BBA 75 816

PHOSPHORYLATION OF BOVINE BRAIN Na^+, K^+-STIMULATED ATP PHOSPHOHYDROLASE BY ADENOSINE [^{32}P]TRIPHOSPHATE STUDIED BY A RAPID-MIXING TECHNIQUE

S. MÅRDH AND Ö. ZETTERQVIST

Institute of Medical Chemistry, University of Uppsala, Box 551, 751 22 Uppsala (Sweden)

(Received July 26th, 1971)

SUMMARY

1. A bovine brain Na^+, K^+-stimulated ATPase preparation was incubated with [γ-^{32}P]ATP by use of a rapid-mixing technique, the reaction time being 8–40 msec. The phosphorylated material was analysed for ^{32}P-labelled acyl-phosphate, phosphoserine, phosphothreonine and phospholipids.

2. With 5 μM [γ-^{32}P]ATP, 3 mM $MgCl_2$ and 120 mM NaCl at pH 7.4 and 21° the acyl-[^{32}P]phosphate appeared at a rate of 0.55 μmole/mg protein per min. At the same conditions the enzyme preparation hydrolysed 0.06 μmole of ATP/mg protein per min. This was the rate of ATP hydrolysis irrespective of whether or not 20 mM KCl was present. After 40 msec the acyl-[^{32}P] phosphate comprised more than 99 % of the [^{32}P]phosphate of the high-molecular-weight material.

3. It is concluded that the [^{32}P]phosphorylenzyme, having a similar pH stability to acyl-[^{32}P]phosphate, is formed rapidly enough to be an intermediate of the Na^+, K^+-dependent ATPase reaction.

INTRODUCTION

The Na^+, K^+-stimulated, ouabain-sensitive ATP phosphohydrolase (EC 3.6.1.3.), which is associated with microsomal material in most mammalian tissues[1,2] incorporates [^{32}P]phosphate into several fractions on incubation with [γ-^{32}P]ATP. Although [^{32}P]phosphate is incorporated into phospholipids[3–6] and protein-bound phosphorylserine[7,8] these have been ruled out as possible intermediates in the Na^+, K^+-stimulated hydrolysis of ATP[9–13]. However, there is also an incorporation of [^{32}P]phosphate into a fraction showing properties consistent with acylphosphate[9,14–16]. This incorporation is dependent on Mg^{2+} and Na^+, is decreased when K^+ is added and is inhibited by ouabain. Furthermore, maximal incorporation is obtained within 0.5 sec which is the shortest reaction time reported so far[17].

These results suggest that protein-bound acyl-phosphate is an intermediate of the Na^+, K^+-stimulated, ouabain-inhibited ATPase reaction. However, alternative mechanisms have also been suggested[1,18,19].

One prerequisite of an enzyme intermediate is that its formation should proceed at least as fast as the over-all reaction. So far no direct measurements have been

reported concerning the rate of formation of acyl-phosphate in preparations of Na^+, K^+-stimulated ATPase. However, by use of a rapid-mixing technique it has been possible to show in the present work that protein-bound acyl-phosphate is formed rapidly enough to be an intermediate of the Na^+, K^+-stimulated ATPase reaction.

EXPERIMENTAL PROCEDURE

Materials

Sephadex was obtained from Pharmacia, Uppsala, Sweden. Adenosine triphosphate (disodium and tris salt) was purchased from Sigma. [γ-^{32}P]ATP was prepared by the method of ENGSTRÖM[20]. The specific radioactivity ranged from 0.2 to 0.6 μC/nmole at the rapid-mixing experiments. Distilled and subsequently deionized water was used throughout the work. Ion exchanger was from Illco-Way, Illinois Water Treatment Company, Rockford, Ill.

