BBA 65857

PURIFICATION OF ARGINYL-RNA SYNTHETASE FROM RAT LIVER

H. IKEGAMI* AND A. C. GRIFFIN

The University of Texas, M. D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas, 77025 (U.S.A.)

(Received September 27th, 1968)

SUMMARY

1. The arginyl-RNA synthetase (L-arginine:tRNA ligase (AMP)) of rat liver has been purified 100-fold. The purified enzyme was stable for several months at $-20°$ in 20% glycerol.

2. The kinetic properties of this enzyme were similar to those of other aminoacyl-RNA synthetases. The K_m values for arginine, ATP and rat liver tRNA were 1.25, 1.9 and 0.37 μM, respectively. The K_m values for tRNA of rat liver, yeast and *Escherichia coli* were 0.37, 0.66 and 0.17 μM, respectively.

3. The 100-fold purified enzyme is heat labile, but is stabilized considerably by the addition of glycerol.

INTRODUCTION

Several of the aminoacyl-RNA synthetases have been obtained in highly purified states and their properties and characteristics investigated. These include the synthetases for arginine[1,2], phenylalanine[3,4], isoleucine[5], tyrosine[6], glutamic acid[7], valine[8], methionine[9] and threonine[10]. The classic study of NORRIS AND BERG[11] demonstrated the formation of an intermediate enzyme–aminoacyl adenylate. Several investigators[10,12–16] have reported that two or more synthetases for specific amino acid may be present in a given species.

In studies including the specificity of the aminoacyl-RNA synthetases and tRNA's of normal cells and malignant tumor cells[17] there was a need for mammalian sources of arginyl-RNA synthetase of high activity and stability. ALLENDE AND ALLENDE[1] reported the isolation of a highly purified but unstable preparation of this enzyme. The present communication describes a further purification of a liver arginyl-RNA synthetase with a high degree of stability. Some of the physical and other properties of this enzyme are included.

* Present address: The Third Department of Internal Medicine, Osaka University Hospital, Osaka, Japan.

MATERIALS

Calcium phosphate gel and hydroxylapatite (Bio gel HTP-dried form) were purchased from Bio-Rad Laboratories; DEAE-cellulose (Whatman DE-52) from Reeve Angel; Sephadex G-25 from Pharmacia Fine Chemicals; ATP from Pabst Laboratories; tRNA of yeast and *Escherichia coli* from General Biochemicals; polyethylene glycol 6000 from Baker Chemical.

Uniformly ^{14}C-labeled L-arginine (specific activity $>$ 220 mC/mM) was obtained from New England Nuclear. A uniformly ^{14}C-labeled L-amino acid kit was purchased from Schwarz Bio Research (specific activity 50 mC/mM).

METHODS

Rat-liver tRNA was prepared according to the method of BRUNNGRABER[18]. The concentration of tRNA was determined by absorbance at 260 nm, assuming that 1 mg of tRNA per ml has an absorbance of 25. Approx. 50 mg of tRNA were obtained from 100 g of liver. Acceptance activity was retained for many weeks when the final preparation (10 mg/ml) was frozen and stored at $-10°$.

Assay procedures

The reaction mixture (0.1 ml) was adjusted to the following concentration: 50 mM Tris–HCl (pH 7.5); 5 mM $MgCl_2$; 5 mM KCl; 3 mM 2-mercaptoethanol; 2.5 mM ATP (adjusted to pH 7.5 with NaOH); 0.1 mM L-[^{14}C]arginine. 0.1 mg of rat-liver tRNA and arginyl-RNA synthetase were added as indicated in the tables. In the assay with purified enzyme, crystalline bovine serum albumin was included in the reaction mixture at a concentration of 1 mg per ml in order to reduce the loss of activity during the reaction.

The reaction mixtures were incubated for 10 min at 37°. A blank tube without enzyme was included in each assay. Following the incubation the samples were cooled in an ice bath and 0.05-ml aliquots were pipetted onto Whatman No. 3 MM paper discs. The discs were washed three times in 5% cold trichloroacetic acid, twice with ethanol, then dried and counted in a Packard scintillation counter[19].

The specific activities of arginyl-RNA synthetase are expressed in pmoles of [^{14}C]arginyl-RNA formed per mg enzyme protein. Protein was determined by absorption at 280 nm and by a modification of the method of LOWRY *et al.*[20].

