STUDIES ON ENZYMES INVOLVED IN THE PURINE NUCLEOTIDE CYCLE IN RAT

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Preface

Ammonia production is inevitable in the body so far as protein or amino acids are metabolized. At present there are two known mechanisms from which ammonia is released in mammals. The first mechanism is direct release of ammonia from amino acids catalyzed by enzymes such as serine-threonine dehydratase, cystine desulfrase, histidine ammonia-lyase, glycine cleavage enzyme, glutaminase, asparaginase and so forth. In our laboratory, much effort has been exerted to make clear the molecular properties of some of these enzymes (2-4).

The second one is so-called transdeamination reaction proposed by Braunstein in 1957 (1). In this reaction, amino groups of various amino acids are transfered to α-ketoglutarate to form glutamate by aminotransferases and then ammonia is released from the glutamate formed by glutamate dehydrogenase.

Recently, Lowenstein proposed the third mechanism of ammonia liberation from amino acids which is called as the purine nucleotide cycle. The reaction is represented as follows;

$$\text{IMP} + \text{GTP} + \text{Aspartate} \xrightarrow[\text{adenylosuccinate synthetase}]{} \text{Adenylosuccinate} + \text{GDP} + \text{Pi} \quad 1)$$

$$\text{Adenylosuccinate} \xrightarrow[\text{adenylosuccinase}]{} \text{AMP} + \text{Fumarate} \quad 2)$$

$$\text{AMP} \xrightarrow[\text{AMP deaminase}]{} \text{IMP} + H_2O + NH_3 \quad 3)$$

1) + 2) + 3)

$$\text{Aspartate} + \text{GTP} \longrightarrow \text{GDP} + \text{Pi} + \text{Fumarate} + H_2O + NH_3$$

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Thus one mole of aspartate is cleaved to form each mole of ammonia and fumarate by consumption of one mole of terminal phosphate bond energy of GTP at each turn of the cycle. It was first shown in crude extract of rat skeletal muscle that this cycle actually worked. Recently evidences are presented that the cycle also works either in the brain, the liver or the kidney. It is also said that the purine nucleotide cycle plays an important role in the liberation of ammonia from the skeletal muscle during exercise.

The idea of the purine nucleotide cycle is originated from the report of Parnas entitled "Über die Ammoniakbildung im Muskel und ihren Zusammenhang mit Funktion und Zustandänderung" in 1929 (5). According to Parnas, ammonia is liberated as a following scheme;

Adeninnucleotid ⟶ Inosinsäure + NH_3 / Zuckung (twitch)

Inosinsäure + O_2 + Aminosäurederivat X... ⟶

Adeninnucleotid + Desaminierungsprodukt von X ...

Erholung (recovery)

In 1928, Schmidt discovered AMP deaminase and found that ammonia produced from the muscle was mainly due to this enzyme (6). Carter and Cohen demonstrated that adenylosuccinate was the intermediate during the enzymatic conversion of IMP to AMP (7). Accordingly it may be said that Lowenstein only renewed Parnas's findings after 40 years. It should be emphasized, however, that extensive works on the purine nucleotide cycle by Lowenstein and his colleague made enable to elucidate a close relationship between

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ammonia production and glycolysis in exercizing muscle.

As for the second mechanism, it has been disputed whether it actually works in a physiological condition, because the equilibrium constant of glutamate dehydrogenase reaction favors remarkably toward the synthesis of glutamate. In fact, the free-energy change of the net reaction by virtue of the purine nucleotide cycle is calculated to be approximately -1.0 kcal, whereas that of glutamate dehydrogenase reaction is 6.5 kcal for NAD and 6.7 kcal for NADP at pH 7.0. Glutamate dehydrogenase is not abundant in the muscle, but in the liver. On the contrary, total activities of AMP deaminase, adenylosuccinate synthetase and adenylosuccinase are higher in the skeletal muscle than any other organs. These findings support Lowenstein's view that the purine nucleotide cycle is more favorable for liberation of ammonia from amino acids than transdeamination at least in the skeletal muscle. Therefore, we attempted to solve the following problems concerning the purine nucleotide cycle with hope that the results obtained will add important informations to our knowledge on nitrogen metabolism so far accumulated in our laboratory.

1. How is the purine nucleotide cycle regulated in the skeletal muscle ? To answer this, the properties of the enzyme must be characterized.

2. Does the cycle work in the organs other than the skeletal muscle actually ? Then what for ?

3. Two enzymes of the cycle are on the main pathway of purine nucleotide synthesis. Then are the enzymes concerning the regulation for ammonia production the same as those concerning

the regulation for the purine nucleotide synthesis ?

In this paper, I would like to describe the results obtained in connection with the question mentioned above.

More knowledges have been accumulated as to molecular mechanism of muscular contraction and relaxation, but interactions of contractile proteins and their surrounding proteins still remain unelucidated. In this sense, our findings obtained during this study showing that AMP deaminase and adenylosuccinate synthetase interacted with actin-myosin may throw light on the relationship between muscular work and ammonia production.

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Chapter I

Purification, Crystallization and Properties of Adenylosuccinate Synthetase from Rat Skeletal Muscle

Purification, Crystallization and Properties of Adenylosuccinate Synthetase from Rat Skeletal Muscle

Summary

Adenylosuccinate synthetase [EC 6.3.4.4] was purified and crystallized from rat skeletal muscle. The purified preparation was shown to be homogeneous by polyacrylamide gel electrophoresis and ultracentrifugal analysis, and by the latter procedure its average molecular weight was estimated as 104,000 and its $S_{20,w}$ value as 5.0.

On SDS-polyacrylamide gel electrophoresis it gave only a single band with a molecular weight of 52,000. Sedimentation equilibrium experiments also indicated that its molecular weight in 6 M guanidine-HCl was 49,000. These findings show that the native enzyme is composed of two subunits with molecular weight of 50,000.

Its optimum pH was 6.8 and its Km values for L-aspartate, IMP and GTP were calculated to be 2.5, 7.0 and 3.8×10^{-4} M, respectively.

Nucleoside mono- and diphosphate, irrespective of whether purine or pyrimidine derivatives, were potent inhibitors, while nucleoside triphosphates were not.

The enzyme activity was inhibited considerably by the succinate derivatives, succinate, adenylosuccinate and argininosuccinate. Succinate caused competitive inhibition with aspartate, and non-competitve inhibitions with IMP and GTP. The Ki value for succinate was 1.9×10^{-3} M.

The purified enzyme preparation showed full activity in the presence of $MgCl_2$ and $MgCl_2$ could be partially replaced by $MnCl_2$, $CoCl_2$ or $CaCl_2$.

INTRODUCTION

From thermodynamic studies, Lowenstein (1) proposed a working hypothesis that the purine nucleotide cycle, involving adenylosuccinate synthetase [IMP:L-aspartate ligase (GDP), EC 6.3.4.4], adenylosuccinase [adenylosuccinate AMP-lyase, EC 4.3.2.2] and AMP deaminase [AMP aminohydrase, EC 3.5.4.6], plays an important role in production of ammonia from amino acids during muscle excercise instead of the transdeamination system involving transaminases and glutamate dehydrogenase [EC 1.4.1.3] (2). Tornheim and Lowenstein also presented evidence that ammonio=genesis by the purine nucleotide cycle might be closely linked to glycolysis. Moreover Ogawa et al.(4) demonstrated that fructose-1,6-diphosphate (FDP) was a potent inhibitor of adenylosuccinate synthetase from rat skeletal muscle. This substantiates the idea that there is a mechanism for synchronous control of the purine nucleotide cycle and glycolysis; FDP is not only a product of the phosphofructokinase [EC 2.7.1.1] reaction but also an activator of the enzyme, while it is an inhibitor of adenylosuccinate synthe=tase. On the other hand, AMP is an intermediate of the cycle, whereas it is an activator of phosphofructokinase. When FDP accumulates owing to increase in phophofructokinase activity by AMP, it will inhibit formation of the latter and this may result in restoration of the initial level of phosphofructokinase activity. This mechanism also explains the control of glycolytic oscillation (5-6).

In addition to its regulatory role in glycolysis as well as ammoniogensis, adenylosuccinate synthetase may be important in regulation of purine biosynthesis in various organs, since it

catalyzes not only the irreversible step in de novo synthesis of adenine nucleotides, but also the branch point of the synthetic pathway for guanine nucleotides.

To examine the detailed mechanism of synchronous control of ammoniogensis and glycolysis and their correlation to regulation of purine nucleotide synthesis, the properties of adenylosuccinate synthetase purified from skeletal muscle must be investigated and compared with those of the enzymes from other Organs. This paper reports the crystallization and molecular and kinetic characteristics of adenylosuccinate synthetase from rat skeletal muscle.

MATERIALS AND METHODS

Animals—— Male Wistar HLA strain rats, weighing 300 to 450 g, were used throughout. Rats were fed a laboratory chow pellet MF from Oriental Yeast Co. Ltd., Osaka. They were killed by decapitation, and their skeletal muscle was removed and stored at -20° until used.

Enzyme Assays

Assay A—— Adenylosuccinate synthetase was assayed by a modification of the method of Lieberman (7). The reaction mixture contained in 250 µl: imidazole-HCl buffer, pH 6.8, 3×10^{-2} M; IMP, 10^{-3} M; GTP, 5×10^{-4} M; L-aspartate, 5×10^{-3} M; $MgCl_2$, 8×10^{-3} M; creatine phosphate, 2×10^{-3} M; creatine kinase, 10 µg (0.25 u at 25°) and enzyme solution. The reaction was started by adding aspartate and stopped by adding 50 µl of 30 % $HClO_4$ after incubation for 5 min at 37°. Denatured materials were removed by centrifugation, and the absorbance of the supernatant at 280 nm was measured in a Beckman, model DB-G, spectrophotometer.

The difference between the molecular absorbances of adenylosuccinate and IMP was 11.7×10^3 per ml per cm at 280 nm (8).

Assay B—— The reaction mixture was the same as for Assay A except that creatine phosphate and creatine kinase were omitted. This assay was used for the purified enzyme which was devoid of GTPase activity. One uint of enzyme activity was defined as the amount catalyzing the formation of 1 μmole of adenylosuccinate either in Assay A or Assay B. The specific activity of the enzyme was expressed as units per mg protein.

Protein was estimated by the method of Lowry et al. (9) with bovine serum albumin as a standard.

Other Enzyme Assays——Adenylosuccinase and AMP deaminase were assayed by the methods of Carter and Cohen (10) and Smiley, Jr. et al. (11), respectively.

Ultracentrifugation——Ultracentrifugation was carried out as described by Chervenka (12). For sedimentation equilibrium experiments, a standard sector cell with a 12 mm centerpiece filled with Epon was used. The rotor speed was 7,176 rpm at 20.5° and Rayleigh interference optics were used. Sedimentation velocity was measured in the conventional way with a single sector cell. The rotor speed was usually 52,640 rpm and Schlieren optics were used.

Materials——The commercial sources of the materials used were as follows: IMP, GMP, AMP, GTP, ITP, and argininosuccinate were from Kyowa Hakko (Tokyo); XMP, GDP, Tris and creatine phosphate were from Sigma (U.S.A.); ADP, ATP, UDP, UTP, CTP and creatine kinase [EC 2.7.3.2] were from Boehringer (Germany); Sephadex G-150 was from Pharmacia (Sweeden); DEAE-cellulose (DE-52) and phosphocellulose (P-11) were from Whatman (U.S.A.); hydroxylapatite (Hypatit C) was Clarkson Chemical Co. (U.S.A.); dithiothreitol (DTT) was from Calbiochem (U.S.A.) and collodion bags were from Sartorius Membrane Filter GmbH (Germany). The other reagents used were purchased from local sources.

Adenylosuccinate was prepared by the method of Carter and Cohen (10).

RESULTS

Purification Procedure——Adenylosuccinate synthetase has been reported to be strongly inhibited by its product GDP (13-14). In confirmation of this, as shown in Fig. 1 no enzyme activity was detected in crude muscle extract when a GTP generating system was omitted from the reaction mixture (Assay B system). Therefore, the GDP formed was recycled to GTP in the presence of phosphocreatine and creatine kinase to increase the forward reaction of the synthetase. That is why Assay A was employed through all purification steps.

All operations were carried out at 0° to 4° unless otherwise stated.

Step I. Preparation of Crude Extract——Approximately 330 g of skeletal muscle were finely chopped up with a pair of scissors and blended with 3 volumes of 5×10^{-2} M Tris-HCl buffer, pH 7.2, containing 2×10^{-4} M DTT for 1 to 1.5 min in a homomixer (Nippon Seiki, Tokyo) operating at maximum speed. The homogenate was centrifuged for 15 min at 15,000 x g and the supernatant was subjected to further purification.

Step II. Heat Treatment——The crude extract (870 ml) was devided into 2 ml portions and each was heated for 1 min at 60° with gentle shaking. The preparations were then cooled and denatured protein was removed by centrifugation for 15 min at 15,000 x g.

Step III. Ammonium Sulfate Fractionation——To the transparent supernatant (840 ml) solid ammonium sulfate was added slowly to a final saturation of 40 % and stirring was continued for 30 min. Then the suspension was centrifuged for 30 min at 15,000 x g. Solid ammonium sulfate was added to the resulting supernatant to a final saturation of 70 %. After mechanical stirring, the mixture was centrifuged for 30 min at 15,000 x g. The precipitate formed was suspended in a minimum volume of buffer A (10^{-2}M potassium phosphate buffer, pH 7.0, containing 2 x 10^{-4}M DTT) and dialyzed overnight against 2 changes of 2 liters of buffer A.

Step IV. Phosphocellulose Column Chromatography——The dialysate (165 ml) was applied to a phosphocellulose column (4 x 25 cm) equilibrated with buffer A. The column was washed throughly with buffer A containing 5 x 10^{-2}M KCl until the effluent did not show any absorbance at 280 nm, and then eluted with a linear concentration gradient of 5 x 10^{-2} to 5 x 10^{-1}M KCl in buffer A, achieved with 1 liter of each buffer. Fractions with activity were combined and solid ammonium sulfate was added to a final saturation of 70 %. The protein precipitated was dissolved in a minimum volume of buffer B (10^{-2}M Tris-HCl buffer, pH 7.2, containing 2 x 10^{-4}M DTT), and dialyzed overnight against 100 volumes of the same buffer.

Step V. Negative Adsorption to DEAE-cellulose——The dialysate (25 ml) was adjusted to pH 7.8 with 1 M Tris solution and passed through a DEAE-cellulose column (2 x 20 cm) equilibrated with 10^{-2}M Tris-HCl buffer, pH 7.8. By this procedure much of the colored

protein was adsorbed to DEAE-cellulose, but adenylosuccinate synthetase was not adsorbed at all.

Step VI. Hydroxylapatite Column Chromatography——The unadsorbed fraction from DEAE-cellulose was directly applied to a hydroxylapatite column (2 x 20 cm) equilibrated with buffer A. The column was washed with 7 x 10^{-2}M potassium phosphate buffer, pH 7.0, containing 2 x 10^{-4}M DTT, and eluted with a linear concentration gradient of 7 x 10^{-2}M to 5 x 10^{-1}M potassium phosphate buffer, pH 7.0 containing 2 x 10^{-4}M DTT, achieved with 200 ml of each buffer. The elution pattern of enzyme activity closely resembled that of protein at this step, as shown in Fig. 2.

Fractions with acitvity were pooled and concentrated by precipitation with solid ammonium sulfate (70 % saturation). The precipitate was dissolved in a minimum volume of buffer A.

Step VII. Sephadex G-150 Gel Colum Chromatography——The preparation (1.5 ml) from Step VI was applied to a Sephadex G-150 column (2 x 90 cm) equilibrated with buffer A containing 5 x 10^{-2}M KCl. The column was eluted with the same buffer at a flow rate of approximately 7 ml per h. The elution profiles of the enzyme activity and protein concentration are shown in Fig. 3. Fractions with activity were combined and concentrated to 1.5 ml in a collodion bag.

A summary of the purification is given in Table I. Adenylosuccinate synthetase was purified approximately 100 fold by this procedure.

Crystallization——Finely triturated ammonium sulfate was slowly added to a final saturation of 50 % to the enzyme solution in a conical tube on an ice-bath with gentle stirring with a glass rod. A faint birefringence began to appear when the mixture had been stored for several minutes in a cold room. The crystals appeared octahedral, as shown in Fig. 4. The specific activity of the crystalline preparation was approximately 5.3 u per mg protein by Assay B. No adenylosuccinase or AMP deaminase was detected in this preparation.

Purity and Molecular Weight

SDS-Plyacrylamide Gel Electrophoresis——The purified protein was subjected to electrophoresis on SDS-polyacrylamide gel (7.5 % acrylamide and 0.1 % sodium dodecylsulfate) following the method of Weber and Osborn (15). As shown in Fig. 5, only one protein band was observed on the gel.

The molecular weight of the subunit was calculated to be 52,000, by comparison of its mobility with those of bovine serum albumin (M.W.=68,000), ovalbumin (M.W.=43,000) and chymotrypsinogen (M.W.=25,000), as shown in Fig. 6. The same result was obtained after the enzyme had been denatured by treatment with 1 % SDS at 50° for 2 h with or without 2-mercaptoethanol.

Sedimentation Velocity Analysis——The crystalline preparation was dissolved in buffer A containing 10^{-1} M KCl and dialyzed over=

night against 100 volumes of the same buffer and then run in a Spinco model E ultracentrifuge equipped with "RTIC" units at 22.0°. The concetration of enzyme solution used was 0.46 %. The typical sedimentation pattern of adenylosuccinate synthetase with a single, symmetrical peak is shown in Fig. 7. The $S_{20,w}$ was calculated to be 4.9 at a protein concentration of 0.46 %. The $S_{20,w}$ value extrpolated to zero enzyme concentration ($S^{\circ}_{20,w}$) was estimated to be 5.0 in the ccncentration range of 0.2 to 0.1 %.

Sedimentation Equilibrium Analysis——A crystalline sample was dissolved in and dialyzed against buffer A, containing 10^{-1} M KCl and subjected to a low speed sedimentation equilibrium analysis. The data were monitored with a computer. The average molecular weight was determined to be 104,000. As shown in Fig. 8, the native enzyme seems to be relatively stable in the dimer form in aqueous solution, since a plot of the logarithm of the fringe displacement with respect to the square of the distance from the axis of rotation gave a concaved curve and the enzyme molecule seemed to dissociate into the monomer form only at high dilution.

A similar plot of values in 6 M guanidine-HCl gave almost a straight line, as shown in Fig. 9, and from the slope the molecular weight of the protein was calculated to be 49,000 in guanidine-HCl solution.

These data together with those obtained by polyacrylamide gel electrophoresis, show that the molecular weight of enzyme subunits is 50,000 and that the native enzyme exists as a stable dimer

in aqueous solution.

Stability——Adenylosuccinate synthetase in crude extract was quite stable when stored at 4°, but in dilute solution the purified enzyme rapidly lost acitvity on storage at either 0° or -20°. However, the crystalline enzyme could be stored in 50 % saturated ammonium sulfate solution at 4° for at least half a year without loss of activity.

Optimum pH——The enzyme activity was determined at various pH values with imidazole buffer (below pH 7.0) and Tris-HCl buffer (above pH 7.0). As shown in Fig. 10, maximum activity was observed at pH 6.8 and more than half maximum activity at between pH 5.5 and 8.5.

Effects of Substrate Concentrations——The effects of substrate concentrations on enzyme activity are shown in Fig. 11. A typical Lineweaver-Burk plot was obtained with each of the three substrates, and from the slopes the apparent Km values for aspartate, IMP and GTP were calculated to be 2.5, 7.0 and 3.8 x 10^{-4} M, respectively.

Effects of Nucleotides and Compounds Related to Nucleotide Biosynthesis——There are many reports that adenylosuccinate synthetase from various species is inhibited by purine nucleotides, such as GMP, GDP, AMP, ADP and adenylosuccinate (13-14). The results in Table II confirm that these nucleotides inhibited the activity of the enzyme from rat skeletal muscle. In addition, pyrimidine nucleotides, such as UDP at a concentraion of 2 x 10^{-3} M also

inhibited the enzyme activity. These data also indicate that nucleoside mono- and diphosphates were potent inhibitors, but nucleoside triphosphates were not.

Effects of Amino Acids and Fatty Acids——If the purine nucleotide cycle is important in ammoniogenesis in skeletal muscle, amino acids might affect the enzyme activity. Accordingly studies were made on the effects of all the amino acids found in the body except tryptophan and tyrosine, which have absorptions at 280 nm and so interfere with the enzyme assay. However, at concentrations of 2×10^{-3} M, none of them had any effect.

Next the effects of free fatty acids were examined, because glycolysis is inhibited by fatty acids and its site of inhibition was found to be phosphofructokinase (17), the regulation of which is closely related with that of adenylosuccinate synthetase, as mentioned previously. However, oleate (10^{-3} M), palmitate (10^{-4} M), laurate (2×10^{-4} M) and linoleate (2×10^{-4} M) did not affect the enzyme activity.