Analytical methods

Radioactivity was measured by use of a Geiger counter as previously described[21] or by measuring the Cerenkov radiation[22–24] of 5–7 ml aqueous samples in a Beckman LS-250 Liquid Scintillation Counter. [^{32}P]Orthophosphate was determined by a micromodification of the method of MARTIN AND DOTY[25], with a final volume of each phase of 2.5 ml. The radioactivity was measured on aliquots of the organic phase.

Protein was assayed as by the method of LOWRY *et al.*[26] with bovine serum albumin as a reference.

Enzyme preparation

Calf brain cortex was used as the enzyme source and the microsomal fraction was purified as described by SKOU AND HILBERG[27]. The preparation was stored at −25° until used. With 1 mM ATP the specific activity of the Na^+, K^+-stimulated ATPase was 3.5 μmoles of ATP hydrolysed/mg protein per min at 37°. At 21° the corresponding activity was 0.5 μmole/mg protein per min (Table I).

Enzyme assay

The assay medium consisted of 3 mM $MgCl_2$, 120 mM NaCl, 20 mM KCl and Tris–HCl buffer, pH 7.4, the Tris concentration being 30 mM. [γ-^{32}P]ATP of low specific radioactivity was used. The final concentration was 5 μM or 1 mM, and the total amount of radioactivity of each assay was about 50 nC. The enzyme concentration was chosen so as to give 5–30 % hydrolysis of [γ-^{32}P]ATP. The assay was performed in a final volume of 1 ml and the temperature was 21° or 37°, as indicated. After 4 min the reaction was interrupted by the addition of 0.5 ml of silicotungstic acid and the [^{32}P]orthophosphate liberated was determined as described under *Analytical methods*.

Phosphorylation by rapid-mixing technique.

Short-time phosphorylation of the enzyme preparation by [γ-^{32}P]ATP was performed in a rapid-mixing apparatus as previously described[28]. The main components of the apparatus are two syringes and a four-jet mixer. Syringe I contained 0.9 ml of 10 μM [γ-^{32}P]ATP and Syringe II 0.9 ml of a solution with 1.5 mg of enzyme

protein. The final concentration of [γ-^{32}P]ATP during the incubation thus was 5 μM. Both solutions contained the same molar concentration of ions and buffer, *i.e.* 3 mM $MgCl_2$ and 30 mM Tris–HCl, pH 7.4, and, as indicated, 120 mM NaCl or 120 mM NaCl *plus* 20 mM KCl. The experiments were carried out at 21° ± 1°.

With experiments including ouabain the enzyme was preincubated at pH 7.4 for 1 h at 21° in a medium containing 0.2 mM ouabain, 3 mM $MgCl_2$, and 30 mM Tris–HCl buffer. 3 min before the rapid-mixing experiment, NaCl and KCl were added to give a final concentration of 120 mM Na^+ and 20 mM K^+, respectively, and the solution was introduced into Syringe II.

5 ml of 5.5 % (w/w) $HClO_4$ were used for quenching at 21°. 30 sec after quenching, 0.8 ml of 10 % sodium dodecyl sulphate was added. After another 30 sec the solution was titrated to about pH 3 (indicator paper) with 2.5 M sodium acetate. Unlabelled ATP and orthophosphate were added to a final concentration of 0.1 mM and 1 mM, respectively. The mixture was chromatographed on a Sephadex G-50 column (2.4 cm × 36 cm) in order to separate the ^{32}P-labelled protein from [γ-^{32}P]-ATP and [^{32}P]orthophosphate. The column was equilibrated and eluted with 0.1 M acetic acid containing 1 % sodium dodecyl sulphate, at pH 3.0.

Phosphorylation experiments at high concentration of [γ-^{32}P]ATP

Incubation with [γ-^{32}P]ATP for 15 sec or 15 min was carried out in a test tube in a mixture containing 3 mM $MgCl_2$, 120 mM NaCl, 1 mM [γ-^{32}P]ATP, 30 mM Tris–HCl buffer, pH 7.4, and 0.75 mg of enzyme protein, in a total volume of 0.9 ml.