Preparation of crude enzyme

Rat livers were homogenized in a teflon–glass homogenizer with 3 vol. of buffer (0.25 M sucrose, 5 mM $MgCl_2$, 25 mM KCl and 50 mM Tris–HCl (pH 7.6)). The homogenate was centrifuged at 15 000 rev./min for 30 min in the Spinco preparative centrifuge, Model L-2, rotor No. 19. The microsomal fraction was removed by centrifugation of the supernatant solution for 60 min at 105 000 $\times$ g.

Calcium phosphate gel fractionation

0.5 vol. of calcium phosphate gel suspension (8%, centrifuged to remove the excess water) was added to the 105 000 $\times$ g supernatant, and stirred 30 min. The suspension was centrifuged at 3000 rev./min for 15 min and the supernatant was

discarded. The gel was stirred for 20 min with one-half the original volume of 0.02 M potassium phosphate buffer (pH 6.8), containing 10% glycerol and 6 mM 2-mercaptoethanol and centrifuged, and the supernatant was discarded. The gel was mixed with one-third the original volume of 0.1 M potassium phosphate buffer (pH 7.5), containing 10% glycerol and 6 mM 2-mercaptoethanol, stirred for 30 min, and centrifuged. The resulting supernatant solution contained most of the enzyme. Glycerol was added to give a final concentration of 20%. The 0.1 M phosphate buffer extracted fraction could be stored for 2 months at −20° without appreciable loss of activity. The gel, which still retained some enzyme activity, was discarded.

For further column chromatography, the enzyme solution was passed through a Sephadex G-25 column (40 cm long and the diameter was adjusted according to the volume of the enzyme solution) and equilibrated with Buffer A (0.005 M potassium phosphate (pH 7.5), 6 mM 2-mercaptoethanol and 10% glycerol).

DEAE-cellulose column chromatography

DEAE-cellulose (Whatman DE-52, 1.0 mequiv/g) was washed with 0.1 M potassium phosphate buffer (pH 6.5) until the pH of the supernatant solution was 6.5, and stored in the same buffer. A column (2 cm × 20 cm) was packed with this material and equilibrated with Buffer A. The enzyme solution was applied, the column was washed with 300 ml of Buffer A at a rate of 60 ml/h, and eluted under pressure with a linear gradient of phosphate ion concentration and of decreasing pH. The mixing chamber contained 300 ml of starting buffer, and the reservoir contained 300 ml of 0.25 M potassium phosphate (pH 6.5), containing 10% glycerol and 6 mM 2-mercaptoethanol. 10 ml fractions were collected and the absorbance at 280 nm was followed. Fractions that had highest activities were pooled and concentrated by dialysis against Buffer A containing 20% polyethylene glycol.

TABLE I

CALCIUM PHOSPHATE GEL FRACTIONATION OF ARGINYL-RNA SYNTHETASE

Specific activity is expressed by pmoles of arginyl-tRNA formed per mg of protein.

Fraction	*Buffer*		*Yield of protein (%)*	*Specific activity*	*Activity yield (%)*	*Relative purification*
	pH	*Phosphate (M)*				
105 000 × *g* supernatant			100	2770	100	1
Non-adsorbed			65.3	20	—	—
1	6.8	0.02	2.2	120	—	—
2	7.5	0.10	9.8	9800	34.6	3.5
3	7.5	0.20	11.5	1700	7.0	—
4	7.5	0.50	7.4	5900	15.9	2.5

Hydroxylapatite column chromatography

Hydroxylapatite was equilibrated overnight in 0.005 M potassium phosphate buffer (pH 6.8) and equilibrated further with Buffer A. The enzyme solution was applied to a column (1.5 cm × 20 cm) and washed with Buffer A, and then eluted with a linear gradient of phosphate 0.005 M to 0.3 M (pH 7.5), containing 10%

glycerol and 6 mM 2-mercaptoethanol, in a total volume of 500 ml. The fractions that had highest activities were pooled and dialyzed overnight against Buffer A. Dialyzed enzyme solution was applied to a column of hydroxylapatite (1 cm × 15 cm) and rechromatographed under the same conditions as described above with an exception of lowering the concentration of phosphate to 0.2 M. Glycerol was added to a final concentration of 20% to the fraction containing highest enzyme activity, and the enzyme was stored at −20°.