Effects of Substrate and Product Analogues——Succinate was found to be a specific inhibitor of this enzyme, like the enzyme from human placenta (14). The inhibition was competitive with aspartate, whereas it was non-competitive with IMP and GTP, as shown in Fig. 11. The Ki value for succinate was calculated to be 1.9×10^{-3} M for a Dixon plot (Fig. 12). Adenylosuccinate, one of the reaction products, was also a potent inhibitor, as shown in Table III, possibly due to its structural similarity to succinate. Argininosuccinate was also inhibitory, although other members of the urea cycle were not. Neither fumarate nor citrate

affected the enzyme activity.

Effects of Divalent Ions——Adenylosuccinate synthetase from skeletal muscle required magnesium ion for full activity. It was found that $MnCl_2$ (43.2 %), $CoCl_2$ (21.3 %) or $CaCl_2$ (16.2 %) could partially replace $MgCl_2$ at a concentration of 2×10^{-3} M. These metal ions did not interfere with the reaction in the presence of $MgCl_2$.

Effects of Other Reagents——Monovalent ions, such as KCl, NaCl and NH_4Cl, did not affect the enzyme activity, but $(NH_4)_2SO_4$ at a concentration of 10^{-2} M caused approximately 50 % inhibition, as shown in Table IV. Potassium sulfate was also inhibitory. These findings indicate that SO_4^{--} has an inhibitory effect on the enzyme as on that from rabbit muscle (13).

Orthophosphate at concentrations of 2×10^{-3} M and 2×10^{-2} M caused 20 % and 59 % inhibition, respectively. Parachloromercuribenzoate at a concentration of 2×10^{-4} M caused 30 % inhibition.

DISCUSSION

Muirhead and Bishop (13) reported the crystallization of adenylosuccinate synthetase from rabbit skeletal muscle. They calculated its Km values for aspartate, IMP and GTP as 3×10^{-4} M, 2×10^{-4} M and 10^{-5} M, respectively. The Km values for aspartate and IMP of the rat enzyme estimated in this work were almost the same as those of the rabbit enzyme. The pH-activity profile of the rat enzyme was also similar to that of the rabbit enzyme, but the Km value of the rat enzyme for GTP was one order higher than that of the rabbit enzyme. Muirhead and Bishop also showed by gel filtration and SDS gel electrophoresis that the molecular weight of rabbit enzyme was 54,000 to 56,000 in both the native and denatured form. In contrast, we found that the rat enzyme was composed of two subunits with molecular weights of 50,000. These findings suggest that the rat enzyme differs from the rabbit enzyme in molecular weight.

Van der Weyden and Kelly (14) partially purified adenylosuccinate synthetase from human placenta. They reported its Km values for aspartate, IMP and GTP as 9.5×10^{-4} M, 3.7×10^{-5} M and 3.1×10^{-5} M, respectively. These values suggest that the muscle enzyme also differs from the placental enzyme. They also demonstrated that the enzyme activity was inhibited by various purine nucleotides in the following order; di- > mono- > triphosphate, and that adenylosuccinate, AMP and XMP were potent competitive inhibitors with respect to IMP, while GMP and GDP were potent competitive inhibitors with respect to GTP. From these kinetical findings, they concluded that the regulation of adenylosuccinate synthetase

was not highly specific for net synthesis of adenine nucleotides, although the synthetase was a shunt enzyme located on the pathway from guanine nucleotide synthesis to adenine nucleotide synthesis.

We found that the rat muscle enzyme was inhibited by pyrimidine nucleotide as well as by purine nucleoside mono- and diphosphate, whereas it was not affected by nucleoside triphosphate, such as UTP and CTP, at concentrations of 2×10^{-3} M. The kinetics of inhibition by the pyrimidine and purine nucleotides was not examined, because these compounds interfered with our spectrophotometric assay. These findings suggest that the mechanism of regulation of the rat muscle enzyme may be different from that of the human placental enzyme, because the latter is only slightly inhibited by pyrimidine nucleoside mono- and diphosphates at concentrations of 2.5×10^{-3} M.

Judging from the results of these inhibition studies, the regulation of the synthetase by nucleotides and related compounds does not seem to be specific for nucleic acid synthesis in skeletal muscle as in case of human placental enzyme. Therefore, we examined the specific regulatory role of the synthetase on metabolic pathways other than purine nucleotide synthesis.

Felig and Wahren (18) showed that the output of alanine from skeletal muscle increased with increase in glucose utilization during excercise (glucose-alanine cycle).

Ahlborg et al. (19) presented evidence that branched chain amino acids (leucine, isoleucine and valine) were preferentially utilized as nitrogen sources for alanine formation in muscle during excercise. As discussed previously, AMP is a critical regulator of glycolysis which produces pyruvate for alanine formation.

Therefore, we tested the effects of various amino acids on adenylo= succinate synthetase. However, none of the amino acids tested had any effect at a concentration of 2×10^{-3}M.

Recently, we found that a physiological concentration of FDP markedly inhibited adenylosuccinate synthetase activity (4), as described in the following paper. In line with these findings, Askari and Rao (20) reported that AMP deaminase in an erythrocyte lysate was inhibited by 2,3-diphosphoglycerate. In this sense, adenylosuccinate synthetase as a member of the purine nucleotide cycle enzymes might be subjected to synchronous control with glyco= lysis in skeletal muscle, as discussed previously. To investigate this working hypothesis further, the regulations of adenylosuccinate synthetase from various organs where nucleic acids are actively formed must be investigated. We found that the isoelectric point of partially purified enzyme from rat liver (pH 6.0) was completely different from that of the enzyme from skeletal muscle (pH 8.8), as will be described later. The problems of whether the liver enzyme is involved in the highly specific mechanism of regulation of purine biosynthesis and of whether the skeletal muscle enzyme is specifically organized for the control of glycolysis will also be discussed in the following papers.

The effects of several free fatty acids on the enzyme activity were also examined to see if they inhibited, because there is a reciprocal relationship between glycolysis and lipolysis. However, they were found to be without effect.

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Fig. 1. Time course of the adenylosuccinate synthetase reaction Ten µl (protein, 150 µg) of crude extract (Step I) were used in experiment (A) and 10 µl (protein, 10 µg) of partially purified enzyme (Step IV) in experiment (B). Closed circles and open circles indicate the enzyme activities assayed in reaction mixture with and without creatine kinase (0.25 u per tube) plus creatine phosphate (2×10^{-3} M). Enzyme activity in the ordinate is expressed as µmoles of adenylosuccinate formed per min per ml.

Fig. 2. Hydroxylapatite column chromatography. The enzyme preparation from Step V (protein, 298 mg) was applied to a hydroxylapatite column (2 x 20 cm) and the column was eluted as described in the text. The solid line with open circles indicates the enzyme activity and that with closed circles, the protein concentration. Enzyme activity in the ordinate is expressed as µmoles of adenylosuccinate formed per min per ml.

Fig. 3. Sephadex G-150 column chromatography. The enzyme preparation from Step VI (protein, 10 mg) was applied to a Sephadex G-150 column (2 x 90 cm). Elution was carried out as described in the text. The solid lines with open circles and closed circles represent enzyme activity and protein concentration, respectively, and the dotted line indicates the specific activity of the enzyme. The elution volume of blue dextran (Vo) was determined from the absorbance at 260 nm. Enzyme activity in the ordinate is expressed as µmoles of adenylosuccinate formed per min per ml.

Fig. 4. Photomicrograph of crystalline adenylosuccinate synthetase. Magnification, x 200.

Fig. 5. SDS polyacrylamide gel electrophoresis of the purified enzyme (Step VII). A portion of enzyme solution (protein, 5µg) was applied to 7.5 % polyacrylamide gel with 0.1 % SDS and electrophoresis was run as described in the text.

Fig. 6. Estimation of the molecular weight of subunits of adenylosuccinate synthetase by electrophoresis on 7.5 % poly= acrylamide gel containing 0.1 % SDS. Bovine serum albumin (BSA), ovalbumin (OVA) and chymotrypsinogen (CHY) were employed as standard markers. The closed circle indicates the position of adenylosuccinate synthetase.

Fig. 7. Ultracentrifugal pattern of crystalline adenylosuccinate synthetase. Photographs were taken 13, 21, 29 and 37 min (from left to right) after the rotor reached 52,640 rpm.

Fig. 8. Equilibrium sedimentation analysis of adenylosuccinate synthetase. The common logarithm of the fringe displacement was plotted versus the square of the distance from the axis of rotation.

Fig. 9. Determination of the molecular weight of subunits of adenylosuccinate synthetase in 6 M guanidine-HCl by sedimentation equilibrium analysis. Crystalline enzyme was dissolved in, and dialyzed for 3 days against 6 M guanidine-HCl solution and then 0.5 % solution was run for 18 h at 12,673 rpm at 20.0°. The ordinate and abscissa are represented as in Fig. 8.

Fig. 10. Optimum pH of adenylosuccinate synthetase. Assays were carried out at 37° for 10 min in 3 x 10^{-2}M Tris-HCl buffer (o) or 3 x 10^{-2}M imidazole-HCl buffer (●). The ordinate shows μmoles of product formed per min per ml.

Fig. 11. Double reciprocal plots of inhibition of adenylosuccinate synthetase by succinate. Enzyme assays were carried out in the presence of fixed concentration of succinate and various concentra= tion of succinate and various concentrations of aspartate (A), IMP (B) or GTP (C).

Fig. 12. Effect of succinate on adenylosuccinate synthetase. The vertical axis shows the percentage inhibition relative to initial velocity without succinate. The inset shows a Dixon plot of the reciprocal of V *versus* the millimolar concentration of succinate (16).

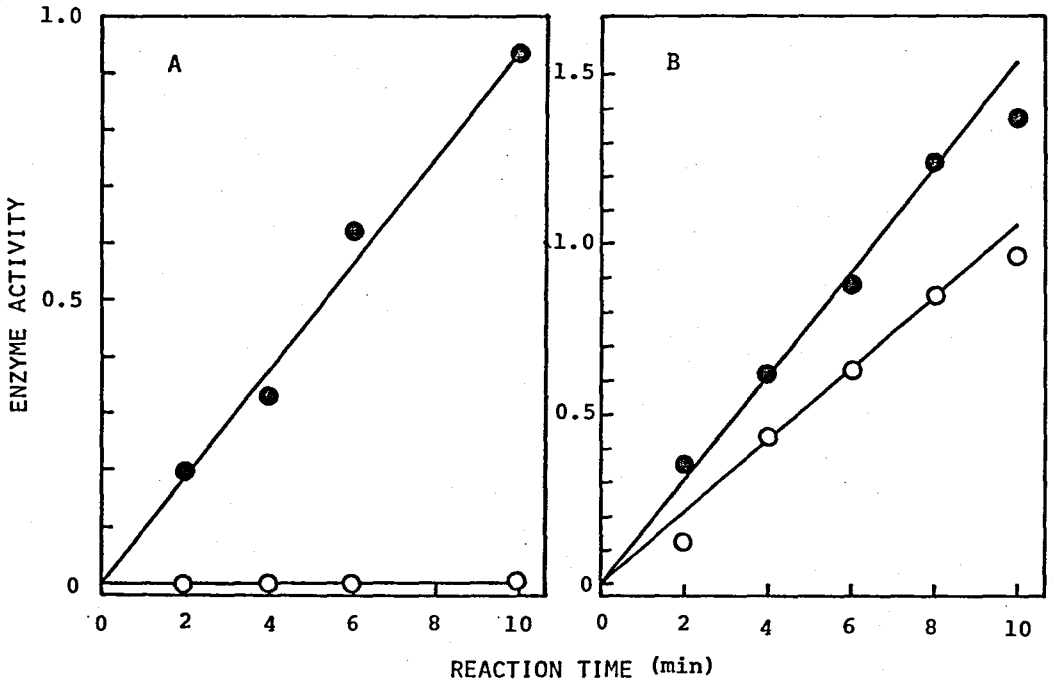
A
B
ENZYME ACTIVITY
1.0
0.5
0
1.5
1.0
0.5
0
0
2
4
6
8
10
REACTION TIME (min)

Fig. 1.

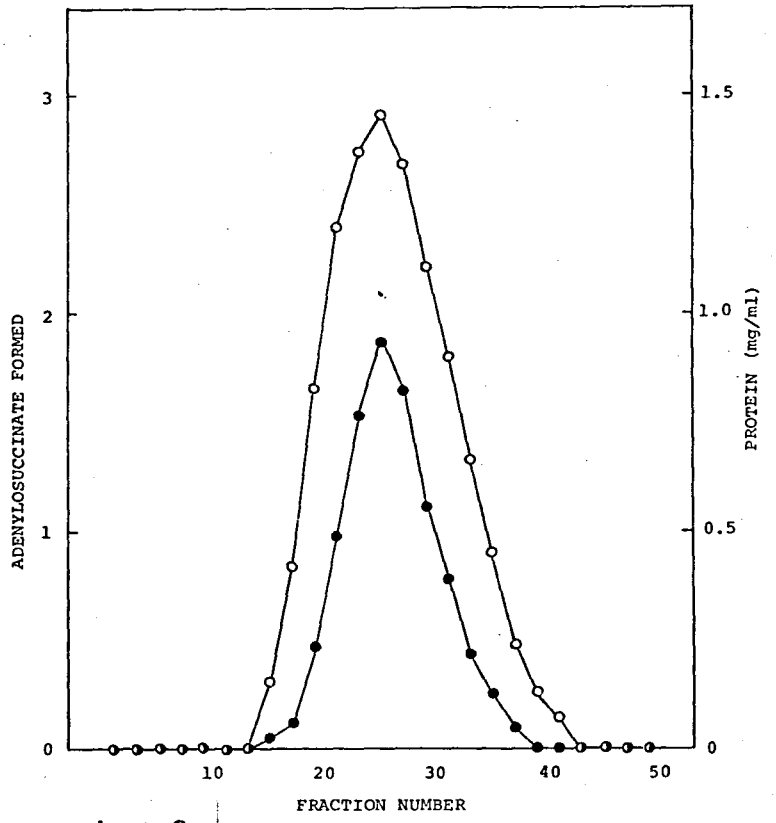
ADENYLOSUCCINATE FORMED
PROTEIN (mg/ml)
FRACTION NUMBER
0
1
2
3
0
0.5
1.0
1.5
10
20
30
40
50

Fig. 2.

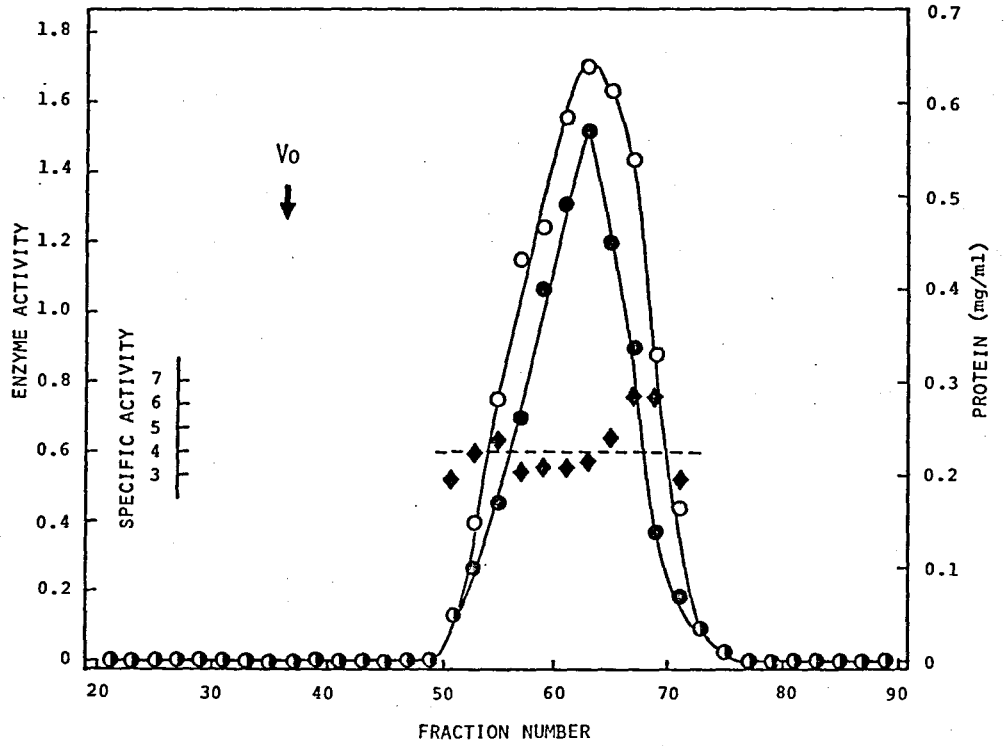
ENZYME ACTIVITY
SPECIFIC ACTIVITY
PROTEIN (mg/ml)
FRACTION NUMBER
Vo
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
3
4
5
6
7
20
30
40
50
60
70
80
90

Fig. 3.

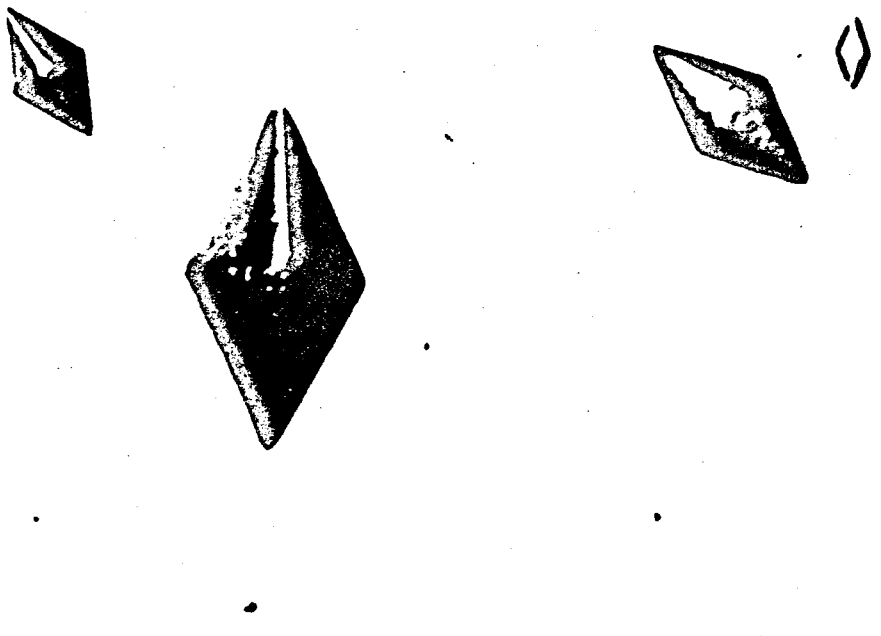

Fig. 4.

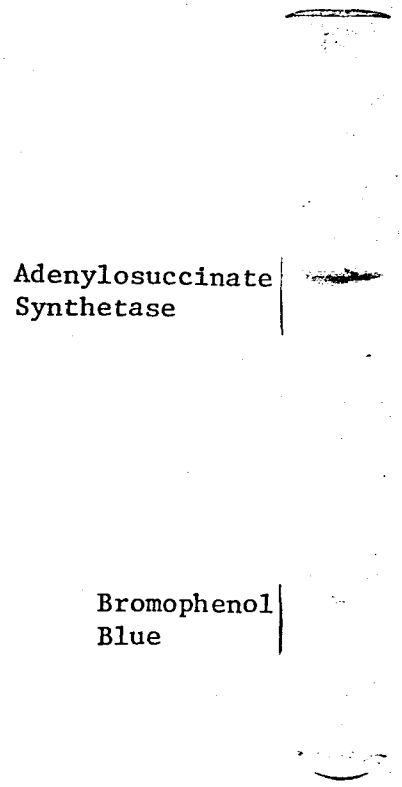
Adenylosuccinate
Synthetase
Bromophenol
Blue

Fig. 5.

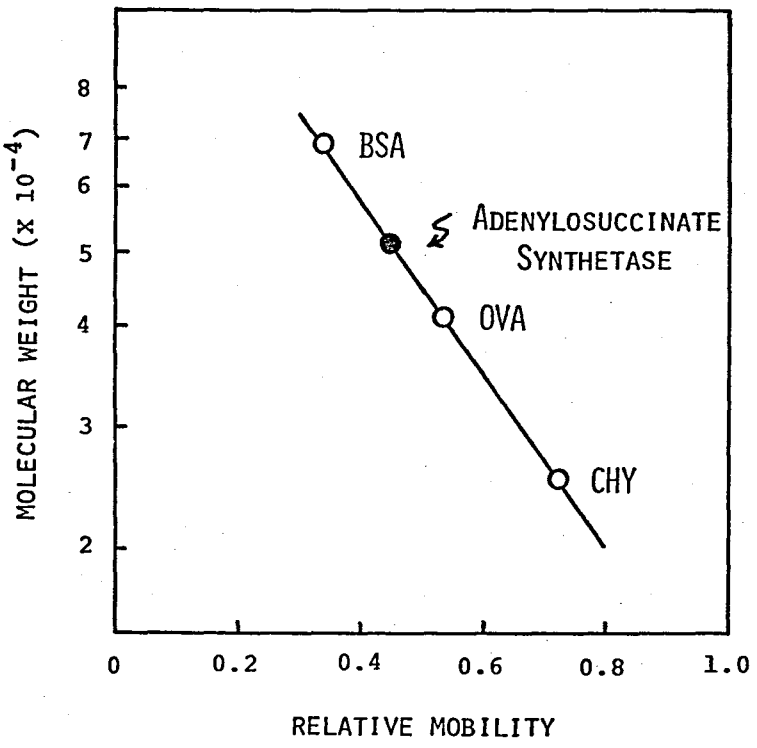
MOLECULAR WEIGHT (x 10⁻⁴)
8
7
6
5
4
3
2
BSA
ADENYLOSUCCINATE
SYNTHETASE
OVA
CHY
0
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

Fig. 6.