The reaction was interrupted by the addition of 5 ml of 5.5 % $HClO_4$. Sodium dodecyl sulphate and sodium acetate were added as described above, and the phosphorylated protein was isolated by chromatography on Sephadex G-50, as described in a preceeding paragraph.

Assay for protein-bound acyl-[^{32}P]phosphate

Acyl-[^{32}P]phosphate was assayed on samples of the void volume fraction from a Sephadex G-50 chromatography. The sample was mixed with 0.2 μmole of unlabelled orthophosphate and 0.5 ml of silico-tungstic acid[25] and then shaken with acid molybdate and isobutanol–benzene for 15 sec as described above for the analysis of [^{32}P]orthophosphate. The radioactivity extracted as [^{32}P]phosphomolybdate was considered to represent protein-bound acyl-[^{32}P]phosphate. In order to evaluate whether part of the extracted radioactivity was due to ^{32}P-containing material different from [^{32}P]phosphomolybdate, *e.g.* [^{32}P]phospholipids, an extraction was also performed without any addition of ammonium molybdate.

Assay for lipid-bound [^{32}P]phosphate

The microsomal material was incubated with 1 mM [γ-^{32}P]ATP as described above. The reaction was interrupted by the addition of 5 ml of 5.5 % $HClO_4$, and the phosphorylated material was collected by centrifugation. The precipitate was then washed 4 times with 5.5 % $HClO_4$ containing 10 mM orthophosphate. The precipitate was suspended in 1 ml of 5 mM HCl and 50 μl of 10 % sodium dodecyl sulphate were added. 2 ml of chloroform–methanol (2:1, v/v) were added and the mixture was stirred for 45 min. The radioactivity which appeared in the organic phase was considered to be lipid-bound [^{32}P]phosphate. The ^{32}P-labelled material remaining in

the inorganic phase was entirely accounted for by acyl-[^{32}P]phosphate, assayed as described above, and by ester-[^{32}P]phosphate, assayed as described below.

Assay for protein-bound ester-[^{32}P]phosphate

Samples of the void-volume fraction from a Sephadex chromatography were treated for 10 min at 100°, either with 0.5 M H_2SO_4 or with 1 M NaOH. The bound [^{32}P]phosphate, that was resistant to the acid treatment but was liberated as [^{32}P]-orthophosphate during the alkaline treatment, was considered as [^{32}P]phosphate bound to seryl and threonyl residues of the protein[29].

Isolation of [^{32}P]phosphoserine and [^{32}P]phosphothreonine

The void volume fraction of a Sephadex G-50 column was mixed with bovine serum albumin which was added to a final concentration of 3 mg/ml. The protein was precipitated with 7 vol. of cold acetone, and then washed 3 times. The precipitate was dried under low pressure and then hydrolysed with 2 M HCl in a sealed test tube for 20 h at 100°. After removal of HCl by repeated evaporation at room temperature in a rotatory evaporator and redissolution by water, the hydrolysate was chromatographed on Dowex-50 X8 and [^{32}P]phosphoserine and [^{32}P]phosphothreonine were isolated as previously described[30]. The phosphoamino acids were separately chromatographed on Dowex-1 X8 and further identified by paper electrophoresis in 1 M acetic acid, pH 2.3, pyridine–acetic acid buffer, pH 3.5 and pH 5.0, 0.1 M with respect to added pyridine, and triethylamine–CO_2 buffer, pH 9.5, 0.1 M with respect to added triethylamine.

After correction for estimated losses during the acid hydrolysis of the ^{32}P-labelled protein[31], [^{32}P]phosphoserine and [^{32}P]phosphothreonine accounted for all of the protein-bound ester-[^{32}P]phosphate, assayed as described above.

