active toward several different types of substrate. A further study of some of the physical and biochemical properties of the enzyme is in progress.

ACKNOWLEDGEMENTS

The author is thankful to Drs. T. L. MCMEEKIN AND H. G. BOMAN for generous gifts of α-casein and Ca(bis(*p*-nitrophenyl)phosphate), respectively. He also wishes to thank Dr. H. MÜLLER-EBERHARD for introducing him to the use of Pevikon as a supporting medium in preparative electrophoresis.

References p. 357.

TABLE II

COMPARISON OF SPLEEN "PHOSPHOPROTEIN PHOSPHATASES"

	Properties	*Present enzyme*	*Sundararajan and Sarma enzyme*	*Hofman enzyme*	*Roche et al. enzyme*
1	Solubility	Highly water-soluble	Precipitates on dialysis against water	Precipitates on dialysis against water	
2	Heat stability	Stable at 70°, 5 min	Stable 70°, 5 min	Stable 70°	
3	pH Inactivation	Partially inactivated 7	Partially inactivated 7	Partially inactivated 7	
4	pH Optimum	pH 6	pH 6	pH 5.0–5.5	pH 6
5	Activators	Sulfhydryl compounds	Sulfhydryl compounds	Sulfhydryl compounds	
6	Effect of Mg^{++}	Negligible	Negligible	Negligible	
7	Substrates:				
	Glycerylphosphate	—	—	—	
	Phosphoramidate	+		+	
	Phenylphosphate	+		+	—
	Diphenylphosphate	—*		—	+
	Inorg. pyrophosphate	+		+	
	ATP	+		+	
	Casein	+ (100%)	+	+ (100%)	+ (50%)

* Ca(bis(*p*-nitrophenyl)phosphate)$_2$ used as substrate.

REFERENCES

1 D. L. HARRIS, *J. Biol. Chem.*, 165 (1949) 541.
2 M. W. FOOTE AND C. A. KIND, *Arch. Biochem. Biophys.*, 46 (1953) 254.
3 R. N. FEINSTEIN AND M. E. VOLK, *J. Biol. Chem.*, 177 (1949) 339.
4 B. NORBERG, *Acta Chem. Scand.*, 4 (1950) 1206.
5 T. ROCHE, N. V. THOAI AND P. PIN, *Compt. rend. soc. biol.*, 147 (1953) 409.
6 M. F. SINGER AND J. S. FRUTON, *J. Biol. Chem.*, 229 (1957) 111.
7 T. A. SUNDARARAJAN AND P. S. SARMA, *Biochem. J.*, 56 (1954) 125.
8 T. HOFMAN, *Biochem. J.*, 69 (1958) 135.
9 T. A. SUNDARARAJAN AND P. S. SARMA, *Biochem. J.*, 65 (1957) 261.
10 J. PORATH, *Arkiv Kemi*, 11 (1957) 97.
11 J. GLOMSET, *Acta Chem. Scand.*, 11 (1957) 512.
12 J. GLOMSET, *Acta Chem. Scand.*, (in press).
13 E. A. PETERSON AND H. A. SOBER, *J. Am. Chem. Soc.*, 78 (1956) 751.
14 H. J. MULLER-EBERHARD AND H. G. KUNKEL, *J. Exptl. Med.*, 104 (1956) 253.
15 H. J. MULLER-EBERHARD, (personal communication).
16 T. ARONSSON, *Scand. J. Clin. & Lab. Invest.*, 9 (1957) 95.
17 J. B. MARTIN AND D. M. DOTY, *Anal. Chem.*, 21 (1949) 965.
18 H. G. BOMAN AND U. KALETTA, *Biochim. Biophys. Acta*, 24 (1957) 619.
19 H. G. BOMAN, *Biochim. Biophys. Acta*, 16 (1955) 245.
20 C. F. CRAMPTON, S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 215 (1955) 787.
21 P. FLODIN AND D. W. KUPKE, *Biochim. Biophys. Acta*, 21 (1956) 368.
22 T. A. SUNDARARAJAN AND P. S. SARMA, *Enzymologia*, 18 (1957) 234.
23 B. v. HOFSTEN, *Acta Chem. Scand.*, 10 (1956) 1432.
24 G. ÅGREN, C.-H. DE VERDIER AND J. GLOMSET, *Acta Chem. Scand.*, 9 (1954) 503.
25 G. ÅGREN, C.-H. DE VERDIER AND J. GLOMSET, *Acta Chem. Scand.*, 8 (1954) 1570.
26 G. ÅGREN, *Acta Univ. Upsaliensis*, 5 (1958.)
27 C.-H DE VERDIER, *Acta Physiol. Scand.*, 39 (1957) 1.

THE ESTIMATION OF INORGANIC PHOSPHATE IN THE PRESENCE OF ADENOSINE TRIPHOSPHATE

B. B. MARSH

Meat Industry Research Institute of New Zealand, Wellington (New Zealand)

(Received July 16th, 1958)

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-

References p. 361.