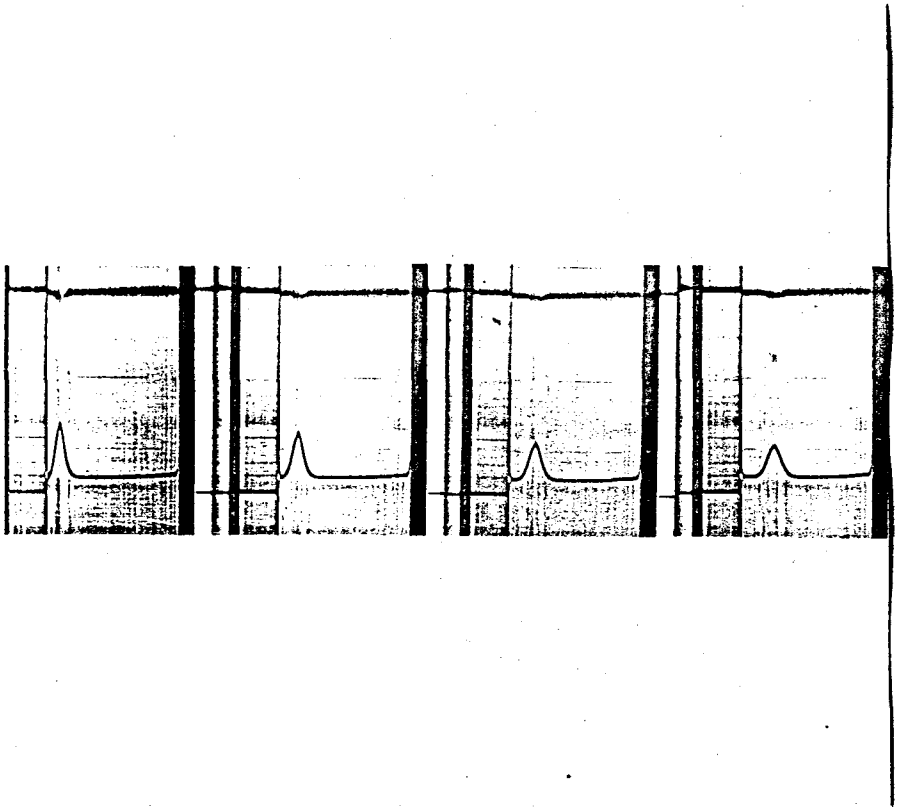

Fig. 7.

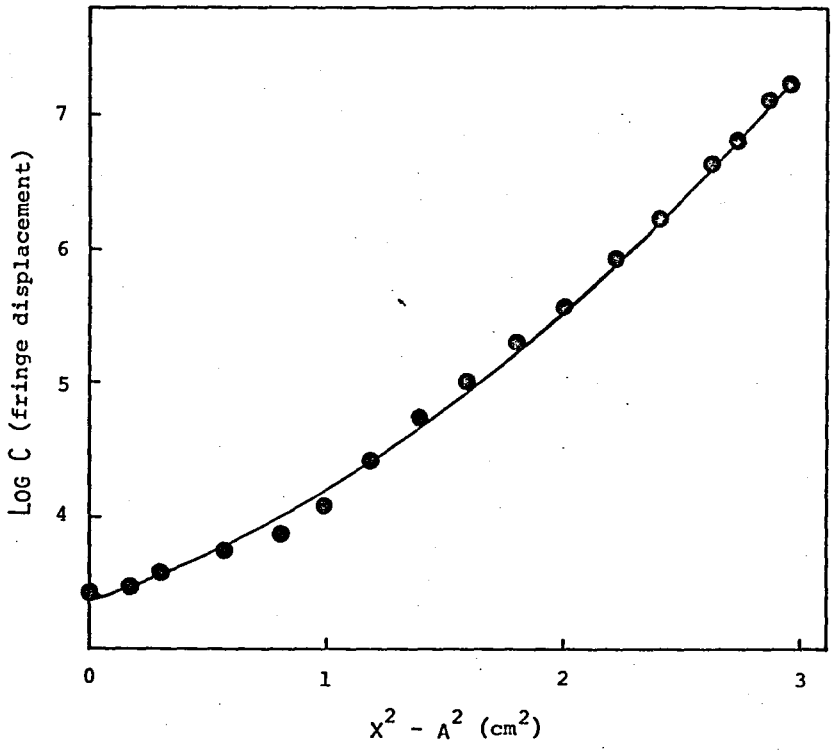
Log C (fringe displacement)
7
6
5
4
0
1
2
3
$X^2 - A^2$ (cm^2)

Fig. 8.

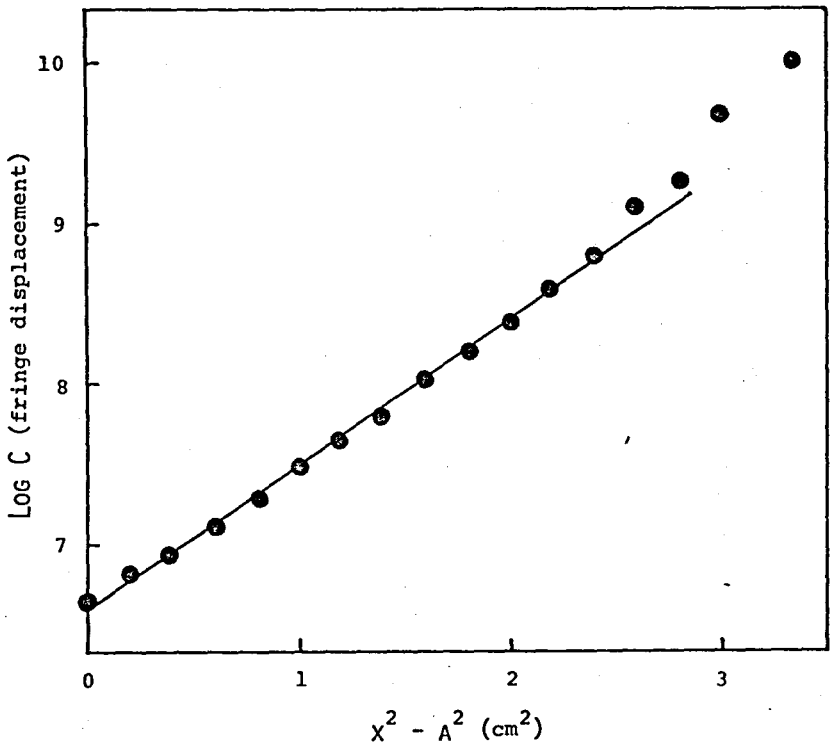
Log C (fringe displacement)
10
9
8
7
0
1
2
3
$X^2 - A^2$ (cm^2)

Fig. 9.

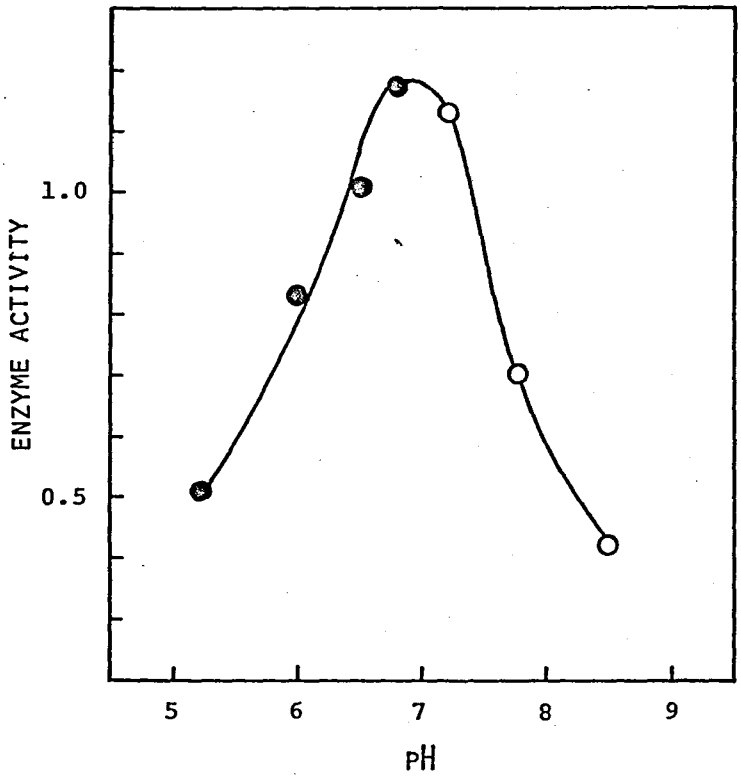

Fig. 10.

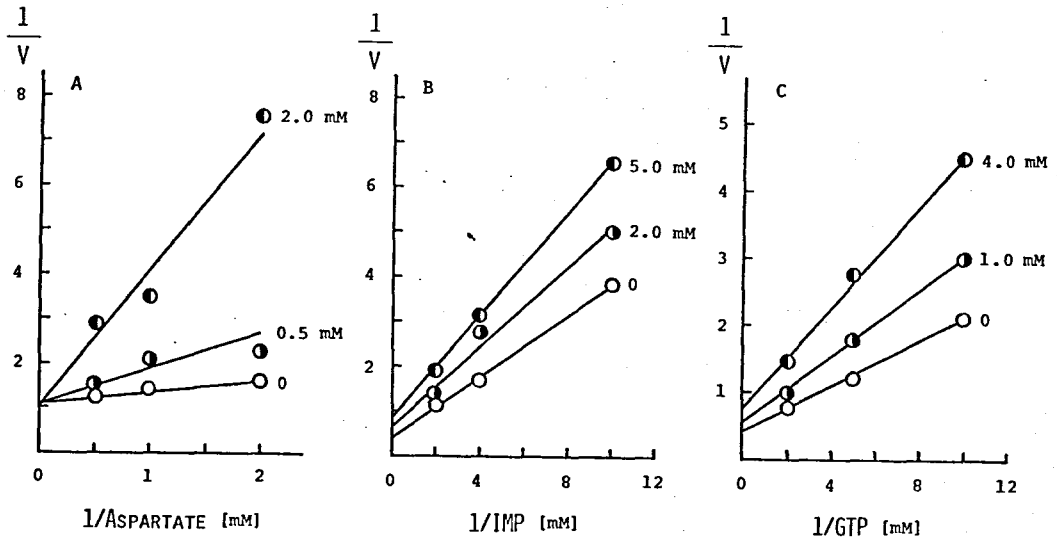
1/V
A
2.0 mM
0.5 mM
0
1/ASPARTATE [mM]
B
5.0 mM
2.0 mM
0
1/IMP [mM]
C
4.0 mM
1.0 mM
0
1/GTP [mM]

Fig. 11.

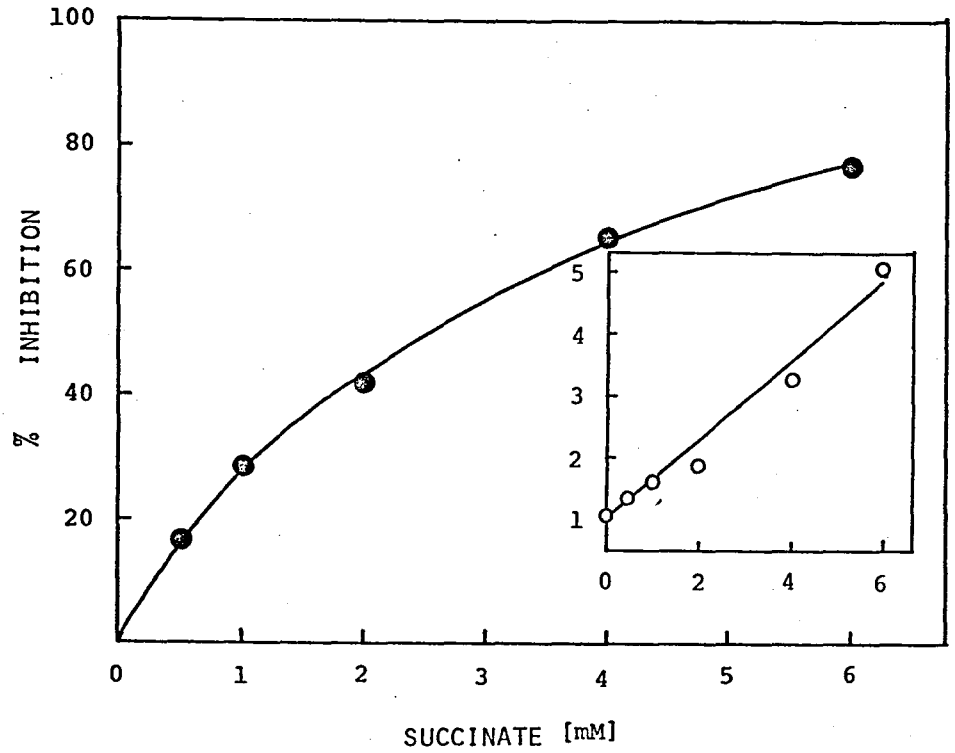
% INHIBITION
SUCCINATE [mM]

Fig. 12.

Table I. Summary of purification of adenylosuccinate synthetase

	Step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity (mu/mg protein)	Purification	Yield (%)
I	Crude extract	870	16.7	14,530	60.8	1	100
II	Heat treatment	840	10.8	9,070	85.1	1.4	87.4
III	Ammonium sulfate fractionation	165	18.5	3,050	212.8	3.5	73.5
IV	Phosphocellulose column chromatography	25	11.9	298	1,425.2	23.4	48.0
VI	Hydroxyapatite column chromatography	1.5	6.7	10	5,463.0	89.9	6.2
VII	Sephadex G-150 column chromatography	2.0	4.0	8	6,238.0	102.6	5.6

Table II. Effects of nucleotides on adenylosuccinate synthetase

Nucleotide added	%Inhibition
AMP	44.3
ADP	53.6
ATP	0
GMP	47.0
GDP	100
ITP	0
UDP	34.6
UTP	6.1
CTP	0

Nucleotides were added to the incubation system at final concentrations of 2×10^{-3} M

Table III. Effects of product analogues and urea cycle members on adenylosuccinate synthetase activity

Addition	Final conc. ($\underline{M} \times 10^3$)	Inhibition of initial velocity (%)
None		0
Adenylosuccinate	0.8	42.0
Succinate	2.0	46.1
Fumarate	2.0	0
Citrate	2.0	0
Argininosuccinate	2.0	39.0
Arginine	2.0	0
Citrulline	2.0	0
Ornithine	2.0	0
Urea	2.0	0
Carbamoylphosphate	2.0	12.4

Table IV. Effects of various salts on adenylosuccinate synthetase

Salt	Percentage of control activity
None	100
KCl	110.4
NH_4Cl	103.0
NaCl	103.5
$(NH_4)_2SO_4$	57.8
K_2SO_4	54.3

Salts were added to the incubation system at final concentrations of 10^{-2} M.

Chapter II

Study on the Regulatory Role of Fructose-1,6-Diphosphate in the Formation of AMP in Rat Skeletal Muscle
—— A Mechanism for Synchronization of Glycolysis and the Purine Nucleotide Cycle

Study on the Regulatory Role of Fructose-1,6-Diphosphate in the Formation of AMP in Rat Skeletal Muscle

——— A Mechanism for Synchronization of Glycolysis and the Purine Nucleotide Cycle

Summary: Fructose-1,6-diphosphate strongly inhibited adenylosuccinate synthetase purified from rat skeletal muscle. This compound was found to be a non-competitive inhibitor of all substrates of the enzyme. No other glycolytic intermediates affected adenylosuccinate synthetase activity. From these findings, it was proposed that this inhibition might play an important role in the oscillation of gylcolysis in skeletal muscle.

INTRODUCTION

Ghosh and Chance (1) first observed a reciprocal oscillation between the concentrations of fructose-6-phophate and fructose-1,6-diphosphate during glycolysis in yeast cells. Later Frenkel (2) reported a similar glycolytic oscillation in beef heart extracts depending on modifiers of the activity of phosphofructokinase [EC 2.7.1.11], a rate-limiting enzyme of glycolysis. Recently, Tornheim and Lowenstein (3) reported that glycolytic oscillation occurred on addition of glucose to particle-free extracts of rat skeletal muscle and suggested that the purine nucleotide cycle (IMP → adenylosuccinate → AMP → IMP) might be closely linked to this oscillation, because the concentration of AMP, an activator of phosphofructokinase, also fluctuated synchronously with oscillation of glycolytic intermediates. Therefore, we tested whether glycolytic intermediates played a critical role in regulation of the AMP concentration.

This paper reports the inhibitory effect of fructose-1,6-diphosphate on adenylosuccinate synthetase (IMP: L-aspartate ligase (GDP), [EC 6.3.4.4]), a member of the purine nucleotide cycle, and discussion of its physiological significance.

MATERIALS AND METHODS

Male Wistar HLA strain rats were used throughout. Adenylosuccinate synthetase was assayed in reaction mixture containing in 250 µl, IMP (1mM), GTP (0.5 mM), L-aspartate (4 mM), imidazole-HCl buffer, pH 6.8 (30 mM), $MgCl_2$ (8 mM) and enzyme solution. The reaction was initiated by addition of aspartate and terminated by addition of perchloric acid after incubation for 5 minutes at 37°. The adenylosuccinate formed in the supernatant was determined by the method of Lieberman (4). Adenylosuccinase (adenylosuccinate AMP-lyase, [EC 4.3.2.2]) and AMP deaminase (AMP aminohydrase, [EC 3.5.4.6]) were assayed by the methods of Carter and Cohen (5) and Smiley et al. (6), respectively. Protein was determined by the method of Lowry et al. (7) with bovine serum albumin as a standard.

Adenylosuccinate synthetase from rat skeletal muscle was purified by the method of Ogawa et al. (8). The purified preparation formed a single band on SDS-polyacrylamide gel electrophoresis. AMP deaminase was crystallized by a modification of the method of Smiley et al. (6). Adenylosuccinase was partially purified by phosphocellulose column chromatography.

IMP and GTP were purchased from Kyowa Hakko (Tokyo). All glycolytic intermediates used were products of Sigma (U.S.A.) except glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-diphosphate which were from Boehringer (Germany).

RESULTS AND DISCUSSION

The effects of glycolytic intermediates on adenylosuccinate synthetase are shown in Table 1. Fructose-1,6-diphosphate was found to be the most potent inhibitor of the intermediates tested.

Triose phosphate seemed to be slightly inhibitory, but inorganic ortho-phosphate was as inhibitory at the same concentration (data not shown). This suggests that triose phosphates may not act as specific inhibitors of the enzyme.

The dose-response curve of the effects of fructose-1,6-di= phosphate on enzyme activity is shown in Fig. 1. The minimal inhibitory concentration of fructose-1,6-diphosphate was 10 μM and 2 mM fructose-1,6-diphosphate caused almost complete inhibition.

The concentration of fructose-1,6-diphosphate in rat skeletal muscle at the crest of glycolytic oscillation has not been accurately determined yet, but the concentration in resting muscle was reported to be 40 to 60 μM (9-10). Thus it is quite possible that fructose-1,6-diphosphate participates in regulation of adenylosuccinate synthetase activity in vivo. Neither fructose-1,6-diphosphate nor other glycolytic intermediates affected the adenylosuccinase and AMP deaminase activities of rat skeletal muscle.

As shown in Fig. 2, the double reciprocal plots of the initial rates with fructose-1,6-diphosphate and aspartate, IMP, and GTP indicate that fructose-1,6-diphosphate is a non-competitive inhibitor with all these substrates.

It is well known that nucleotides, such as AMP and ADP, and glycolytic intermediates, such as fructose-1,6-diphosphate and fructose-6-phosphate, activate phosphofructokinase, whereas ATP is inhibitory. According to Frenkel (2) and Tornheim and Lowenstein (3), the levels of AMP and ADP were highest when that of ATP was lowest during the glycolytic oscillation. Tornheim and Lowenstein (11) also observed that the activating effect of

fructose-1,6-diphosphate on phosphofructokinase depended strongly on the presence of AMP. These findings explain the sudden increase in phosphofructokinase activity. However, this mechanism cannot explain the oscillatory fluctuation of phosphofructokinase activity, because when once the enzyme activity has increased it does not return to the previous level while the concentration of AMP remains high.

Our findings give a clue to this problem, since they show that the accumulation of fructose-1,6-diphosphate inhibits adenylosuccinate synthetase, reducing AMP formation. ATP might also participate in this mechanism as an allosteric effector of AMP deaminase. When fructose-1,6-diphosphate is degraded by fructose diphosphate aldolase [EC 4.1.2.13], the inhibition of adenylosuccinate synthetase is relieved. Accordingly the inhibitory effect of fructose-1,6-diphosphate on adnylosuccinate synthetase seems to be critical for oscillation of glycolysis.