RESULTS

Optimal concentration of [γ-^{32}P]ATP and microsomal ATPase at the rapid-mixing experiments

Due to the limits set by the specific radioactivity of the [γ-^{32}P]ATP, an enzyme concentration of about 0.8 mg of protein/ml had to be used to ensure sufficient accuracy in the determination of the radioactivity bound to the enzyme preparation in the rapid-mixing experiments. With this enzyme concentration it was necessary to use fairly low concentrations of [γ-^{32}P]ATP. When experiments were performed at approximately saturating concentrations of [γ-^{32}P]ATP, *e.g.* 1 mM, maximal phosphorylation was obtained already after the shortest possible reaction time, *i.e.* 8 msec. Thus, lower concentrations were tried, and 5 μM [γ-^{32}P]ATP was found to be optimal with the available rapid-mixing apparatus.

Effect of ATP, temperature and ions on ATPase activity

Since it was necessary to compare the rate of phosphorylation with the rate of the over-all reaction, the ATPase activity was assayed at the ATP concentration and temperature that was chosen for the rapid-mixing experiments. With 5 μM ATP there was no additional stimulation of the activity when K^+ was present with Na^+ (Table I). However, the Na^+-dependent activity was inhibited by ouabain.

TABLE I

THE EFFECT OF TEMPERATURE, IONS, OUABAIN AND CONCENTRATION OF [^{32}P]ATP ON THE HYDROLYSIS OF [^{32}P]ATP BY THE MICROSOMAL ATPase

The assay of the ATPase activity was performed as described under EXPERIMENTAL PROCEDURE, except that the metal ions were added as indicated in the table. At those experiments which included ouabain, the enzyme was preincubated at pH 7.4 for 1 h at 21° in a medium containing 0.2 mM ouabain, 3 mM $MgCl_2$ and 30 mM Tris–HCl buffer. 3 min before the addition of [γ-^{32}P]ATP, NaCl and KCl were added to a final concentration of 120 mM and 20 mM, respectively.

Temperature (°)	*Ions and ouabain*	*Hydrolysis of* [^{32}P]*ATP* (*nmoles·mg*$^{-1}$*·min*$^{-1}$)	
		5 μM [^{32}P]*ATP*	*1 mM* [^{32}P]*ATP*
21	Mg^{2+}	26	77
21	Mg^{2+}, ouabain	22	80
21	Mg^{2+}, Na^+	59	115
21	Mg^{2+}, Na^+, ouabain	23	90
21	Mg^{2+}, Na^+, K^+	60	570
21	Mg^{2+}, Na^+, K^+, ouabain	28	110
37	Mg^{2+}	67	520
37	Mg^{2+}, Na^+	207	680
37	Mg^{2+}, Na^+, K^+	280	4050

A slight extra stimulation was obtained with K^+, if the reaction was performed at a higher temperature. To obtain an appreciable effect of K^+ on the ATPase activity, a higher concentration of ATP seemed to be necessary. This was the case irrespective of the temperature. This dependence of the effect of K^+ on the concentration of ATP seems to be consistent with experiments of NEUFELD AND LEVY[32,33] and CZERWINSKI *et al.*[34].

Phosphorylation by rapid-mixing technique

The results of the rapid-mixing experiments are shown in Fig. 1. A low level of phosphorylation was obtained when Mg^{2+} was the only metal ion. When Na^+ was present in addition to Mg^{2+}, a half-maximal phosphorylation (0.1 nmole/mg protein) was reached within 15 msec. This corresponds to a pseudo-first-order rate constant of 46 sec^{-1}. When K^+ was present in addition to Mg^+ and Na^+ the steady-state level of phosphorylation did not exceed 1/5 that with Mg^{2+} and Na^+. After preincubation of the enzyme with 0.2 mM ouabain the level of the phosphorylation was very low, irrespective of which ions were present.

Nature of rapidly formed [^{32}P]*phosphate*

The phosphorylated material isolated after incubation for 40 msec with 5 μM [γ-^{32}P]ATP was analysed for ^{32}P-labelled acyl-phosphate, phosphoserine and phosphothreonine as described under EXPERIMENTAL PROCEDURE. All of the protein-bound [^{32}P]phosphate showed properties of acyl-[^{32}P]phosphate.