TABLE II

SUMMARY OF PURIFICATION OF ARGINYL-RNA SYNTHETASE

Specific activity is expressed by pmoles of arginyl-tRNA formed per mg of protein.

Fraction	*Total protein (mg)*	*Specific activity**	*Total activity (× 10^3)*	*Yield (%)*	*Relative purification*
1. 105 000 × *g* supernatant fraction	6600	2 796	18 480	100	1
2. Calcium phosphate gel eluate	647	9 800	6 341	34.3	3.5
3. DEAE-cellulose eluate	38	44 426	1 688	9.0	16
4A. Hydroxylapatite, eluate I	2.9	200 425	581	3.2	72
4B. Hydroxylapatite, eluate II*	1.6	273 350	431	2.3	98

* Three preparations were combined for the hydroxylapatite rechromatography and the yield of enzyme was calculated as one preparation.

RESULTS

Activities at the various stages of enzyme preparation are shown in Table II. The enzyme was finally purified 100-fold from the original 105 000 × *g* liver supernatant fraction with a recovery of 2.3% (Fraction 4B). A typical pattern of hydroxylapatite rechromatography is shown in Fig. 1.

As shown in Table III, the purified arginyl-RNA synthetase (Fraction 4B) contained low activities of the synthetases for lysine, glutamic acid, aspartic acid and proline. No activity was detected for other aminoacyl synthetases.

Acrylamide-gel electrophoresis of purified arginyl-RNA synthetase, using 5% gels running at pH 7.3, was carried out on a vertical gel electrophoresis cell (EC-470, E-C Apparatus). The purified enzyme migrated as a single major band with two additional, faster moving components in trace amounts. These faint bands may reflect minor heterogeneity which represents the existence of low activities of few other synthetases as indicated in Table III.

The formation of arginyl-RNA requires ATP, Mg^{2+}, tRNA and enzyme. The purified enzyme (Fraction 4B) was stable, and could be stored at least for 4 months without appreciable loss of activity at −20° with added 20% glycerol.

Kinetic properties of arginyl-RNA synthetase

The amount of arginyl-RNA formed in 10 min was linear over the enzyme concentration range of 0.1 to 2.0 μg of protein per assay. The effect of varying the concentration of arginine on the formation of arginyl-RNA by the purified enzyme is shown in Fig. 2a. The K_m value was determined from the Lineweaver–Burk plots

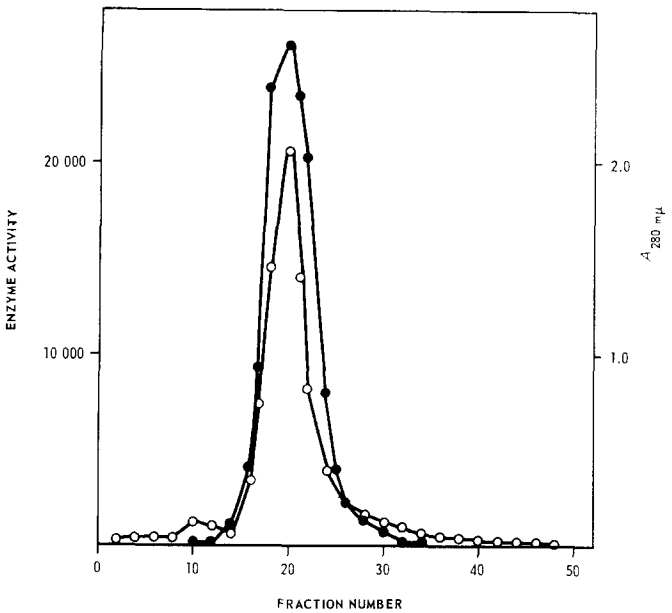


Fig. 1. Rechromatography of arginyl-RNA synthetase on hydroxylapatite. The enzyme was eluted with a linear gradient as described in the text. ●—●, enzyme activity (counts/min). 10 μl of each fraction was added to the assay. ○---○, absorbance at 280 nm.