REFERENCES

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4. Lieberman, I., J. Biol. Chem., 222, 17 (1956)
5. Carter, C. E., & Cohen, L. H., J. Biol. Chem., 222, 17 (1956)
6. Smiley, Jr., K. L., Berry, A. J., & Suelter, C. H., J. Biol. Chem., 242, 2502 (1967)
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9. Dale, R. A., J. Physiol., 181, 701 (1965)
10. Edington, D. W., Ward, G. R., & Saville, W. A., Amer. J. Physiol., 224, 1375 (1973)
11. Tornheim, K., & Lowenstein, J. M., J. Biol. Chem., 250, 6304 (1975)

Fig. 1. Effects of various concentration of fructose-1,6-diphosphate (FDP) on adenylosuccinate synthetase activity. The amount of enzyme used was the same as that given in the legend to Table I.

Fig. 2. Double reciprocal plots of the initial velocities of adenylosuccinate synthetase with various substrate concentrations in the presence of a fixed concentration.

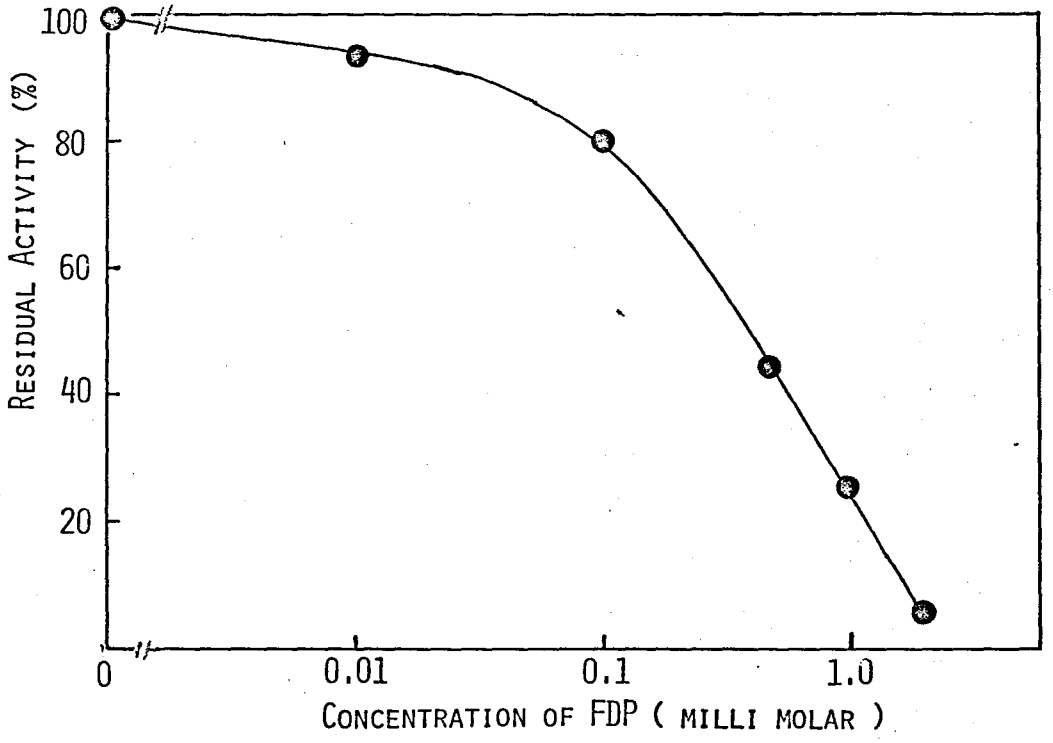

100
80
60
40
20
RESIDUAL ACTIVITY (%)
0
0.01
0.1
1.0
CONCENTRATION OF FDP (MILLI MOLAR)

Fig. 1.

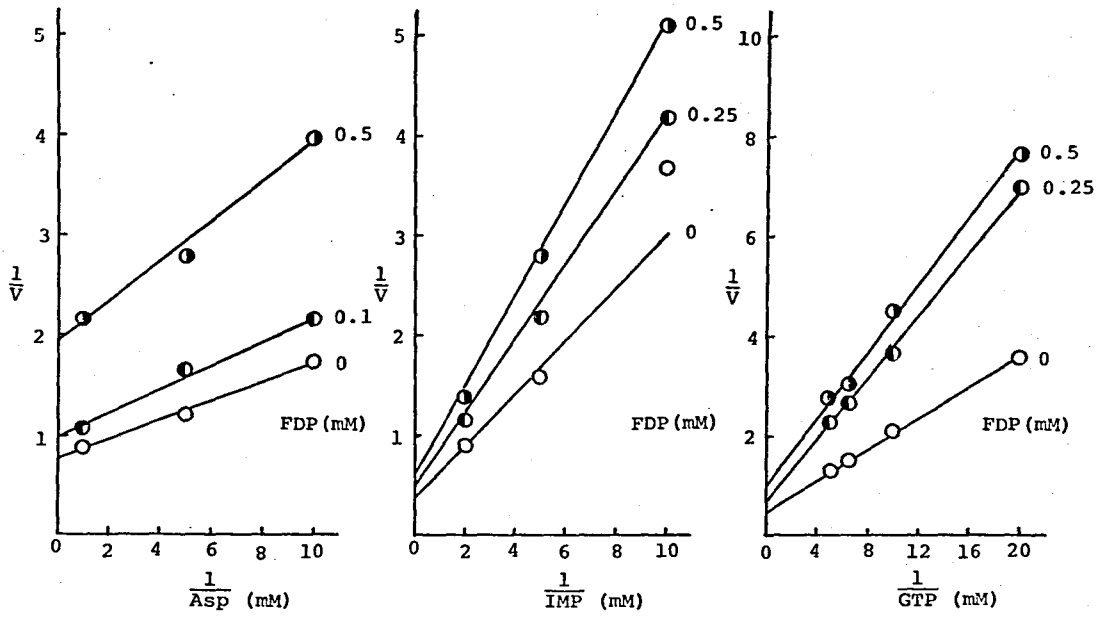

1/V
0.5
0.1
0
FDP (mM)
1/Asp (mM)
0.5
0.25
0
FDP (mM)
1/IMP (mM)
0.5
0.25
0
FDP (mM)
1/GTP (mM)

Fig. 2.

Table I. Effects of glycolytic intermediates on adenylosuccinate synthetase activity

Addition	inhibition (%)
Glucose	5
Glucose-6-phosphate	2
Fructose-6-phosphate	8
Fructose-1,6-diphosphate	80
Dihydroxyacetone phosphate	3
Glyceraldehyde-3-phosphate	0
2,3-Diphosphoglycerate	18
3-Phosphoglycerate	17
2-Phosphoglycerate	16
Phosphoenolpyruvate	16
Pyruvate	0
Lactate	6

Enzyme activity was determined in the presence of 2 mM of each glycolytic intermediate. The results are expressed as percentage inhibitions of the activity without glycolytic intermediates. Each reaction system contained 16 munits of enzyme. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmole of adenylosuccinate per mg protein.

Chapter III

Distributions of Enzymes Involved in the Purine Nucleotide Cycle and Isozymes of Adenylosuccinate Synthetase in Various Organs in Rat

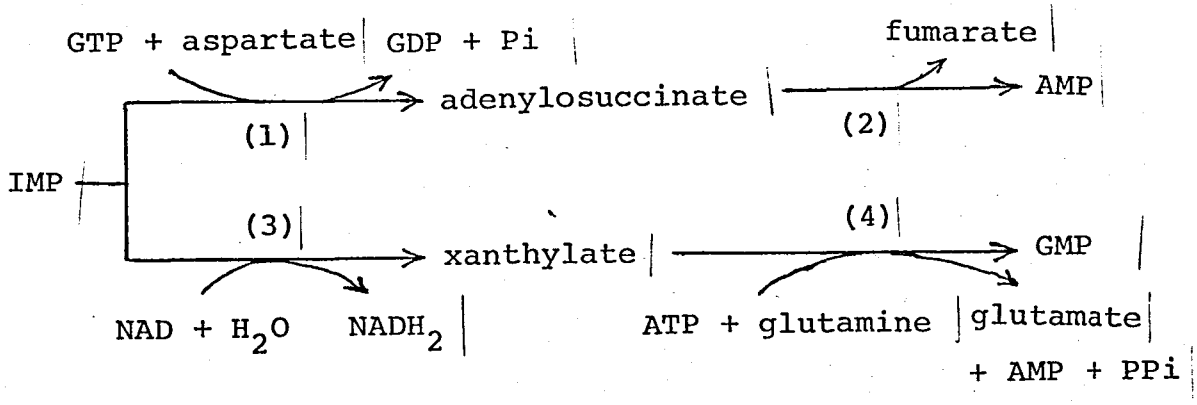
Distributions of Enzymes Involved in the Purine Nucleotide Cycle and Isozymes of Adenylosuccinate Synthetase in Various Organs in Rat

Summary: The distributions of adenylosuccinate synthetase and adenylosuccinase were determined in various organs. Both enzyme activities were highest in the skeletal muscle together with AMP deaminase activity. Two isozymes were found in adenylosuccinate synthetase, one enzyme found in the skeletal and cardiac muscles with isoelectric point of pH 8.8, while the other found in other non-contractile organs with isoelectric poin of pH 6.0.

INTRODUCTION

In 1955, Arams and Bently found the activity of adenylo= succinate synthetase catalyzing the conversion of IMP to adenylo= succinate in rabbit bone marrow extracts (1). Since then several investigators have been interested in this enzyme (2-6), because it resides in the branch point of main pathway of purine nucleotide synthesis together with IMP dehydrogenase [EC 2.4.2.14] catalyzing the formation from IMP to xanthylate. In the former reaction (1), AMP is formed at the expenses of GTP as an energy source and of aspartate as a nitrogen donor. In the latter reaction xanthylate is formed and then converted to GMP by GMP synthetase [EC 6.3.4.1]. In those two sequential reactions ATP and glutamine are employed as an energy source and a nitrogen donor, respectively. It should be noteworthy that the formation of AMP from IMP is dependent on GTP, while that of GMP from IMP is dependent on ATP. This cross-dependency of energy source may play an important role in the control mechanism for the maintenance

of the balance of synthesis of both adenine and guanine nucleotides;



As previously stated, Tornheim and Lowenstein (7) showed that adenylosuccinate synthetase together with adenylosuccinase [EC 4.3.2.2] (reaction 2) and AMP deaminase [EC 3.5.4.6] played an critical role in ammonia liberation from the skeletal muscle in higher animals.

Then questions arise as to whether one enzyme is involved in both nucleotide synthesis and ammonia metabolism, and if so, one enzyme is controlled to elicit such dual functions.

In the previous chapter, it was shown that adenylosuccinate synthetase obtained from the skeletal muscle had an isoelectric point (I.P.) of pH 8.8 (type M), while that of other organs had an I.P. of pH 6.0 (type L). In this chapter, distributions of enzymes involved in the cycle was examined in the various organs. On the basis of these findings type M and type L isozymes were compared to explore their physiological meanings.

MATERIALS AND METHODS

Animals: Male Wistar strain rat, weighing 300 to 450 g was used for chromatographic studies of adenylosuccinate synthetase. Rat was decapitated and tissues were stored at -70°C until used.

Tumors: Walker carcinosarcoma and Yoshida sarcoma were gifts from Shionogi Pharmaceutical Co. (Osaka), and transplanted into the neck of male Wistar rat or into the abdomen of Donryu strain rat by 4 weeks after birth, respectively. The former was killed 10 days and the latter 6 to 7 days after transplantation of the tumor. The tissue or cells were processed and used for experiments immediately after the animals were sacrificed.

Biochemicals: Biochemical reagents and materials employed were the same as described in Chapter I. Protein was determined by the microbuiret method (8).

Enzyme assay: Assays for enzymes involved in the purine nucleotide cycle were described in Chapters I and II.

Isoelectric focussing: Isoelectric focussing was carried out by the method by Matsuo et al. (9). Each sample eluted from a column of DEAE-cellulose or phosphocellulose was concentrated with $(NH_4)_2SO_4$, desalted, and applied on a column (120 ml volume) with a linear gradient of 0 to 50 % (w/v) sucrose containing 0.75 ml and 2.25 ml of 40 % ampholine (pH range from 3.5 to 10, a product of LKB, Sweeden), respectively, and it was subjected to electrophoresis for 40 hours at 700 volts. After running, 40 drops were collected into each test tube and pH of each fraction was measured on ice bath with a pH meter.

RESULTS AND DISCUSSION

Distributions of Enzymes Involved in the Purine Nucleotide Cycle——Distribution of adenylosuccinate synthetase in various organs of rat is shown in Table I. The enzyme activity is expressed by units per g fresh tissue or mg protein. As expected (12), the value was strikingly high in the skeletal muscle, whereas those were generally low in other organs. Among tissues tested, the brain showed the minimum activity. As shown in Table II, adenylosuccinase activity was rather widely distributed and higher than adenylosuccinate synthetase activity, but the enzyme activity was the lowest in the kidney notwithstanding that the synthetase showed the second highest activity. Noteworthy is that the brain shows high specific activity. In combination of these findings, the reason why adenylosuccinate synthetase activity in the brain is almost undetectable may result from degradation of the product, adenylosuccinate, by adenylosuccinase. This is also supported by the fact that the synthetase activity in brain extract increased from 0.7 to 3.9 nmoles per mg protein 24 hours after preparation of the extract because of extreme lability of adenylosuccinase. The specific inhibitors for the synthetase was not so far detected in the crude extracts.

It is well known that skeletal muscle of rat is consisted of white and red muscle (and strictly, its intermediate muscle). Then the activities of enzymes were compared in red and white muscles of quadriceps of behind leg and it was found that adnylosuccinate synthetase and adenylosuccinase in red muscle were

26.3 and 38.5 % higher than those of white muscle, respectively, though AMP deaminase in both muscles was not quantitatively different (Table III). Taken the abundancy of AMP deaminase in the muscle into consideration, the purine nucleotide cycle seems to highly organized in the muscle. It also seems likely that the cylce works more actively in red muscle than in white muscle.

Recently Moss and McGivan reported the presence of the cycle in the liver cytosol (4). Lowenstein presented evidences of the cycle in the brain (10) and the kidney extract (personal communi= cation). However, the efficiency of the cycle in these organs are presumed to be far less than that in the muscles.

Raggi et al. (11) reported AMP deaminase in red and white muscle is different in chromtographic behavior on phosphocellulose. It was also reported that AMP deaminases independently isolated from red and white muscles showed a slightly different elution profile on phosphocellulose column chromatography. The features became more distinct in AMP deaminase from rabbit muscle (11). Lactate dehydrogenase (14), phosphorylase kinase (15) and myosin (16) are also known to be qualitatively different between red and white muscles. It was shown, however, that the synthetase behaved as one form either on phosphocellulose or DEAE-cellulose chromato= graphy (see Chapter I) or isoelectric focussing as described later. Moreover, it was confirmed that there was no qualitative difference in the synthetase from red and white muscle on isoelectric focussing profile, even if extracts were prepared separately from two muscles.

Dual Forms of Adenylosuccinate Synthetase——Chromatographic profiles of adenylosuccinate synthetase from various organs were compared. High-speed supernatant of the extract obtained from the skeletal muscle, the heart, the liver, the kidney, the testis, and the tumors such as Walker carcinosacoma and Yoshida ascites cells were dialyzed overnight against 100 volumes of 10 mM Tris-HCl buffer pH 7.2, and the dialysate was adjusted to pH 8.5 with 1 M Tris and then applied on a column of DEAE-cellulose. The enzyme activities from the liver, the kidney, the testis, and the tumors were retained on DEAE-cellulose and eluted with Tris buffer containing 300 mM KCl. In contrast, the activities of the muscle and the heart did not adsorb on DEAE-cellulose, but adsorbed on phosphocellulose and eluted from it with potassium phosphate buffer (10 mM, pH 7.0) containing 500 mM KCl. Each concentrated active fraction eluted from DEAE-cellulose or phosphocellulose column was applied to an ampholine column and then subjected to electrophoresis . The results are presented in Figs. 1 and 2. Isoelectric point (I.P.) of adenylosuccinate synthetase in the muscle and the heart was determined to be pH 8.8 and that of the liver and Yoshida ascites tumor cells, pH 6.0. From these data, two synthetases can be classified; one is a basic protein contained in contractile tissues (type M) and the other is a weak acidic protein contained in non-contractile tissues (type L).

Kinetic properties of type M and type L enzymes are shown in Table IV. The enzyme of the liver was partially purified by the following procedures; 1. $(NH_4)_2SO_4$ fractionation (40 to 70 % saturation), 2. chromatography on DEAE-cellulose (DE 52) (elution with a linear gradient of KCl from 0 to 300 mM at pH 7.2),

3. isoelectric focussing (pH 3.5-10 ampholine). The preparation thus obtained did not include the interfering enzymes, though it contained much impure materials yet.

Km values of two isozymes for IMP and GTP were almost the same, but Km value for aspartate was about two times higher in the liver enzyme than the muscle enzyme. As previously mentioned, we found that FDP was a potent inhibitor of the muscle enzyme. The inhibition of the liver enzyme by FDP was also observed. So far as examined, however, the liver enzyme tends to be less sensitive to substrates or inhibitor than the muscle enzyme.

De novo synthesis of purine nucleotides is started from the synthesis of 5-phosphoribosyl-1-pyrophosphate and 12 reaction steps are included up to formation of AMP and GMP. However, only several studies have been performed on enzymes catalyzing these reactions. Accordingly almost no report on isozymes involved in the purine nucleotide synthesis has so far been presented. In this sense, the discovery of the isozymes of adenylosuccinate synthetase in contractile and non-contractile tissues might give us a clue to solve the dual control mechanism of the enzyme. The fact that rapid proliferating cells such as ascites tumor has type L suggests that type L enzyme may be responsible for key enzyme of the main pathway of purine nucleotide synthesis. As stated previously, Moss and McGivan reported that ammonia was produced from amino acids via aspartate even in liver extract, though Lowenstein doubted that the purine nucleotide cycle was operated in the liver because of low activity of AMP deaminase (see Preface).

If this is the case, previous hypothesis that type L and type M isozymes are responsible for the purine nucleotide synthesis and ammonia poduction, respectively, becomes invalidated. Doesn't the previous findings that the inhibition kinetics of the liver enzyme is different from that of the muscle enzyme have any physiological meaning then ? This problem must await further investigations.

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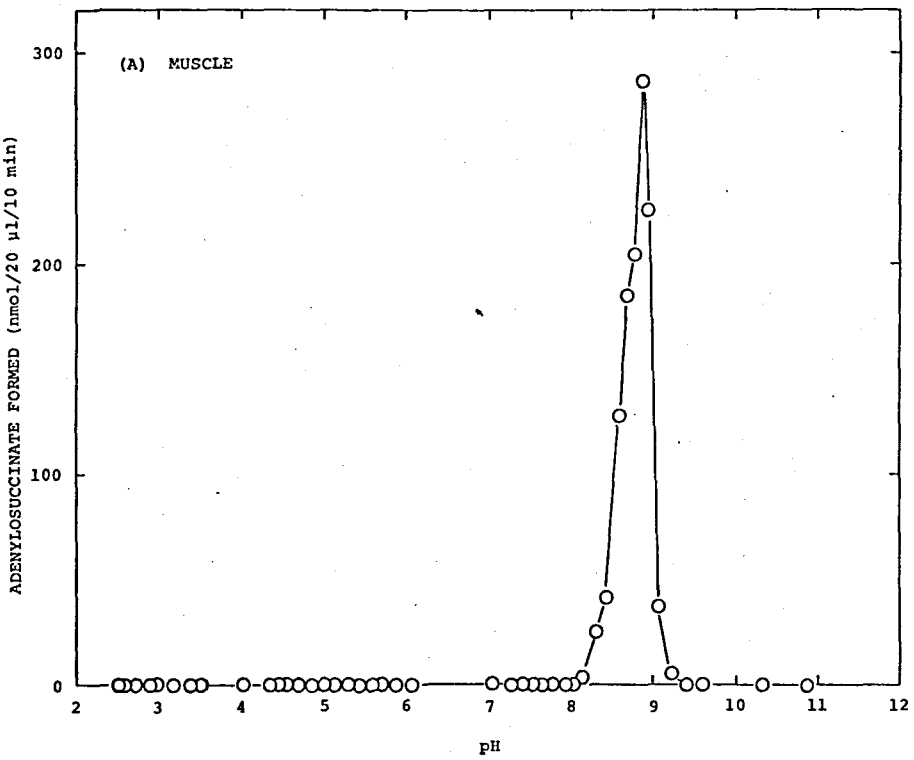
Fig. 1. Isoelectric focussing profiles of adenylosuccinate synthetase of the muscle and the heart. Active fractions eluted from a column of phosphocellulose were collected and concentrated with $(NH_4)_2SO_4$ (40 and 70 % saturated fraction). The precipitate was dissolved in small volume of 5 mM potassium phosphate buffer, pH 7.0 containing 0.2 mM DTT and diaylzed against the same buffer. The dialyzed preparation was applied on a column of 120 ml volume as described in materials and methods. The initial current was usually 20 mA and the final current was 1 mA. The enzyme activity is indicated as (o).
(A); Pattern of the enzyme obtained from the whole muscle of 30 g.
(B); Pattern of the enzyme obtained from the hearts of 10 rats.