In order to make this test more stringent the same analyses were performed on ^{32}P- labelled material obtained after incubation at a saturating concentration of [γ-^{32}P]ATP, *i.e.* 1 mM. In addition, the content of [^{32}P]phospholipids was assayed

(Table II). Again acyl-[^{32}P]phosphate was the only ^{32}P-labelled compound to be detected in significant amounts after 40 msec.

The amount of acyl-[^{32}P]phosphate obtained when the protein was precipitated

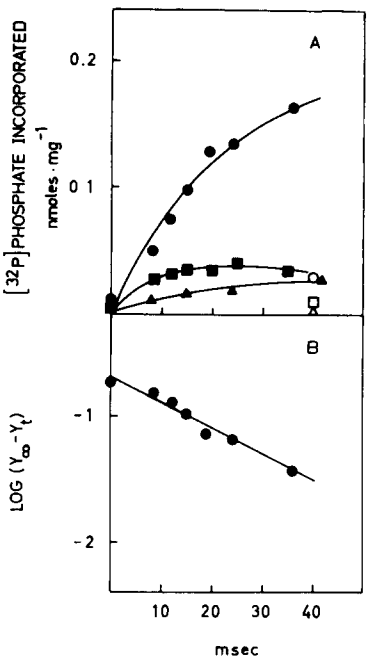

Fig. 1. Rapid phosphorylation of microsomal ATPase. Rapid-mixing experiments were performed as described under EXPERIMENTAL PROCEDURE. The values represent total incorporation of [^{32}P]-phosphate into the microsomal ATPase. A. Phosphorylation in the presence of different ions and of ouabain. ▲—▲, 3 mM Mg^{2+}; ●—●, 3 mM Mg^{2+} and 120 mM Na^+; ■—■, 3 mM Mg^{2+}, 120 mM Na^+ and 20 mM K^+. Open signs represent phosphorylation experiments in the presence of ouabain, the shape of the sign having the same meaning as above. B. Pseudo-first-order plot of the phosphorylation in the presence of Mg^{2+} and Na^+. Y_∞ represents the amount of acyl-[^{32}P]phosphate after 15 sec at 1 mM [γ-^{32}P]ATP (Table II) and Y_t represents the amount of [^{32}P]phosphate incorporated after a varying time at 5 μM [γ-^{32}P]ATP.

TABLE II

PHOSPHORYLATION OF MICROSOMAL ATPase WITH 1 mM [γ-^{32}P]ATP FOR DIFFERENT PERIODS OF TIME. INCORPORATION OF [^{32}P]PHOSPHATE INTO ACYL-[^{32}P]PHOSPHATE, LIPID-BOUND [^{32}P]-PHOSPHATE, [^{32}P]PHOSPHOSERINE, AND [^{32}P]PHOSPHOTHREONINE

Incorporation of [^{32}P]phosphate was measured as described under EXPERIMENTAL PROCEDURE. When ouabain was included, preincubation was performed as described in Table I. The values of [^{32}P]phosphoserine and [^{32}P]phosphothreonine are those obtained after correction for estimated losses during the acid hydrolysis of the ^{32}P-labelled protein[31].

Time	*Incorporation of [^{32}P]phosphate (nmoles·mg^{-1})*			
	Acyl-[^{32}P]phosphate	*Lipid-[^{32}P]phosphate*	*Seryl-[^{32}P]phosphate*	*Threonyl-[^{32}P]phosphate*
40 msec	0.2	<0.01	<0.001	<0.001
15 sec	0.2	<0.01	0.036	0.004
15 min	0.1	0.23	0.330	0.030
15 min + ouabain	<0.05	0.25	0.325	0.025

and washed with $HClO_4$ was in good agreement with the value obtained by the chromatographic method.