TABLE III

SUMMARY OF OTHER AMINOACYL-RNA SYNTHETASES PRESENT IN PURIFIED ARGINYL-RNA SYNTHETASE

Assays were carried out under conditions of the standard assay except 2.0 nmoles of labeled amino acids were used. The specific activity of each of amino acid was 50 μC per μmole. The reaction mixture contained 4 μg of purified enzyme and was incubated for 15 min at 37°.

Amino acid	*Aminoacyl RNA-formed (pmoles)*	*Relative activity (%)*
Arg	176.8	100
Lys	4.9	2.7
Glu	4.6	2.6
Asp	2.5	1.4
Pro	1.6	0.9
Tyr	0	0
Thr	0	0
Leu	0	0
Val	0	0
Gly	0	0
Ala	0	0
Ser	0	0
His	0	0
Ileu	0	0
Phe	0	0

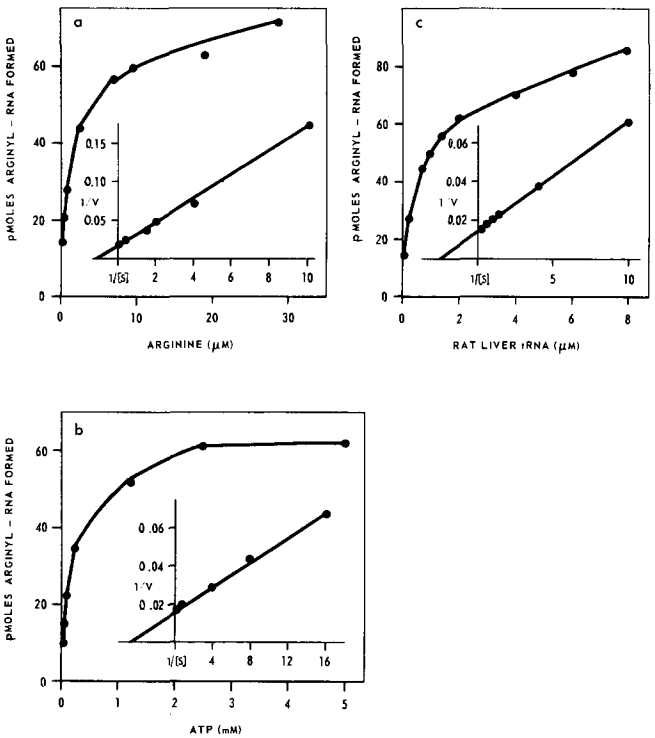


Fig. 2. Kinetic data on the rate of arginyl-RNA formation. Effect of the concentration of arginine (a), ATP (b), and rat-liver tRNA (c). Incubations were carried out for 5 min with 1.45 μg of purified enzyme.

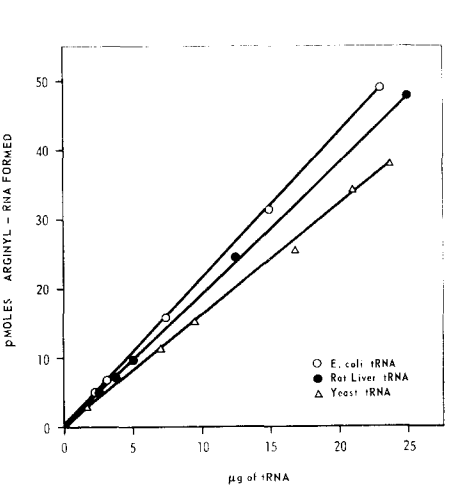
of these data as 1.25 μM for arginine. Fig. 2b shows the effect of ATP concentration on the formation of arginyl-RNA. The K_m value for ATP calculated from these data was 1.9 μM.

The effect of rat-liver tRNA concentration upon the formation of arginyl-RNA is shown in Fig. 2c. From Fig. 3 it is apparent that the extent of the acceptance of [^{14}C]arginine, in the presence of excess enzyme, was proportional to the amount of tRNA added within limiting concentrations. It was possible to calculate the molar quantity of $tRNA^{Arg}$ per μg of tRNA, assuming that one arginyl-RNA ester formed per $tRNA^{Arg}$ molecule. From these data, 1.0 μg of rat-liver tRNA was equivalent to 1.92 pmoles of $tRNA^{Arg}$, and the apparent K_m for arginyl-RNA formation was calculated to be 0.37 μM.