Fig. 2. Isoelectric focussing profiles of adenylosuccinate synthetase of the liver and tumor.
(A); The supernatant of liver extract obtained by high-speed centrifugation was desalted and applied on a DEAE-cellulose column (2 x 20 cm) and then eluted with 300 mM KCl as described in the text. Active fractions were treated as shown in the legend to Fig. 1.
(B); Yoshida ascites tumor cells were collected and washed once with 5 volumes of 50 mM Tris-HCl, pH 7.2 containing 0.25 M sucrose and 0.2 mM DTT. The pellets (10 g) were suspended in the same buffer and sonicated with a Branson sonifier at a maximal power (70 W) for 15 sec and the suspension was centrifuged at 100,000 g for 60 min. The supernatant fraction was dialyzed overnight against 10 mM Tris-HCl, pH 7.2. After the dialysate was adjusted to pH 8.5 with 1 M Tris, it was applied on a DEAE-cellulose cloumn (2 x 20 cm) equilibrated with 10 mM Tris-HCl, pH 8.5 containing 0.2 mM DTT (the buffer), washed with the same buffer, and then eluted with the buffer containing a linear gradient of 0 to 300 mM KCl. Subsequent procedures were the same as (A). The enzyme activity is expressed as (o).


(A) MUSCLE
ADENYLOSUCCINATE FORMED (nmol/20 µl/10 min)
300
200
100
0
2
3
4
5
6
7
8
9
10
11
12
pH

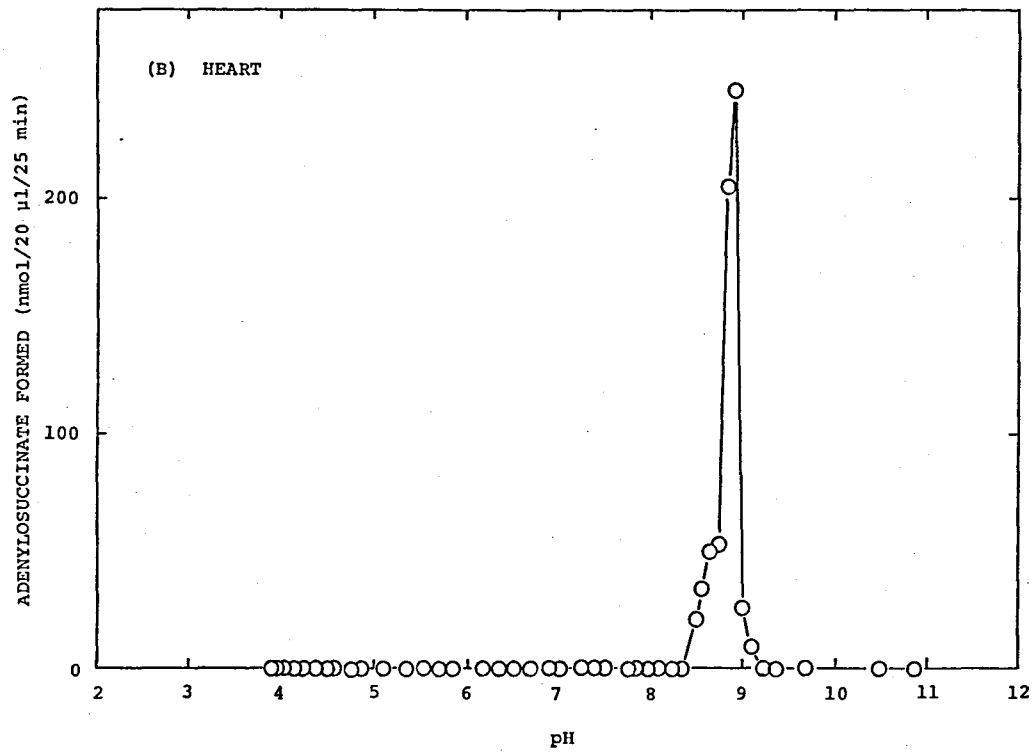
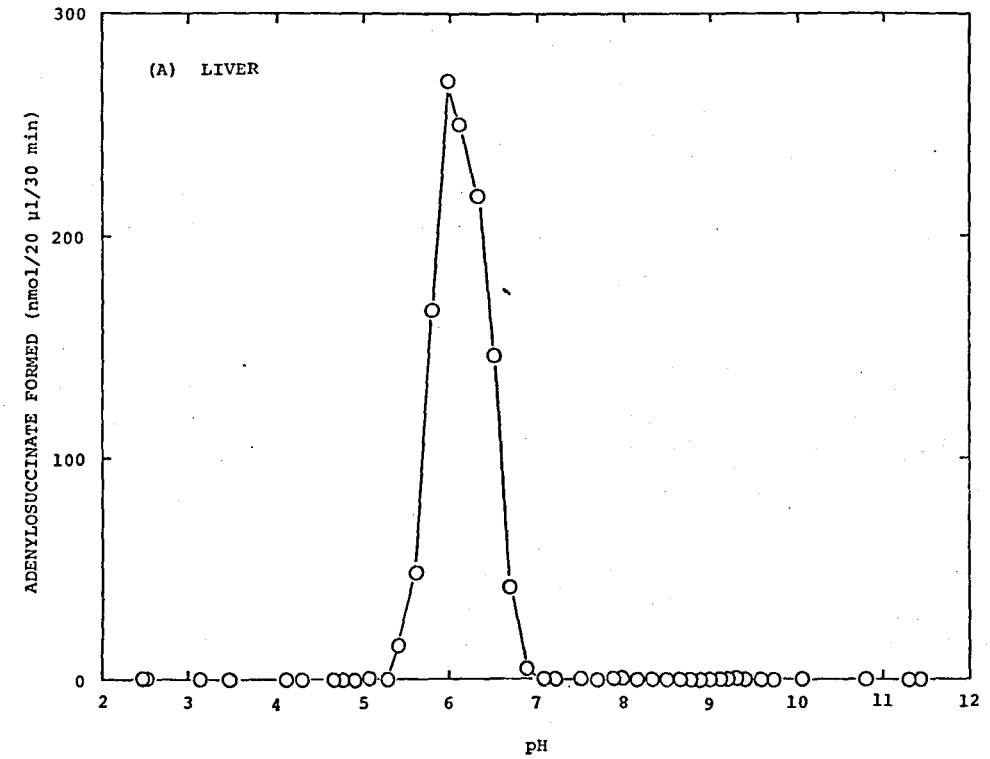

(B) HEART
ADENYLOSUCCINATE FORMED (nmol/20 µl/25 min)
200
100
0
2
3
4
5
6
7
8
9
10
11
12
pH

Fig. 1.


(A) LIVER
ADENYLOSUCCINATE FORMED (nmol/20 µl/30 min)
300
200
100
0
2
3
4
5
6
7
8
9
10
11
12
pH

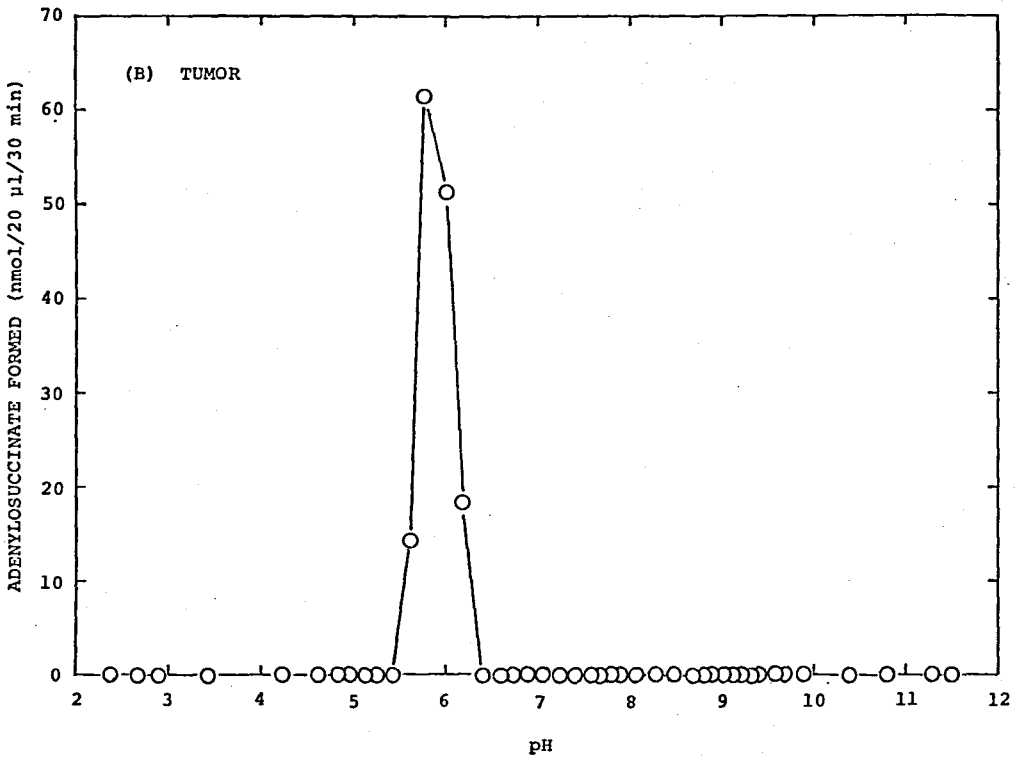

(B) TUMOR
ADENYLOSUCCINATE FORMED (nmol/20 µl/30 min)
70
60
50
40
30
20
10
0
2
3
4
5
6
7
8
9
10
11
12
pH

Fig. 2.

Table I. Adenylosuccinate synthetase activity in various rat organs

Organs	nmoles per g wet tissue per min	nmoles per mg protein per min
Muscle	1366.9 ± 157.4	42.5 ± 7.4
Kidney	284.0 ± 58.1	5.7 ± 1.5
Liver	267.7 ± 16.5	3.7 ± 0.6
Spleen	194.0 ± 51.4	3.2 ± 1.0
Heart	185.7 ± 15.3	4.6 ± 0.7
Lung	101.0 ± 40.1	1.8 ± 0.6
Testis	68.4 ± 27.2	2.0 ± 1.8
Brain	14.4 ± 7.6	0.7 ± 0.4
Tumor	156.9	1.5

The skeletal muscle and the heart were blended with a Homomixer (Nihon Seiki) for 1 min with 3 vol of 50 mM Tris-HCl, pH 7.2 containing 0.2 mM dithiothreitol. Other organs were homogenized with a Potter-Elvehjem homogenizer with 10 strokes with the same buffer. The homogenate of Yoshida ascites tumor cells was prepared as described in the legend to Fig. 2 (B). Each homogenate was centrifuged at 100,000 g for 60 min. An aliquot of 20 µl of the supernatant was used for enzyme assay. The reaction was initiated by adding enzyme and terminated by adding 30 % perchloric acid 20 min after incubation at 37°. In case of muscle enzyme assay, the incubation time was shorten to 5 min. Then the absorbance of the supernatant after removal of denatured materials was measured at 280 nm. The detail of the assay method and definition of unit of enzyme activity were described in materials and methods of Chapter I. The value is expressed as a means ± standard deviation of means of three rats except tumor.

Table II. Adenylosuccinase activity in various rat organs

Organs	nmoles per g wet tissue per min	nmoles per mg protein per min
Muscle	1737.1 ± 270.8	53.0 ± 4.9
Heart	1138.5 ± 104.5	27.8 ± 4.3
Liver	857.9 ± 68.3	12.0 ± 4.3
Spleen	808.1 ± 90.8	13.0 ± 2.2
Brain	444.0 ± 83.5	20.1 ± 4.8
Lung	292.1 ± 40.6	5.4 ± 1.2
Testis	248.1 ± 140.3	8.1 ± 4.4
Kidney	162.5 ± 76.1	3.2 ± 1.5
Tumor	1124.9	10.6

The sample used were the same as given in Table I. Twenty µl of the supernatant except muscle and liver (10 µl) were used for adenylosuccinase assay. The reaction mixture was incubated for 20 min at 37° and stopped by adding 30 % perchloric acid. The absorbance of the supernatant after removal of denatured materials was read at 280 nm. Unit of enzyme was defined as described by Carter and Cohen (17). The values in the table is the same as given in the legend to Table I except tumor.

Table III. Specific activities of the enzymes involved in the purine nucleotide cycle in red and white quadriceps.

	skeletal muscle	
	red	white
adenylosuccinate synthetase	38.9 ± 2.8	30.8 ± 4.7
adenylosuccinase	55.4 ± 5.0	40.0 ± 6.2
AMP deaminase	2700 ± 520	2800 ± 173

The red and white portions of quadriceps of behind legs were separated and 2.5 g of each were subjected to preparation of crude extract (the method is given in the legend to Fig. 1). AMP deaminase was assayed by the method of Smiley et al. (18). The enzyme activity is represented as nmoles of product formed per min per mg protein and the values are expressed as a means ± standard deviation of means of three rats.

Table IV. Comparison of kinetical properties of adenylosuccinate synthetase from the muscle (type M enzyme) and the liver (type L enzyme)

	MUSCLE	LIVER
Km for IMP (mM)	0.7	0.5
Km for aspartate (mM)	0.3	0.7
Km for GTP (mM)	0.4	0.4
Ki for FDP (mM)	0.4	0.9
Ki for succinate (mM)	1.9	N.D.
Optimum pH	6.8	6.8

The assay conditions were as mentioned in Chapters I and II.
N.D.= not determined

Chapter IV

Characterizations of Adenylosuccinase in Rat Organs

Characterizations of Adenylosuccinase in Rat Organs

Summary

1. Heat treatment for 1.5 min at 55° C was found to be effective for stabilization of adenylosuccinase activity in crude extract obtained from the skeletal muscle. Glycerol and sucrose also protected the enzyme against inactivation by salts.
2. The enzyme was partially purified by phosphocellulose and DEAE-cellulose chromatographies.
3. Adenylosuccinse, irrespective of crude extract or partially purified one, was inhibited by Zn^{++}, Cd^{++}, Cu^{++}, and Hg^{++} at the concentrations of 100 μM. The inhibition was fully restored by addition of excess EDTA or SH reagents. These suggest that SH group is prerequisite to catalytic activity of the enzyme. In line with these findings, it was shown that the operation of the purine nucleotide cycle was suppressed by adding these metals.
4. Ampholine-isoelectrofocussing technique revealed that I.P. of the enzyme was nearly neutral either in the skeletal, the cardiac muscle or the liver. A minor peak was occasionally detected in the neighborhood of acidic area.

INTRODUCTION

According to working hypothesis proposed by Lowenstein (1), adenylosuccinase [EC 4.3.2.2], catalyzing the conversion of adenylosuccinate to AMP and fumarate, as a member of the purine nucleotide cycle plays, an important role in release of ammonia from the excersized muscle.

It was reported that the enzyme catalyzed the cleavage of 5-amino-4-imidazole-N-succino-carboxamide ribonucleotide (SAICAR) to fumarate and 5-amino-4-imidazole-carboxamide ribonucleotide (AICAR). Both nucleotides are intermediates of purine nucleotide

synthesis. In 1957, Miller et al. (2) demonstrated with partially purified enzymes from chick liver and yeast that these dual reactions were catalyzed by a single enzyme. Giles et al. (3) and Gots and Gollab (4) reached the same conclusion with adenine-requiring mutants of Neurospora crassa and Escherichia coli, respectively. In 1966, Woodward and Braymer (5) reported firstly the subunit structure of the enzyme purified from Neurospora.

In higher animals, however, no one has been succeeded in isolation of homogeneous preparation of adenylosuccinase. Brox (6) described preliminarily the kinetics of enzyme purified partially from Ehlrich ascites tumor cells by phosphocellulose column chromatography. He also reported that the enzyme was labile and likely different from that of Neurospora in molecular structure.

In order to know about molecular properties more precisely, we attempted to purify adenylosuccinase from the skeletal muscle or other tissues.

MATERIALS AND METHODS

Animals: Wistar HLA strain rats, weighing 300 to 450 g, were used throughout the experiments. Rat was decapitated and whole skeletal muscles including white and red muscles were removed and chilled on ice. Freshly prepared muscles were usually used, because adenylosuccinase activity was extremely lowered, when the muscle was frozen for a long time in a deep freezer.

Biochemicals: Biochemical reagents and materials used here were the same as described in the previous chapter.

Enzyme assay: Adenylosuccinase activity was determined spectrophotometrically. Reaction mixture (250 µl) contained; 20 mM Tris-HCl, pH 7.2, 0.5 mM adenylosuccinate(NH_4 salts) and enzyme solution. Adenylosuccinate was prepared by the method of Carter and Cohen (7). Reaction system was incubated for 10 min at 37° C and the reaction was terminated by adding 50 µl of 30 % PCA. For a control, PCA was added to one test tube immediately after the addition of the enzyme. Denatured protein was removed by centrifugation for 10 min at 3,000 g , then the absorbance at 280 nm of the supernatant was determined. The extinction coefficient difference between AMP and adenylosuccinate is 10,7000 under the condition employed in the experiment.

RESULTS

Effect of Heat Treatment—— As in case of Ehlrich ascites tumor cells, adenylosuccinase from rat skeletal muscle was labile. Almost all enzyme activity in crude extract was lost within a day after its preparation, whether the extract was kept at 4° C or at room temperature. However, the enzyme became rather stabilized when it was heated at 55° C in 20 % glycerol solution as shown in Fig. 1. The addition of glycerol to the enzyme solution resulted in the increase in the stability

30 to 40 % more than without addition. It seems likely from Fig. 1 that adenylosuccinase consists of two components; unstable fraction (40 %).and stable fraction (60 %). Fig. 2 shows the residual activity 2 days after heating. The results indicate that heat treatment for 1.5 min in glycerol is most effective for the stabilization of the enzyme.

Salts such as KCl, $(NH_4)_2SO_4$ and $MgCl_2$ inactivated rapidly the enzyme even if it was previously heated for 1.5 min. Sucrose (20 % w/v) as well as glycerol (20 %) protected the enzyme activity, but 100 mM EDTA was without effect, as shown in Fig. 3.

pH Stability——The supernatant after heat treatment was fractionated with $(NH_4)_2SO_4$. Thirty five to 60 % saturated fraction was dissolved in potassium phosphate buffer (KPB), pH 7.0, and the solution was divided into several portions. Each aliquot was dialyzed overnight against KPB or Tris buffer with various pH. As shown in Fig. 4, adenylosuccinase was stable at the ranging from pH 6.5 to pH 7.5.

Phosphocellulose and DEAE-cellulose Column Chromatographies—— All subsequent procedures were carried out at 4° C and enzyme solution contained 20 % glycerol unless otherwise stated. After fractionation of heat-treated enzyme with $(NH_4)_2SO_4$ (35-60 % saturation) the enzyme solution was desalted and applied on a phosphocellulose column and then eluted with a linear gradient of 10 mM KPB (pH 7.0) containing 10 to 150 mM EDTA. The active fractions eluted were pooled and concentrated by addition of $(NH_4)_2SO_4$ to 60 % saturation. The sample was desalted again, put on a DEAE-cellulose column and then eluted with a linear

gradient of Tris-HCl, pH 7.2 contining 0 to 300 mM KCl. At this step, the yield of the activity was only approximately 10 % in spite of the addition of glycerol and specific activity increased 20 fold. Further purification was attempted, but failed. The peak fraction eluted from a DEAE-cellulose column was subjected to SDS polyacrylamide gel electrophoresis. As shown in Fig. 5, one major band (M.W.≒57,000), two minor bands (M.W.= 77,000, 97,000) and several faint bands were detected.

Effect of Metal Ions—— The effects of metal ions on adenylo= succinase activity were examined. The fraction eluted from a phosphocellulose column with 200 mM KCl was used for this experiment. Divalent inos such as $MgCl_2$, $MnCl_2$, $BaCl_2$, $CaCl_2$, $CoCl_2$ and $FeSO_4$ were without effect at the concentrations of 2 mM, but those such as $HgCl_2$, $CdCl_2$, $ZnCl_2$, $CuSO_4$ and $Hg(CH_3COO)_2$ caused complete inactivation of the enzyme at the concentrations of 200 μM. However, the enzyme fraction eluted from phospho= cellulose with 100 mM EDTA was not affected by any metal ions except $HgCl_2$. The inhibitory effect of $HgCl_2$ was protected by cysteine or dithiothreitol. These suggest that sufhydryl ligand is prerequisite to catalytic activity of adenylosuccinase. The same phenomenon was observed in crude extract. $CuSO_4$ was most potent inhibitor among metals tested, causing complete inhibition at the concentration of 20 μM (Table I).

Lowenstein first demonstrated with crude extract of rat skeletal muscle that adenylosuccinate was gradually accumulated as IMP decreased, but approximately 30 min after the start of the reaction adenylosuccinate formed began to convert to AMP and fumarate by adenylosuccinase. This observation was confirmed by

us. As shown in Fig. 6, degradation of adenylosuccinate formed was completely blocked by the addition of low concentration of $HgCl_2$ or $CuSO_4$. Consequently adenylosuccinate synthetase reaction proceeded linearly. As previously described (Chapter I), $HgCl_2$ inhibited considerably adenylosuccinate synthetase activity at the concentration of 200 μM, but $CuSO_4$ and $HgCl_2$ were not inhibitory for the synthetase activity at the concentrations of 20 μM.

Isoelectric Focussing of Adenylosuccinase—— The existence of isozymes of adenylosuccinate synthetase was shown in Chapter III. In order to analyze further the physiological meaning of the purine nucleotide cycle in various organs, the isozymes of adenylosuccinase were examined. As shown in Fig. 7, two peaks were detected on isoelectric focussing, whether crude extract from skeletal muscle applied was heated or not. More clear peaks were observed in case of the cardiac muscle. It cannot be concluded, however, that the major peak (I.P.=7.0) is the main enzyme in the skeletal or the cardiac muscle strictly, because the recovery of the enzyme activity is extremely low (below 20 %).