[^{32}P]phosphoserine, [^{32}P]phosphothreonine and [^{32}P]phospholipids only appeared after longer periods of incubation and this phosphorylation was shown to be insensitive to ouabain.

Due to the high background of other [^{32}P]phosphoryl compounds the accuracy of the values of acyl-[^{32}P]phosphate obtained after 15 min is low. With ouabain no acyl-[^{32}P]phosphate was detected, the estimated limit of detection being about 0.05 nmole. The low but detectable amount of acyl-[^{32}P]phosphate obtained after 15 min in the absence of ouabain may be explained by the fact that almost all of the [^{32}P]-ATP was hydrolysed within this period.

DISCUSSION

Neither protein-bound [^{32}P]phosphoserine nor [^{32}P]phospholipids were formed rapidly enough to account for intermediates of the ATPase reaction (Table II). This is in accordance with previous reports (9–13). The present results show this is also true for [^{32}P]phosphothreonine.

All of the [^{32}P]phosphate bound to the enzyme preparation at the rapid-mixing experiments, had stability properties consistent which acyl-[^{32}P]phosphate, as defined by others[9,14–16]. From Fig. 1 the velocity of the incorporation of acyl-[^{32}P]-phosphate with 5 μM [γ-^{32}P]ATP and 21° in the presence of Mg^{2+} and Na^+ is calculated to be 0.55 μmole/mg protein per min. At the same [γ-^{32}P]ATP concentration and temperature, about 0.06 μmole/mg protein per min was hydrolysed, in the presence of Mg^{2+} and Na^+, irrespective of whether K^+ was present or not. Thus, at 5 μM [γ-^{32}P]-ATP and 21° the [^{32}P]phosphoryl enzyme is formed rapidly enough to be an intermediate in a Na^+-dependent, ouabain-sensitive hydrolysis of ATP.

With 1 mM [γ-^{32}P]ATP, preliminary experiments showed that steady-state phosphorylation was reached already after 8 msec. This means that the rate of phosphorylation, at this concentration of [γ-^{32}P]ATP, is several times above 0.55 μmole/mg protein per min. The Na^+, K^+-dependent ATPase reaction, at 1 mM ATP and 21°, proceeded at a rate of about 0.5 μmole/mg protein per min according to Table I. Consequently, the[^{32}P] phosphoryl enzyme, having stability properties of acyl-[^{32}P]-phosphate, is formed rapidly enough to be an intermediate of the Na^+, K^+-dependent, ouabain-sensitive ATPase.

The rapid-mixing technique, as used in the present work, may be a valuable tool to obtain further insight into the mechanism of reaction of Na^+, K^+-dependent ATPase. The results show that, under the conditions used, the Na^+-dependent phosphorylation is not rate limiting in the hydrolysis of ATP. Thus the phosphoenzyme satisfies one criterion of an intermediate. However, it also must be investigated whether the dephosphorylation is at least as rapid as the Na^+, K^+-dependent hydrolysis of ATP. Since the native phosphoenzyme seems to persist only as long as ATP is present[1], one way to measure the rate, *e.g.* of K^+-dependent dephosphorylation, would be to use a rapid-mixing apparatus with consecutive mixing chambers and more than two syringes. The development of such an equipment is at progress at this laboratory.

ACKNOWLEDGEMENTS

The authors wish to express their appreciation to Mrs. Gunilla Grindsäter and Mrs. Christina Stafberg for skillful technical assistance. This work was supported by the Swedish Medical Research Council (Project No B71-13X-50).