The purified rat-liver arginyl-RNA synthetase was capable of forming arginyl-RNA, not only with rat-liver tRNA but also with *E. coli* and yeast tRNA. However, the greatest acceptance was obtained with *E. coli* tRNA and the least with yeast tRNA. To obtain an adequate reaction velocity, the K_m values for yeast, rat-liver, and *E. coli* tRNA were determined by the assay with 3.6, 1.45 and 0.72 μg of purified enzyme, respectively. The K_m values calculated according to the method described above were 0.66 μM for yeast tRNA and 0.17 μM for *E. coli* tRNA.

Heat lability of arginyl-RNA synthetase

The purified enzyme was preincubated prior to the standard assay. The preincubation mixture contained 10 μl (8 μg of protein) of purified enzyme and 10 μl of 0.5 M Tris–HCl buffer (pH 7.5) in a total volume of 0.1 ml. The enzyme solution (Fraction 4B) was used without further dialysis. The final concentrations of each



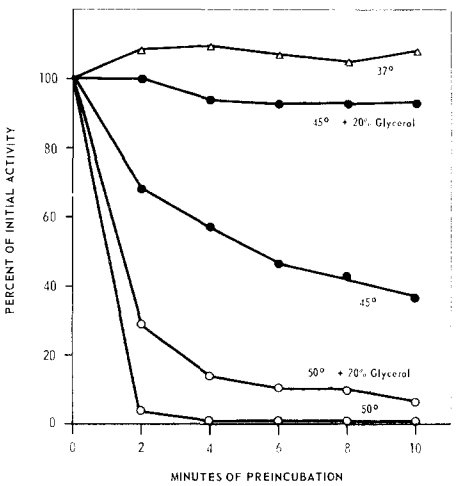


Fig. 3. Effect of tRNA concentration on the yield of arginyl-RNA formation. Incubations were carried out for 10 min according to the standard assay described in the text. 4.0 μg of purified enzyme was added for rat-liver and *E. coli* tRNA assays and 16.0 μg of the enzyme was used for yeast-tRNA assay.

Fig. 4. The effect of glycerol upon the heat lability of arginyl-RNA synthetase. Purified enzyme was preincubated at the temperatures and times indicated at a concentration of 0.08 mg per ml. At given times, aliquots (10 μl) of preincubated enzyme was assayed for activities as described in the text.

component in the preincubation mixture were: 0.05 M Tris–HCl (pH 7.5); 0.01 M potassium phosphate (pH 7.5); 1% glycerol; 0.6 mM 2-mercaptoethanol, and 80 μg/ml of the enzyme. After the preincubation, 10 μl aliquots (0.8 μg of protein) were added to the standard assay tubes and enzyme activities were assayed in the same method as described earlier.

As shown in Fig. 4, the purified enzyme was extremely heat labile, losing over 50% of its activity in 2 min at 45° and all activity in 2 min at 50°. The addition of 20% glycerol in the preincubation mixture protected completely the enzyme against heat inactivation at 45°.

DISCUSSION

Without the addition of 10% glycerol in all solutions used for the preparation of enzyme, the arginyl-RNA synthetase of rat liver was extremely labile at any steps of the purification and lost over 50% of its activity after 24 h at −10°. However, the 105 000 × *g* supernatant fraction was stable for 2 months at −20°. Although the enzyme was stabilized to some extent by the addition of 2-mercaptoethanol or

by the storage in the solution of sodium or potassium salt at a high concentration, glycerol was required for maximum stability.

Arginyl-RNA synthetase was especially sensitive to certain of the procedures that have been employed for the purification of other aminoacyl synthetases. These steps include: $(NH_4)_2SO_4$ fractionation; the dialysis against the buffer of low salt concentration[2-9]. The pH 5 fraction of 105 000 $\times$ g supernatant could not be used as a starting material because of its instability.

The arginyl-RNA synthetase, although very labile as described above, was stabilized considerably by the use of glycerol. No major difficulties were encountered with the stability of enzyme in the procedure reported in this paper. The stabilizing effect of sucrose and glycerol in the purification of other aminoacyl-RNA synthetases has been reported[4,9,21,22].

The kinetic properties of arginyl-RNA synthetase of rat liver were similar to those of other aminoacyl synthetases[2,8,23]. The K_m value for tRNA, 0.1 μM, was lower than the K_m of arginine, 1.0 μM. K_m values varied for the different tRNA's derived from yeast, *E. coli* and rat liver.