In liver extract, only the activity with I.P. of pH 7.0 was detectable (Fig. 7).

The Km values for adenylosuccinate of adenylosuccinase in major and minor peaks were the same (0.2 mM) (Fig. 8). No other kinetical differences have so far been detected between two peaks.

DISCUSSION

Adenylosuccinase from rat skeletal muscle was unstable like that in Ehlrich ascites tumor cells. Heat treatment in the presence of glycerol were found to be effective for stabilization of the enzyme. The reason why adenylosuccinase was stabilized by such treatments is not clear yet, but heat treatment may destroy heat-stable proteolytic enzymes. Or it may alter the conformation of the enzyme to be more stable, though isoelectric point of the enzyme did not change even after heat treatment (Figs. 7 (A) and (B)).

Low recovery of the enzyme during chromatogrphies or electrophoretic procedure made further purification extremely difficult. The fact that adenylosuccinase is unstable in the presence of high concentration of salts may reflect highly dissociability of its subunits.

A gel filtration of adenylosuccinase on Sephadex G-200 in the presence of 20 % glycerol indicated that the enzyme protein had molecular weight of more than 100,000 (data not shown). SDS polyacrylamide gel electrophoresis showed that major band had molecular weight of 57,000 of the most purified preparation. These findings suggest that adenylosuccinase from rat skeletal muscle consisted of two isozymes, assuming that the major band is a component of the enzyme.

Woodwars and Braymer reported that adenylosuccinase of Neurospora had a molecular weight of 200,000 to 220,000 and dissociated into several identical subunit forms (5).

Ratner also reported that crystallized argininosuccinase had a molecular weight of 202,000 and its subunit was not dissociated at room temperature, but at low temperature where the enzyme was inactivated (8-9). These findings tempted us to speculate that adenylosuccinase is cold sensitive like arginino= succinase. However, neither substrate (adenylosuccinate) nor products (AMP and fumarate) protected the enzyme against inactivation during maintenace for 24 hours at 0° C or room temperature.

For further investigations, more purified sample should be obtained.

Some metals strongly inhibited adenylosuccinase activity and the inhibition was fully restored by adding excess EDTA or dithiothreitol. This suggests the enzyme requires SH group(s) for catalytic activity like yeast adenylosuccinase. This characteristics was applied to crude extract to stop the operation of the purine nucleotide cycle. This was also applicable to determine actual activity of adenylosuccinate synthetase in crude extract. Then this will become very useful means to develop the study on regulation mechanism of the synthetase *in vivo*.

So far examined, there are no tissue specific isozymes, though multiforms were observed in cases of adenylosuccinate synthetase and AMP deaminase. Isoelectric point of main peak in the same tissue fluctuated from pH 6.8 to pH 7.3. Minor peak did not constantly observed. Moreover, total recovery of activity was only 20 %. Accordingly, this technique did not permit the detection of real isozymes.

The Km value for the enzyme was 210 μM. This value was approximately 100 fold higher than that of any species so far examined (3.2 μM in yeast and 1.2 μM in Neurospora and 8.4 μM in Ehlrich ascites tumor cells). Buchanan et al. reported that Km value was 190 μM in chick liver enzyme (2). Is this high Km value favorable for the regulation of ammonia production then ? General speaking, the Km values for adenylosuccinate or SAICAR of adenylosuccinase are low.

The effects of various amino acids on enzyme activity at the concentrations of 2 mM were examined, but without effect. The effects of various nucleotides could not be measured by this spectrophotometric assay, because nucleotides had high absorbance at 280 nm , interfering the assay.

Accordingly the problem of whether adenylosuccinase of the skeletal muscle is specifically organized to operate the purine nucleotide cycle to produce ammonia or to control purine nucleotide synthesis won't be solved unless the enzyme is more purified and other sensitive assay method is developed.

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Fig. 1. Heat stability of adenylosuccinase. Rat skeletal muscle was homogenized in homomixer for 1 min with 3 volumes of 50 mM Tris-HCl, pH 7.2, containing 0.2 mM DTT. The homogenate was centrifuged at 10,000 g for 15 min and the supernatant was adjusted to pH 7.0 with 1 M Tris solution. Each 2 ml ailquot was heated for the time indicated in abscissa in the presence (o) or absence (⊙) of 20 % glycerol. The ordinate was expressed as the residual activity relative to unheated control.

Fig. 2. Residual activity of adenylosuccinase 2 days after heating. The samples obtained in Fig. 1 were kept for 2 days at 4° and their residual activities were plotted versus unheated sample.

Fig. 3. Effects of salts, EDTA, sucrose and glycerol on stability of adenylosuccinase. Two ml aliquot of crude extract were heated for 1.5 min at 55°. Then salts, EDTA, sucrose and glycerol were added and kept at 4° for days indicated in the abscissa. The ordinate was expressed as the residual activity relative to the activity of the initial day.

Fig. 4. pH stability of adenylosuccinase. 45 g muscle was used. The heated supernatant was fractionated with $(NH_4)_2SO_4$ of 35 to 60 % saturation and the precipitate was dissolved to 10 ml water and it was dialyzed overnight against 30 mM of buffer indicated.
(⊙); potassium phosphate buffer (o); Tris-HCl buffer

Fig. 5. SDS polyacrylamide gel electrophroresis of partially purified adenylosuccinase. The procedure for the preparation of the sample was as described in the text. Approximately 20 μg protein was used for each run. CHY (chymotripsiongen, 25,000), OVA (ovalubmin, 45,000) and BSA (bovine serum albumin, 68,000) were used as marker enzymes.

Fig. 6. Effects of the metal ions on the purine nucleotide cycle. Time course of adenylosuccinate synthetase activity freshly prepared extract was followed in the presence or the absence of metals.

Fig. 7. Profiles of isoelectric focussing of adnylosuccinase from the muscle, the heart and the liver.
(A); Crude extract of 20 g muscle was heated for 1.5 min and centrifuged. The supernatant was dialyzed against 10 mM Tris-HCl, pH 7.2, containing 0.2 mM DTT and applied to a column containing 1 % ampholine (pH range, 3.5 to 10).
(B); Unheated crude extract of muscle was treated as (A).
(C); Crude extract of 13 g heart was treated as (A).
(D); Forty two g liver were homogenized and centrifuged at 100,000 g for 60 min. The supernatant was heated for 1.5 min at 55° and centrifuged after cooling. The heat-treated supernatant was dialyzed for 4 hours and then the dialysate was applied to an ampholine column.

Fig. 8. Lineweaver-Burk plot of adenylosuccinase of the major and minor peaks obtained in the experiment shown in Fig. 7 (A).
(⊙); Activity of the minor peak (fraction number 19).
(●); Activity of the major peak (fraction number 25).

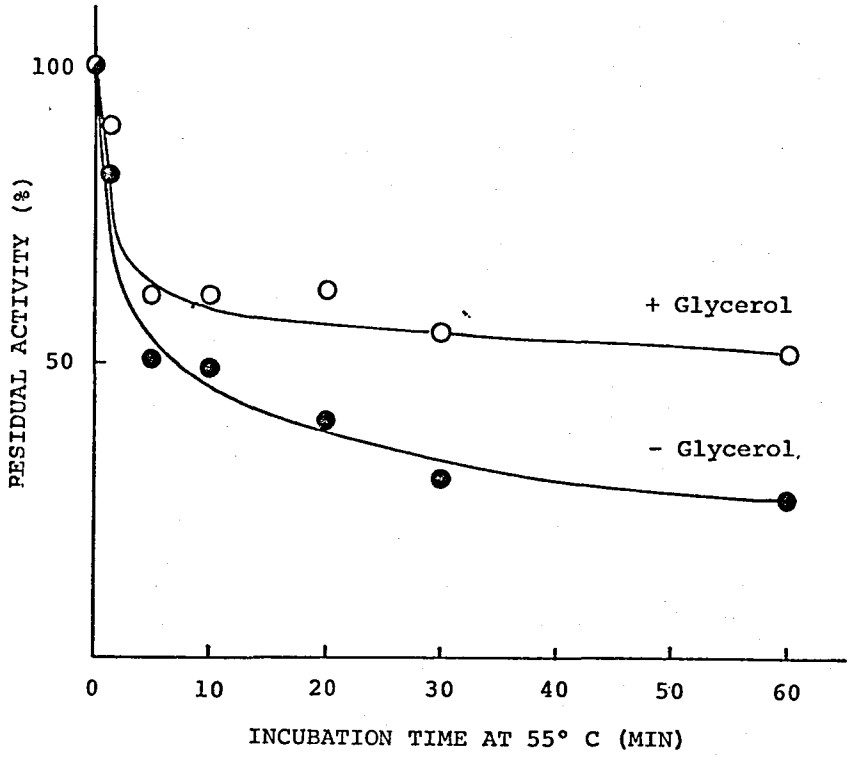
100
50
RESIDUAL ACTIVITY (%)
+ Glycerol
- Glycerol
0
10
20
30
40
50
60
INCUBATION TIME AT 55° C (MIN)

Fig. 1.

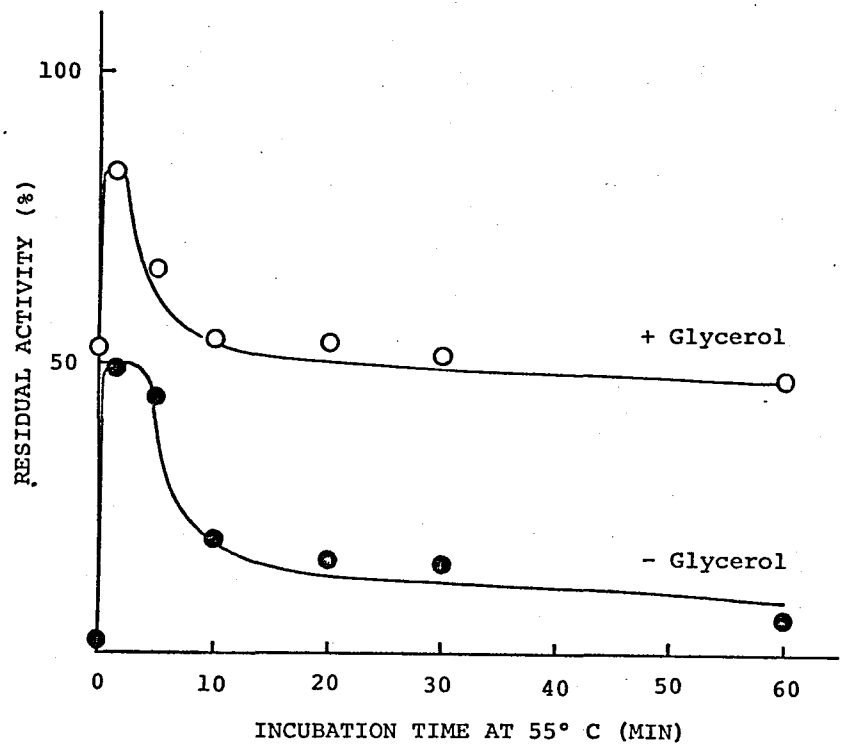
100
50
RESIDUAL ACTIVITY (%)
+ Glycerol
- Glycerol
0
10
20
30
40
50
60
INCUBATION TIME AT 55° C (MIN)

Fig. 2.

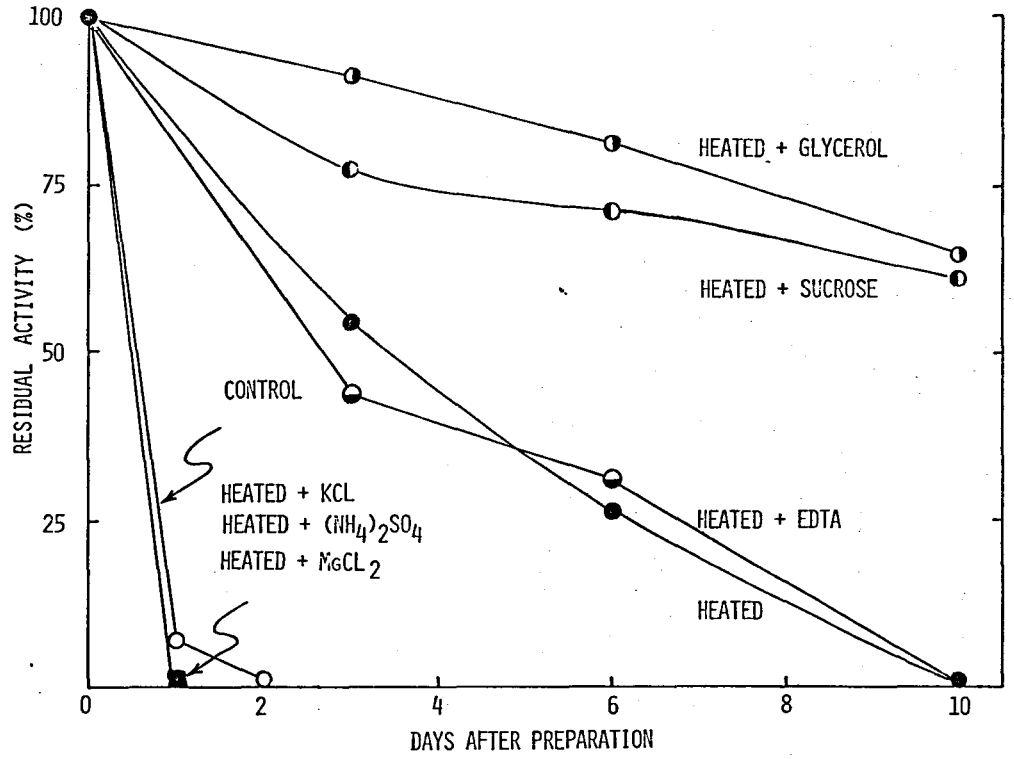
100
75
50
25
RESIDUAL ACTIVITY (%)
HEATED + GLYCEROL
HEATED + SUCROSE
CONTROL
HEATED + KCL
HEATED + $(NH_4)_2SO_4$
HEATED + $MgCL_2$
HEATED + EDTA
HEATED
0
2
4
6
8
10
DAYS AFTER PREPARATION

Fig. 3.

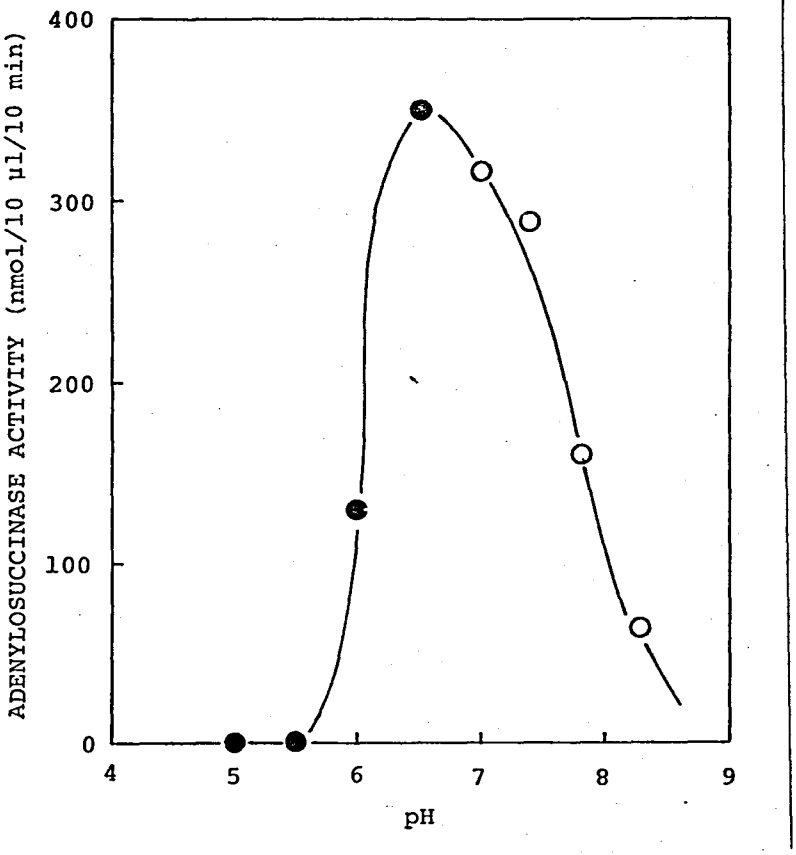
ADENYLOSUCCINASE ACTIVITY (nmol/10 µl/10 min)
400
300
200
100
0
4
5
6
7
8
9
pH

Fig. 4.

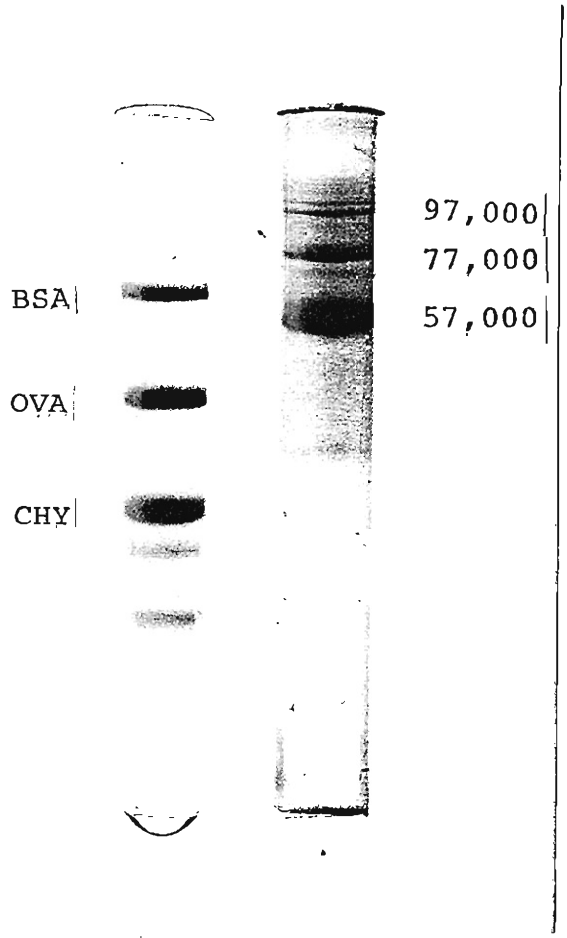

Fig. 5.

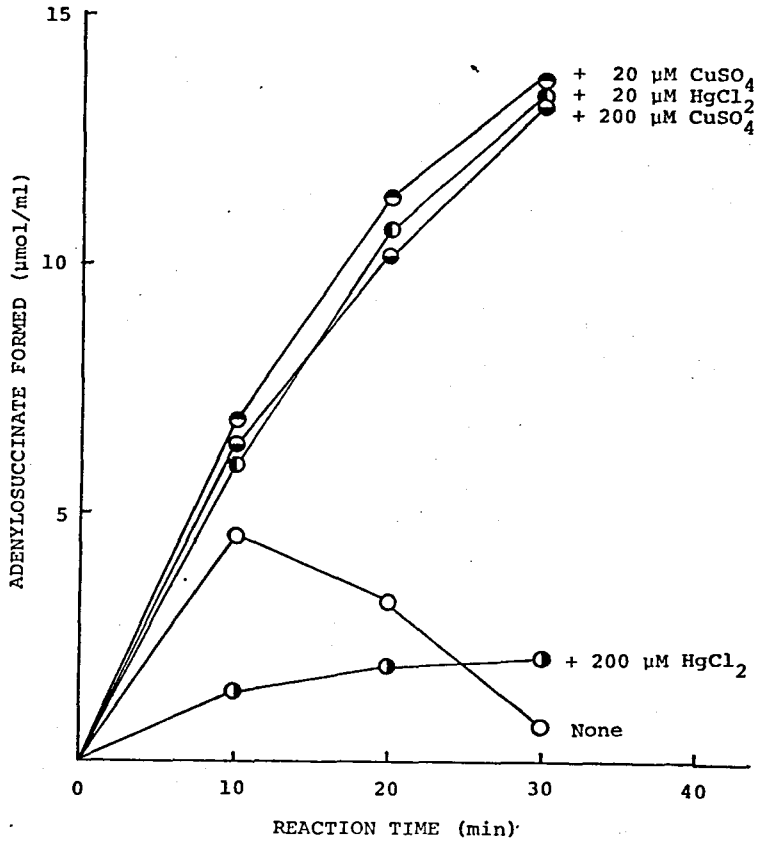
15
10
5
0
10
20
30
40
ADENYLOSUCCINATE FORMED (µmol/ml)
REACTION TIME (min)
+ 20 µM CuSO4
+ 20 µM HgCl2
+ 200 µM CuSO4
+ 200 µM HgCl2
None

Fig. 6.