REFERENCES

1 J. C. SKOU, *Physiol. Rev.*, 45 (1965) 596.
2 R. W. ALBERS, *Annu. Rev. Biochem.*, 36 (1967) 727.
3 L. E. HOKIN AND M. R. HOKIN, *J. Gen. Physiol.*, 44 (1960) 61.
4 R. B. ELLIS AND J. N. HAWTHORNE, *Acta Neurol. Scand.*, 38, suppl. 1 (1962) 33.
5 W. THOMPSON AND R. M. C. DAWSON, *Acta Neurol. Scand.*, 38, suppl. 1 (1962) 34.
6 L. E. HOKIN AND M. R. HOKIN, *Annu. Rev. Biochem.*, 32 (1963) 553.
7 P. J. HEALD, *Nature*, 193 (1962) 451.
8 K. AHMED AND J. D. JUDAH, *Biochim. Biophys. Acta*, 104 (1965) 112.
9 H. BADER, A. K. SEN AND R. L. POST, *Biochim. Biophys. Acta*, 118 (1966) 106.
10 J. JÄRNEFELT, *Biochim. Biophys. Acta*, 59 (1962) 655.
11 M. R. HOKIN AND L. E. HOKIN, *J. Biol. Chem.*, 239 (1964) 2116.
12 L. E. HOKIN AND M. R. HOKIN, *Biochim. Biophys. Acta*, 84 (1964) 563.
13 I. M. GLYNN, C. W. SLAYMAN, J. EICHBERG AND R. M. C. DAWSON, *Biochem. J.*, 94 (1965) 692.
14 K. NAGANO, T. KANAZAWA, N. MIZUNO, Y. TASHIMA, T. NAKAO AND M. NAKAO, *Biochem. Biophys. Res. Commun.*, 19 (1965) 759.
15 L. E. HOKIN, P. S. SASTRY, P. R. GALSWORTHY AND A. YODA, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 177.
16 A. KAHLENBERG, P. R. GALSWORTHY AND L. E. HOKIN, *Arch. Biochem. Biophys.*, 126 (1968) 331.
17 K. NAGANO, N. MIZUNO, M. FUJITA, Y. TASHIMA, T. NAKAO AND M. NAKAO, *Biochim. Biophys. Acta*, 143 (1967) 239.
18 W. SCHONER, R. KRAMER AND W. SEUBERT, *Biochem. Biophys. Res. Commun.*, 23 (1966) 403.
19 T. KANAZAWA, M. SAITO AND Y. TONOMURA, *J. Biochem.* (Tokyo), 61 (1967) 555.
20 L. ENGSTRÖM, *Ark. Kemi*, 19 (1962) 129.
21 O. WÅLINDER, Ö. ZETTERQVIST AND L. ENGSTRÖM, *J. Biol. Chem.*, 243 (1968) 2793.
22 J. V. JELLEY, *Cerenkov Radiation and its Applications*, Pergamon Press, London, 1958.
23 T. CLAUSEN, *Anal. Biochem.*, 22 (1968) 70.
24 R. H. ELRICK AND R. P. PARKER, *Int. J. Appl. Radiat. Isotopes*, 19 (1968) 263.
25 J. B. MARTIN AND D. M. DOTY, *Anal. Chem.*, 21 (1949) 965.
26 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
27 J. C. SKOU AND C. HILBERG, *Biochim. Biophys. Acta*, 185 (1969) 198.
28 O. WÅLINDER, Ö. ZETTERQVIST AND L. ENGSTRÖM, *J. Biol. Chem.*, 244 (1969) 1060.
29 M. RABINOWITZ AND F. LIPMANN, *J. Biol. Chem.*, 235 (1960) 1043.
30 L. ENGSTRÖM, *Biochim. Biophys. Acta*, 52 (1961) 49.
31 L. RASK, O. WÅLINDER, Ö. ZETTERQVIST AND L. ENGSTRÖM, *Biochim. Biophys. Acta*, 221 (1970) 107.
32 A. H. NEUFELD AND H. M. LEVY, *J. Biol. Chem.*, 244 (1969) 6493.
33 A. H. NEUFELD AND H. M. LEVY, *J. Biol. Chem.*, 245 (1970) 4962.
34 A. CZERWINSKI, H. J. GITELMAN AND L. G. WELT, *Am. J. Physiol.*, 213 (1967) 786.