These results suggest that the tRNA from different species vary in chemical or physical structures. The yeast tRNA does not function optimally as a substrate for the rat-liver arginyl-RNA synthetase. However ALLENDE AND ALLENDE[1] used the yeast tRNA in the assay to follow the purification of the same enzyme.

The extractions of the enzyme from calcium phosphate gel were carried out with sodium and potassium phosphate buffer at different concentration and pH (Table I). 0.1 M potassium phosphate (pH 7.5) was the best buffer to extract the enzyme, however, the extract by 0.5 M potassium phosphate still contained some enzyme activity. It is possible therefore, to consider the existence of two kinds of arginyl-RNA synthetase in rat liver. This possibility was also supported by the fact that the main peak of enzyme activity on DEAE-cellulose column chromatography was followed by another small peak of activity. The existence of two or more enzymes for several aminoacyl-RNA synthetases has been reported[10,12-16]. In the present study only the main arginyl-RNA synthetase was purified and studied in any detail. The other form of this enzyme will require further investigation in order to determine the structural difference that may exist between two enzymes.

ACKNOWLEDGEMENTS

This work was supported by American Cancer Society Grant E-312 and The Robert A. Welch Foundation Grant G-035.

REFERENCES

1 C. C. ALLENDE AND J. E. ALLENDE, *J. Biol. Chem.*, 239 (1964) 1102.
2 S. K. MITRA AND A. H. MEHLER, *J. Biol. Chem.*, 242 (1967) 5490.
3 T. W. CONWAY, E. M. LANSFORD, JR. AND W. SHIVE, *J. Biol. Chem.*, 237 (1962) 2850.
4 M. P. STULBERG, *J. Biol. Chem.*, 242 (1967) 1060.
5 A. N. BALDWIN AND P. BERG, *J. Biol. Chem.*, 241 (1966) 831.
6 R. CALENDAR AND P. BERG, *Biochemistry*, 5 (1966) 1681.
7 M. P. DEUTCHER, *J. Biol. Chem.*, 242 (1967) 1123.
8 U. LAGERKVIST AND J. WALDENSTRÖM, *J. Biol. Chem.*, 242 (1967) 3021.
9 F. LEMINE, J-P. WALLER AND R. VAN RAPENBUSCH, *European J. Biochem.*, 4 (1968) 213.

10 C. C. ALLENDE, J. E. ALLENDE, M. GATICA, J. CELIS, G. MORA AND M. MATAMALA, *J. Biol. Chem.*, 241 (1966) 2245.
11 A. T. NORRIS AND P. BERG, *Proc. Natl. Acad. Sci. U.S.*, 52 (1964) 330.
12 F. INAMOTO, T. YAMANE AND N. SUEOKA, *Proc. Natl. Acad. Sci. U.S.*, 53 (1965) 1456.
13 W. E. BARNETT AND J. L. EPLER, *Proc. Natl. Acad. Sci. U.S.*, 55 (1966) 184.
14 C. T. YU AND H. P. RAPPAPORT, *Biochim. Biophys. Acta*, 123 (1966) 134.
15 B. L. STREHLER, D. D. HENDLEY AND G. P. HIRSH, *Proc. Natl. Acad. Sci. U.S.*, 57 (1967) 1751.
16 A. VESCIA, *Biochem. Biophys. Res. Commun.*, 29 (1967) 496.
17 A. C. GRIFFIN, B. HOLLAND AND L. CANNING, *Developmental and Metabolic Control Mechanisms and Neoplasia*, Williams and Wilkins, Baltimore, Maryland, 1965, p. 475.
18 E. F. BRUNNGRABER, *Biochem. Biophys. Res. Commun.*, 8 (1962) 1.
19 M. A. O'NEAL AND A. C. GRIFFIN, *Cancer Res.*, 23 (1963) 628.
20 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
21 K. H. MUENCH AND P. BERG, *Procedures in Nucleic Acid Research*, Harper and Row, New York, 1966, p. 375.
22 H. GEORGE AND A. MEISTER, *Biochim. Biophys. Acta*, 132 (1967) 165.
23 M. P. DEUTCHER, *J. Biol. Chem.*, 242 (1967) 1132.