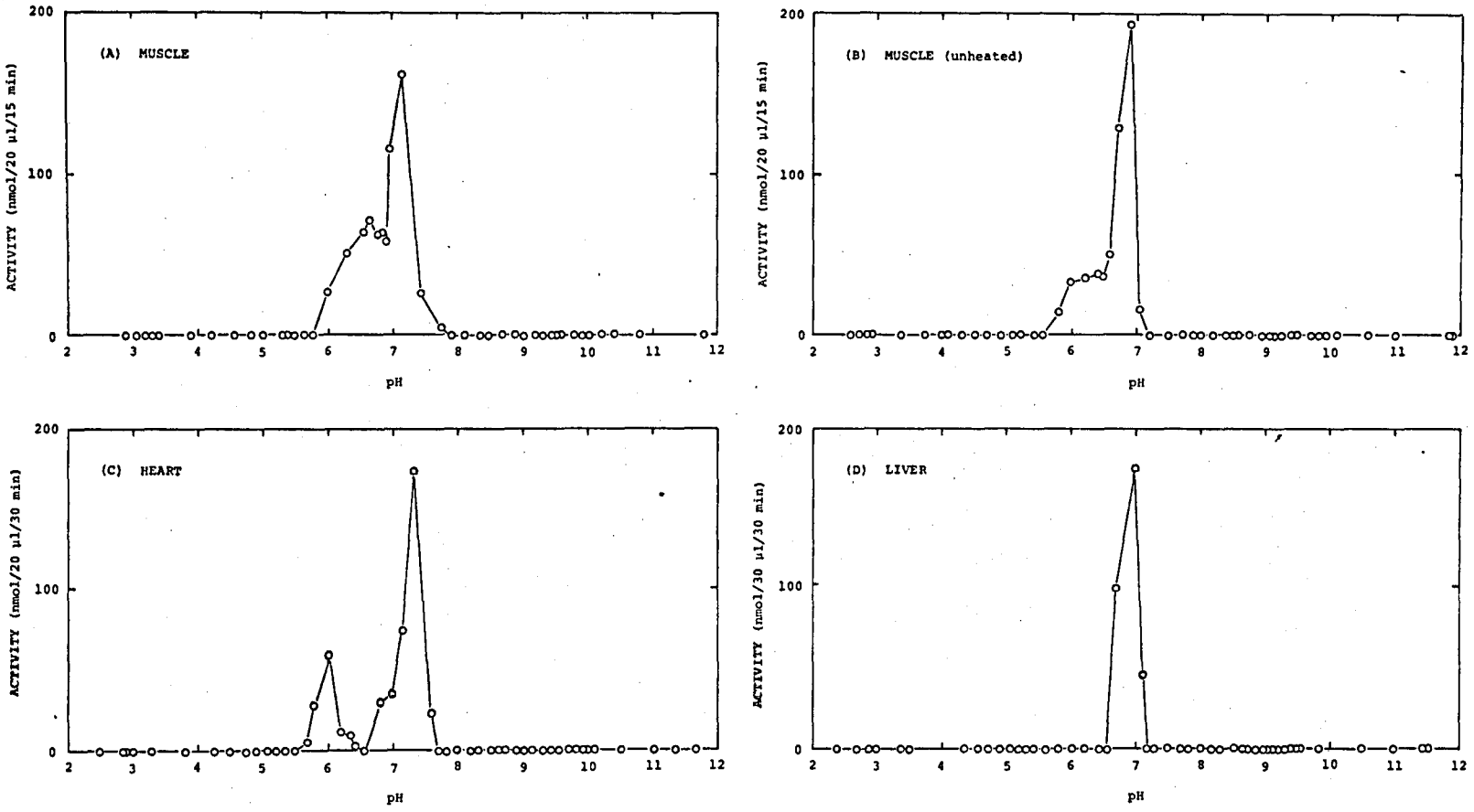
(A) MUSCLE
ACTIVITY (nmol/20 µl/15 min)
200
100
0
2 3 4 5 6 7 8 9 10 11 12
pH
(B) MUSCLE (unheated)
ACTIVITY (nmol/20 µl/15 min)
200
100
0
2 3 4 5 6 7 8 9 10 11 12
pH
(C) HEART
ACTIVITY (nmol/20 µl/30 min)
200
100
0
2 3 4 5 6 7 8 9 10 11 12
pH
(D) LIVER
ACTIVITY (nmol/30 µl/30 min)
200
100
0
2 3 4 5 6 7 8 9 10 11 12
pH

Fig. 7.

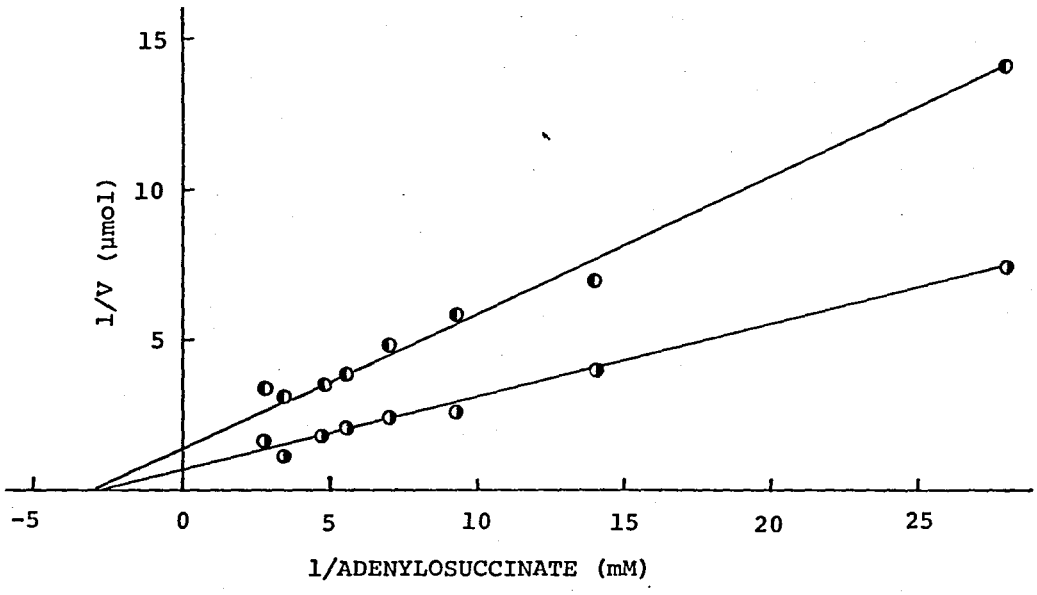

Fig. 8.

Table I. Effects on metal ions on adenylosuccinase activity in crude extract

Metals (M)	Residual activity (%)			
	2×10^{-6}	2×10^{-5}	2×10^{-4}	2×10^{-3}
$MgCl_2$				105
$MnCl_2$				99
$BaCl_2$				104
$CaCl_2$				94
$FeSO_4$				100
$CdCl_2$	95	73	0	0
$ZnCl_2$	96	61	0	0
$CuSO_4$	98	7	0	0
$HgCl_2$	70	29	0	0
$Hg(CH_3COO)_2$	96	28	0	0
EDTA				118

Adenylosuccinase was partially purified by phospho= cellulose chromatography. The activity without addition of metal ion is expressed as 100.

Chapter V

Interaction of Adenylosuccinate Synthetase and Actin

Interaction of Adenylosuccinate Synthetase and Actin

Summary

Adenylosuccinate synthetase was extracted from the debris of muscle homogenized with water. The total activities in the supernatant and precipitate varied with volume of water of homogenization. It was found that the extractability of the enzyme depended on ionic strength in homogenizing medium. It was also found that the enzyme was adsorbed to actin, but not to myosin. The molar ratio of adenylosuccinate synthetase to actin in the complex formed in the absence of KCl was 1 to 6. The complex was dissociated completely in the presence of 210 mM KCl.

From these evidences it was suggested that the purine nucleotide cycle operated actually on the contractile proteins, in other word, ammonia is produced in connection with muscular contraction.

RESULTS AND DISCUSSION

During the works described in Chapters I to IV, kinetical properties of purified adenylosuccinate synthetase and partially purified adenylosuccinase and their characteristics in various organs were examined. These studies indicated that there was difference in molecular properties of adenylosuccinate synthetase between contractile and non-contractile tissues. Thus, it has been examined what this difference means physiologically in this chapter.

Adenylosuccinate synthetase was originally regarded as a cytoplasmic enzyme. In fact, the enzyme was purified to be crystalline from the soluble fraction obtained from rat skeletal muscle

homogenate prepared with 50 mM Tris-HCl, pH 7.2, containing 0.2 mM DTT (Chapter I). When the muscle was homogenized with 3 volumes of water, enzyme activity was observed in a high-speed supernatant of the homogenate (Sup I). In addition, almost a half activity of Sup I could be extracted from the debris of the same preparation with 3 volumes of 0.6 M KCl (Sup II).

As shown in Fig. 1, Sup I showed a conical-shape reaction with time, but Sup II obtained from 0.6 M KCl-homogenate showed almost linear time course. This suggests that adenylosuccinase activity is freed from Sup II. Treatment with 0.5 % Triton X-100 was unable to liberate the enzyme from the residues, but the subsequent treatment with 0.6 M KCl released the enzyme. Detailed studies indicated that optimal concentration range of KCl liberating the enzyme was 0.1 to 0.4 M KCl and treatment with 0.6 M KCl was rather less effective. Buffer suspension of debris itself showed no activity (Fig. 1).

Then a question arises as to whether adenylosuccinate synthetase in the supernatant is different qualitatively from that of the precipitate. Are there two kinds of adenylosuccinate synthetase in the skeletal muscle originally ?

This was examined by recombination of various fractions as shown in Diagram 1. In this experiment, the skeletal muscle was first homogenized with 3 volumes of water and the homogenate was separated by centrifugation into supernatant (Sup I) and precipitate (Ppt I). Sup I was dialyzed for 7 hours against 100 volumes of 10 mM potassium phosphate buffer, pH 6.6 (Buffer A).

Ppt I was further suspended in 3 volumes of Buffer A containig 0.1 M KCl (Buffer B) and stirred well with a glass rod. This suspension was separated by centrifugation into supernatant (Sup II) and precipitate (Ppt II). Sup II showed enzyme activity, as shown in Fig. 2. This fraction was then dialyzed for 7 hours against 100 volumes of Buffer A.

Ppt II was washed well with Buffer A and the suspension was divided into two portions. After centrifugation, the precipitate of one portion was combined with dialyzed Sup I and that of the other portion with Sup II. The suspensions were centrifuged and their supernatants were designated as Sup I' and Sup III, respectively, as shown in Diagram I. As shown in Fig. 2, little activity was found either in Sup I' or Sup III. Ppt I' and Ppt III were again extracted with 3 volumes of Buffer B and their supernatant were designated as Sup I'' and Sup IV, respectively. Enzyme activities were considerably restored in Sup I'' and Sup IV. (Fig. 2). These results indicate that adenylosuccinate synthetase isolated from debris of the muscle (Sup II) is the same as that of the supernatant (Sup I). Such a possibility was also supported by the following experiments.

Table I shows the change in distribution ratio of adenylo= succinate synthetase activity in the supernatant and the precipitate when the muscle was homogenized with various volumes of water. When the muscle was homogenized with 6 volumes of water, only 36 % of total activity (Sup I and II in diagram I) was found in the supernatant. However, the less extraction volume of water,

the more the enzyme activity in the supernatant. These findings suggest that the distribution ratio of the activity in the supernatant and the precipitate depends on ionic strength in the extraction medium, since it can be considered that the less the extraction volume, the more ionic strengh in the medium. It should be noteworthy, in this connection, that homogenization of the muscle with 3 volumes of 0.1 M KCl solution is the most efficient for extraction of the enzyme (83 %) as far as we examined (Table I).

What component(s) is the enzyme bound to ? It is known that these precipitates contain contractile proteins such as myosin, actin and others. Judging from the data from Fig. 1, it seems probable that the enzyme binds to myosin.

On the other hand, it is well known that AMP deaminase, a member of the purine nucleotide cycle, is a sticky contaminant of myosin preparations till the last purification step. Moreover, Suelter et al. showed that purified AMP deaminase could be recombined with pure myosin. Namely, mixture of myosin and AMP deaminase in 0.6 M KCl was associated to form gel when it was diluted with water and this gel exhibited both ATPase and AMP deaminase activities. This method was applied to the synthetase, but the enzyme did not undergo the same behavior as AMP deaminase (1-3).

Next, the association of adenylosuccinate synthetase and actin was examined. Actin was isolated by the method of Spudich and Watt (4). This preparation was shown to be almost homogeneous on SDS polyacrylamide gel electrophoresis. Fig. 3 depicts interaction of adenylosuccinate synthetase and actin.

Various amounts of actin were added into a Nalgen tube containing a constant amount adenylosuccinate synthetase. The mixture was allowed to stand for 30 min at 0° C and centrifuged for 1 hour at 100,000 g. Residual activity of the enzyme in the supernatant was plotted against the amount of actin added. In this experimental condition, 1,000 µg of actin was required for the coprecipitation with 200 µg of the synthetase. Assuming that the molecular weights of actin and the synthetase are 42,000 and 50,000, respectively, one molecule of the synthetase binds to 6 molecules of actin.

The coprecipitates obtained in the experiment shown in Fig. 3 were homogenized well with various concentrations of KCl in a homomixer. The suspension was spun with high speed and the enzyme activities in the supernatant were measured (Fig. 4). With 210 mM KCl all enzyme activity could be released from actin and with 75 mM KCl half an activity was solubilized. As shown in Fig. 5, 600 µg of purified actin was necessary for complete precipitation of the synthetase from crude extract of skeletal muscle containing 5,000 µg protein and no more than 100 µg of actin was required for 50 % coprecipitation. In the latter case, almost no decrease in protein concentration in the crude extract was detected. This suggests that the synthetase is selectively trapped by a small amount of actin.

The interaction of the synthetase and actin is considered to be electrostatic, because dissociation of the synthetase from actin is affected by low ionic strength and the synthetase, purified from the liver, having the isoelectric point of pH 6.0, was not bound to muscle actin under the same condition.

Assuming that the ionic strength in the muscle is 0.1 to 0.15 µ, approximately 30 % of total synthetase activity is

calculated to be bound to actin *in vivo*. AMP deaminase was also extracted with 0.6 M KCl from the debris after homogenization with 3 volumes of water. In this case, 34 % of AMP deaminase included in 0.6 M KCl extract, but not in 0.1 M KCl extract.

From the results mentioned above, it was demonstrated that adenylosuccinate synthetase and AMP deaminase of the purine nucleotide cycle interacted with contractile components of the muscle, actin and myosin, respectively. This suggests that the functions of the cycle may work closely linked with muscle contractile proteins.

As previously mentioned, adenylosuccinate synthetase might have dual functions; one as a key enzyme for ammonia production, the other as a key enzyme for purine nucleotide biosynthesis. If these two functions are controlled by a single enzyme protein, it can be speculated that the kinetical properties of free and bound forms of adenylosuccinate synthetase might be different. However, no difference in Km values for IMP and aspartate and Ki value for fructose-1,6-diphosphate were found between free and bound forms.

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Fig. 1. Release of adenylosuccinate synthetase from the debris of muscle homogenate by treatment with KCl. The muscle was homogenized with 3 volumes of water by a homomixer for 1 min at full speed. Homogenates were once washed with 10 mM potassium phosphate buffer, pH 6.6. The debris was then suspended in 3 volumes of the same buffer containing KCl and Triton X-100 to make final concentrations of 0.6 M or 0.5 % (v/v), respectively. Thirty min after standing on ice, the suspension was separted by centrifugation into the supernatant and the precipitate. The precipitates obtained after extraction with 0.6 M KCl were resuspended in 3 volumes of 0.5 % Triton and vice versa. Each supernatant was assayed for adenylosuccinate synthetase activity.
(o); original sup (⊙); 0.6 M KCl (⊕); Triton → 0.6 M KCl (⊖); Triton (⊘); 0.6 M KCl → Triton (⊛); the first buffer suspension (⊜); sup washed with the buffer

Diagram 1. Schematic presentation of recombination of adenylo= succinate synthetase with muscle debris.

Fig. 2. Rcombination of adenylosuccinate synthetase in the Sup I and Sup II with muscle debris. The details are given in text and diagram 1.

Fig. 3. Interaction of adenylosuccinate synthetase and actin. A half ml of 200 µg purified adenylosuccinate synthetase and various amounts of actin were mixed in a Nalgen tube to make final volumes of 1 ml. The mixtures were incubated for 30 min in an ice-bath and then centrifuged for 60 min at 100,000 g. Residual adenylo= succinate synthetase activity in the supernatant was plotted against the amount of actin added.

Fig. 4. Release of adenylosuccinate synthetase from the enzyme-actin complex by the various concentrations of KCl. The complex (see legend to Fig. 3) precipitated (1,500 μg of actin were added) was vigorously stirred with 1 ml of buffer containing various concentrations of KCl and centrifuged for 60 min at 100,000 g. The supernatants were taken for assay of the enzyme activity.

Fig. 5. Interaction of purified actin and adenylosuccinate synthetase in crude extract. The experiment was carried out by the same method as described in the legend to Fig. 4, except that each 0.5 ml aliquot of the solutions containing various amounts of actin was mixed with each 0.5 ml aliquot of crude extract (5 mg protein) dialyzed for 2 days against the buffer A in order to inactivate adenylosuccinase.

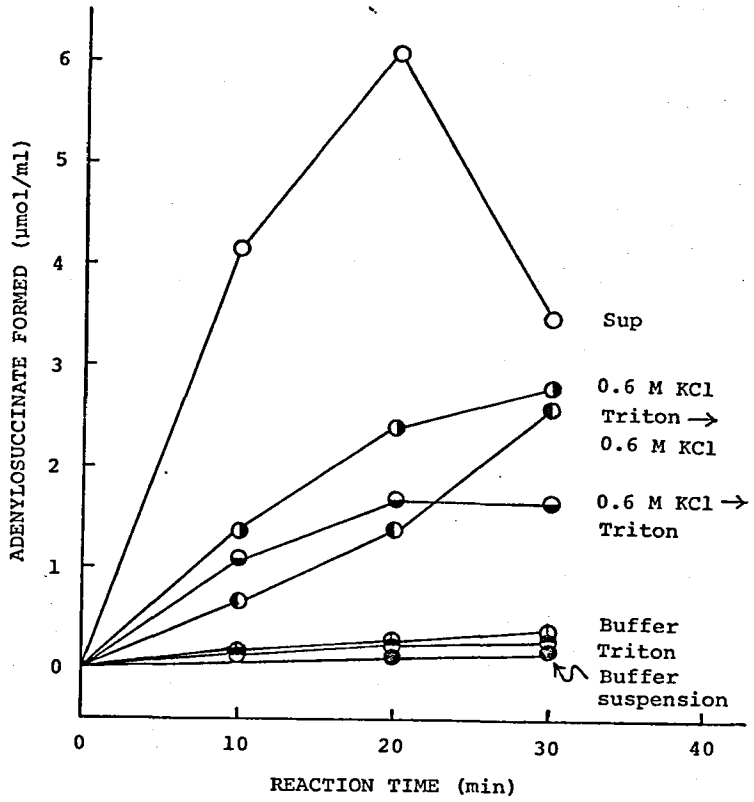

ADENYLOSUCCINATE FORMED (µmol/ml)
6
5
4
3
2
1
0
0
10
20
30
40
REACTION TIME (min)
Sup
0.6 M KCl
Triton →
0.6 M KCl
0.6 M KCl →
Triton
Buffer
Triton
Buffer
suspension

Fig. 1.

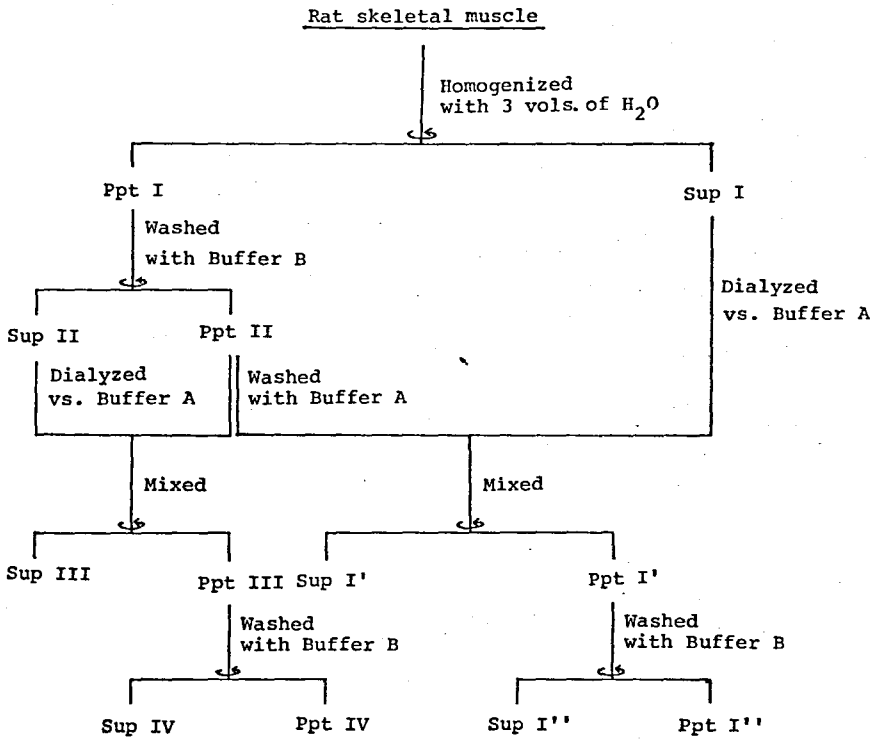


Diagram 1.

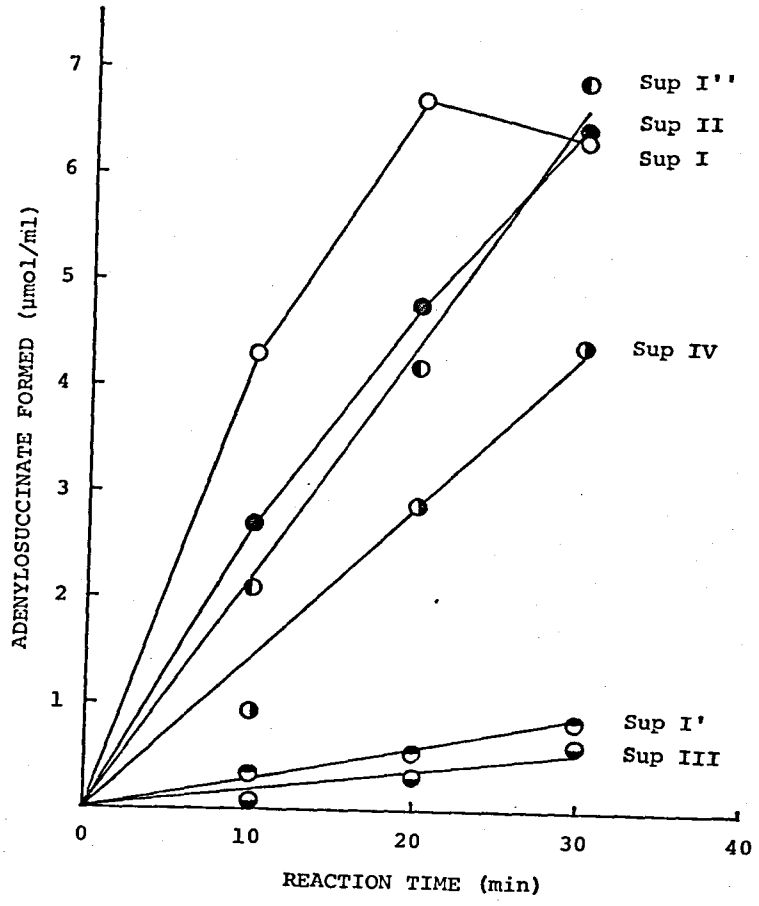


Fig. 2.

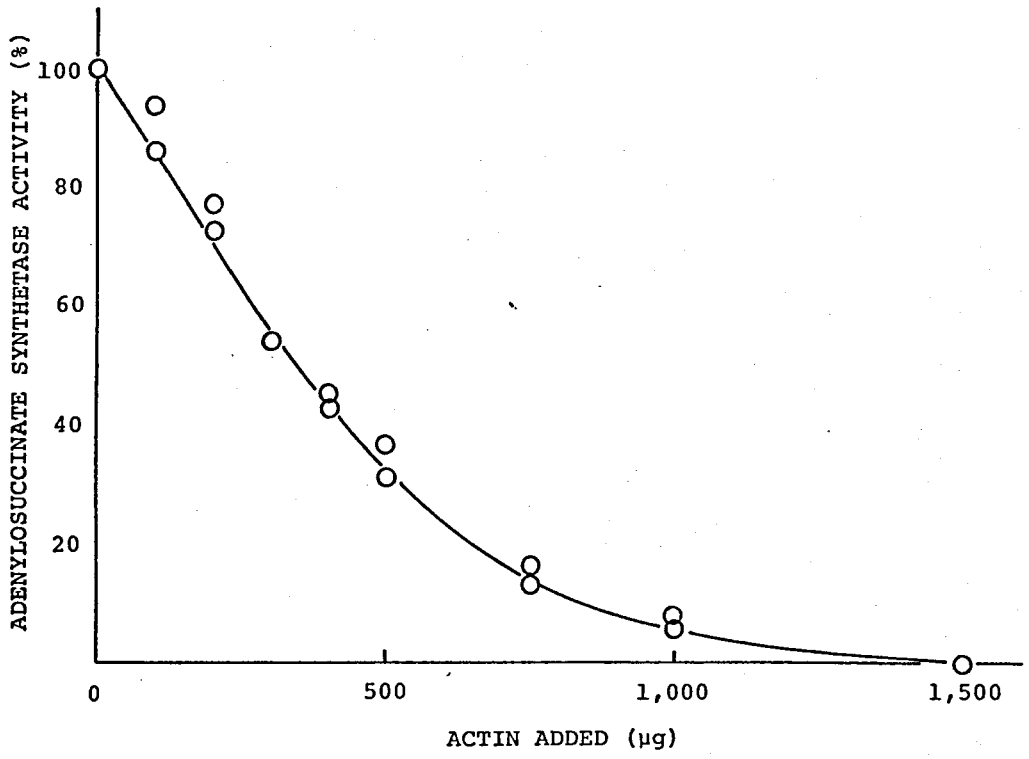

ADENYLOSUCCINATE SYNTHETASE ACTIVITY (%)
100
80
60
40
20
0
500
1,000
1,500
ACTIN ADDED (µg)

Fig. 3.

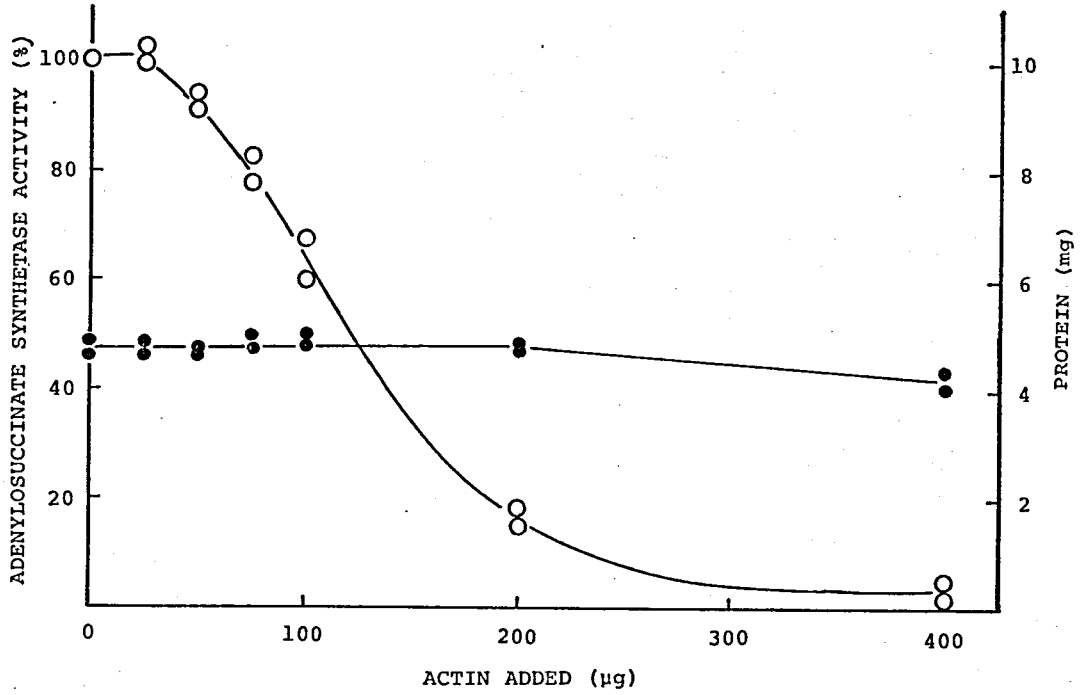

ADENYLOSUCCINATE SYNTHETASE ACTIVITY (%)
100
80
60
40
20
0
100
200
300
400
ACTIN ADDED (µg)
PROTEIN (mg)
10
8
6
4
2

Fig. 5.

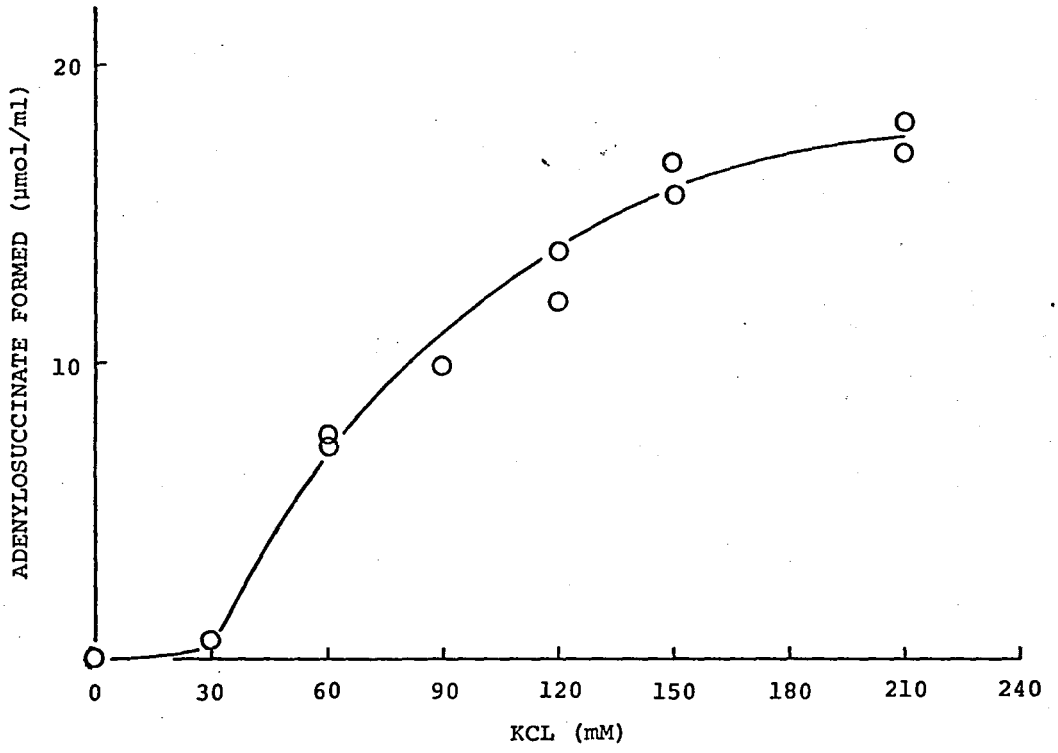

ADENYLOSUCCINATE FORMED (µmol/ml)
20
10
0
30
60
90
120
150
180
210
240
KCL (mM)

Fig. 4.

Table I. Effect of volumes of water or KCl solution on the extraction of adenylosuccinate synthetase from the muscle

Volumes	(A) Supernatant (units)	(B) Precipitate (units)	(B)/(A)
6	112	201	64/36
3	212	156	43/57
1.5	236	133	36/64
3 (0.1 M KCl)	382	77	17/83

Ten g of minced muscle were used in each experiment. Total activity was expressed as units (μmoles adenylosuccinate formed/min/ml enzyme). (A) and (B) mean the total activities in the Sup I and Sup II, respectively. For the precise determination of the enzyme activity in Sup I, the reaction mixture contained 50 μM $CuSO_4$ in order to avoid interference by adenylosuccinase.

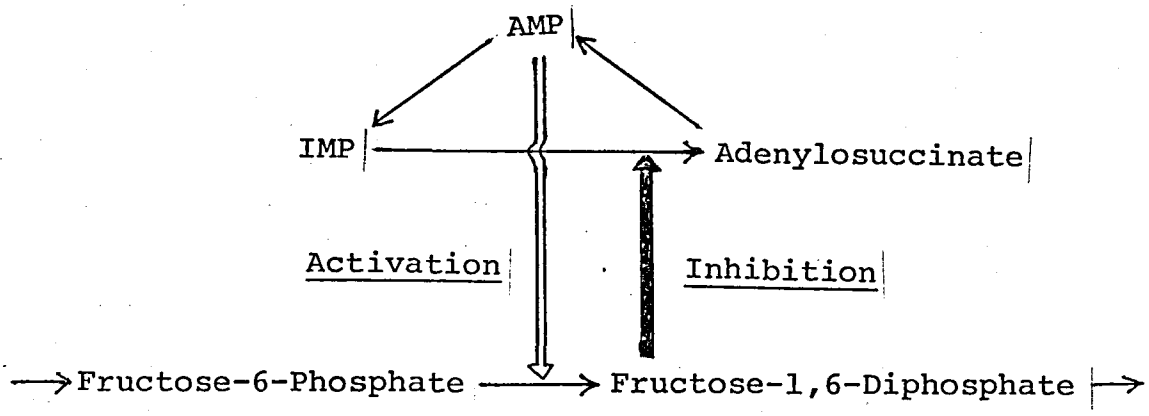
Concluding Remarks

1. Enzymic chemistry of adenylosuccinate synthetase...

During our investigations (1973-1976), only several papers on adenylosuccinate synthetase have been published from other laboratories. Bishop and Muirhead firstly purified and crystallized the enzyme from rabbit skeletal muscle (1). Kelly et al. partially purified the enzyme from human placenta and studied its kinetics in details (2). Eyzaruirre and Atkinson studied the regulatory properties of the enzyme partially purified from Escherichia coli (3).

From these data, it can be concluded that the enzyme, from whatever species it is isolated, is controlled by nucleoside mono- and diphosphates such as AMP, XMP, GMP, ADP, and GDP and reaction product such as adenylosuccinate, but these compounds do not seem to be specific for the regulation of the enzyme resided at the branch point of purine nucleotide synthesis.

We found that the enzyme activity was specifically inhibited by fructose-1,6-diphosphate, a glycolytic intermediate. This suggests that the enzyme might be interrelated with glycolysis, as shown in the following scheme;



This inhibitory effect by fructose-1,6-diphosphate may also in part account for the synchronizatioh beween the oscillations of the purine nucleotide cycle and the glycolysis (4).

2. Dual functions of adenylosuccinate synthetase or adenylo= succinase.

It was substantiated in Chapter III that adenylosuccinate synthetase was able to be classified into two forms, type M and type L forms. The enzyme in ascites tumor cells is type L enzyme.

Recently, Clark and Rudolf reported that Km value for aspartate and Ki value for AMP of the enzyme partially purified from Novikoff ascites tumor cells was slightly higher than those of normal liver and the tumor enzyme was more sensitive to hadacidine, a specific inhibitory antibiotics than normal liver enzyme (5). They also suggested that the molecular weight of the enzyme was 55,000 to 60,000 whether it was extracted from tumor and the liver.

We also preliminarily showed by gel filtration on Sephadex G-150 that molecular weight of rat liver enzyme was the same as the tumor enzyme.

Many investigators have reported metabolic deviations in tumor-bearing cells. For example, IMP dehydrogenase is induced in a rapid proliferating hepatoma and in regenerating liver (6). GMP synthetase is also abundant in Ehlrich ascites tumor cells, notwithstanding that the level of the enzyme is very low in normal liver (7). Amido phosphoribosyltransferase [EC 2.4.2.14], a key

enzyme in the purine nucleotide synthesis de novo is altered in quality in hepatoma (8). Isoelectric point of adenylosuccinase of Ehlrich ascites tumor cells is the same as that of the liver but Km value for adenylosuccinate of adenylosuccinase of Ehlrich tumor cells is strikingly different from that of the liver. From these findings, it may be speculated that adenylosuccinase is responsible for deviation of purine nucleotide synthesis in tumor-bearing cells.

3. Physiological role of the purine nucleotide cycle (1)

Does the purine nucleotide cycle really turn over more rapidly in the excersized muscle than sedantary one ? Hollowzy et al. determined the enzyme activities of the cycle in rats subjected to treadmill running for 12 weeks (9). According to them, AMP deaminase and phosphofructokinase activities decreased 29.6 and 28.1 %, respectively, and adenylosuccinase and citrate synthetase increased 16.0 and 94.4 %, respectively, in red muscle in comparison with those of sedantary rats. On the contrary, Hryniewiecki reported that AMP deaminase activity became almost three fold higher in leg muscles of rat subjected to swimming for 30 days (4h/day) than in the muscles of sedantary controls (10).

Anyway, glycolysis and the purine nucleotide cycle are considered to be closely linked. Lowenstein indicated that the cycle worked in brain and kidney extracts. Moss and McGivan, also reported that the cycle worked in liver extracts, as mentioned in Chapter III. Hence, the purine nucleotide cycle seems to be furnished in all tissues as an apparatus of ammonia producer.

4. Physiological role of the purine nucleotide cycle (2)

The evidences were presented that adenylosuccinate synthetase of contractile tissues was different from that of non-contractile tissues. Most ammonia is liberated from the muscle especially during exercise, so the purine nucleotide cycle must work more efficiently in the muscle than other organs. If enzymes of the cycle are directly associated with actin-myosin system, it seems quite likely that ammonia production occurs concomitantly with muscle contraction.

Many investigators have been suffered from the contamination of AMP deaminase during purification. However, the authors think that these phenomena should not be neglected.

$$ATP \longrightarrow ADP + Pi \quad \text{(myosin-actin ATPase)} \qquad 1)$$

$$ADP + \text{creatine phosphate} \longrightarrow ATP + \text{creatine} \quad \text{(creatine kinase)} \qquad 2)$$

$$2ADP \longrightarrow ATP + AMP \quad \text{(adenylate kinase)} \qquad 3)$$

1) + 2) + 3) are called as Lohmann's reaction.

$$AMP \longrightarrow IMP + H_2O + NH_3 \quad \text{(AMP deaminase)} \qquad 4)$$

$$IMP + GTP + \text{aspartate} \longrightarrow \text{adenylosuccinate} + GDP + Pi \quad \text{(adenylosuccinate synthetase)} \qquad 5)$$

$$\text{adenylosuccinate} \longrightarrow AMP + \text{fumarate} \quad \text{(adenylosuccinase)} \qquad 6)$$

4) + 5) + 6) are called as the purine nucleotide cycle.

When the muscle contracts, ATP is employed and ADP formed is quickly converted to ATP by the reaction 2. A part of ADP is regenerated to ATP and AMP by the reaction 3. In fact, the concentration of AMP increased 2 to 3 times at the oneset of contraction of flight muscle of the blowfly (11). Opie et al.

also reported the increase of the concentration of AMP in the cardiac muscle when the heart of the perfused rat beated (12). Thus the elevated contents of AMP is deaminated by the reactions (4-6) to produce ammonia. Gerez and Kirsten observed a signifi= cant rise of the ammonia level from 275 x 10^{-9}moles per g fresh weight to 945 x 10^{-9}moles per g fresh weight in tetanized leg muscles of the rat (13).

Then it can be considered that ammonia produced may be useful for the neutralization of acid caused by glycolysis by which ATP is supplied for muscle contraction. The experiments in line with this idea are now in progress.

Anyhow, without a further investigation on the interrelation-ship between the contractile proteins and their surrounding proteins such as glycolytic enzymes, the purine nucleotide cycle enzymes and cytoplasmic organella, muscle physiology in vivo will be failed to understand.

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Supplement

The amino acid composition of adenylosuccinate synthetase is given in Table I. The results are expressed as the averages of 24-, 48-, and 72-hour hydrolysates except half-cystine, threonine and serine. The values of threonine and serine were determined by extrapolation of values obtained from 24-, 48-, and 72-hour hydrolysates. Tryptophan was determined spectrophotometrically to be 21.9 moles per 50,000 g of enzyme.

In Chapter V, we suggested a possible relationship between the synthetase and actin. To know further structural relationship of the two proteins star-diagrams of the amino acid composition were compared. Though the data on the amino acid composition of actin of rat skeletal muscle have not been presented yet, but comparison was made on the assumption that the amino acid composition of actin of rat skeletal muscle is the same as that of rabbit skeletal muscle whose primary structure has already been identified.

As shown in Diagram 1, distributions of amino acids in the two proteins are similar to each other except glycine, valine, methionine and leucine. In particular amino acids in the first and the second quadrants are almost the same. It is uncertain at present what this similarity in the amino acid compositions of the synthetase and actin means. There is a possibility, however, that dissimilarity of the fourth quadrant may be concerned with the interaction of the two proteins, though we suggested previously that the electrostatic interaction took an important role in binding of the synthetase and

actin. To clearify it more precisely, not only primary structure , but also higher structure of the synthetase will have to be analysed.

Acknowledgements——We are due to Dr. T. Iwanaga and Miss K. Fukunishi, Division of Plasma Protein of our institute for performing amino acid analyses.

Table I. Amino acid composition of adenylosuccinate synthetase

Amino acid	Residues per subunit	Nearest integer residues per subunit
Lysine	29.42	29
Histidine	10.54	11
Arginine	21.88	22
Aspartic acid	43.27	43
Threonine	22.40	22
Serine	17.60	18
Glutamic acid	47.84	48
Proline	17.60	18
Glycine	50.91	51
Alanine	30.56	31
Half-cystine		
Valine	40.17	40
Methionine	7.06	7
Isoleucine	26.71	27
Leucine	43.15	43
Tyrosine	13.06	13
Phenylalanine	19.67	20
Tryptophan	21.86	22

Analyses were performed with a JEOL JLC-5AH amino acid analyzer according to the procedure of Spackman et al. (Spackman, D. H., Stein, W. H., & Moore, S., Anal. Chem., 30, 1190 (1958)). Samples of approximately 0.3 mg were hydrolyzed with 6 N HCl in evacuated sealed tubes for 24 to 72 hours at 110°. Tryptophan was determined spectrophotometrically by the method of Goodwin and Morton (Goodwin, T. W., & Morton, R. A., Biochem. J., 40, 628 (1946)). Half-cystine was not detected in the samples of 0.3 mg.

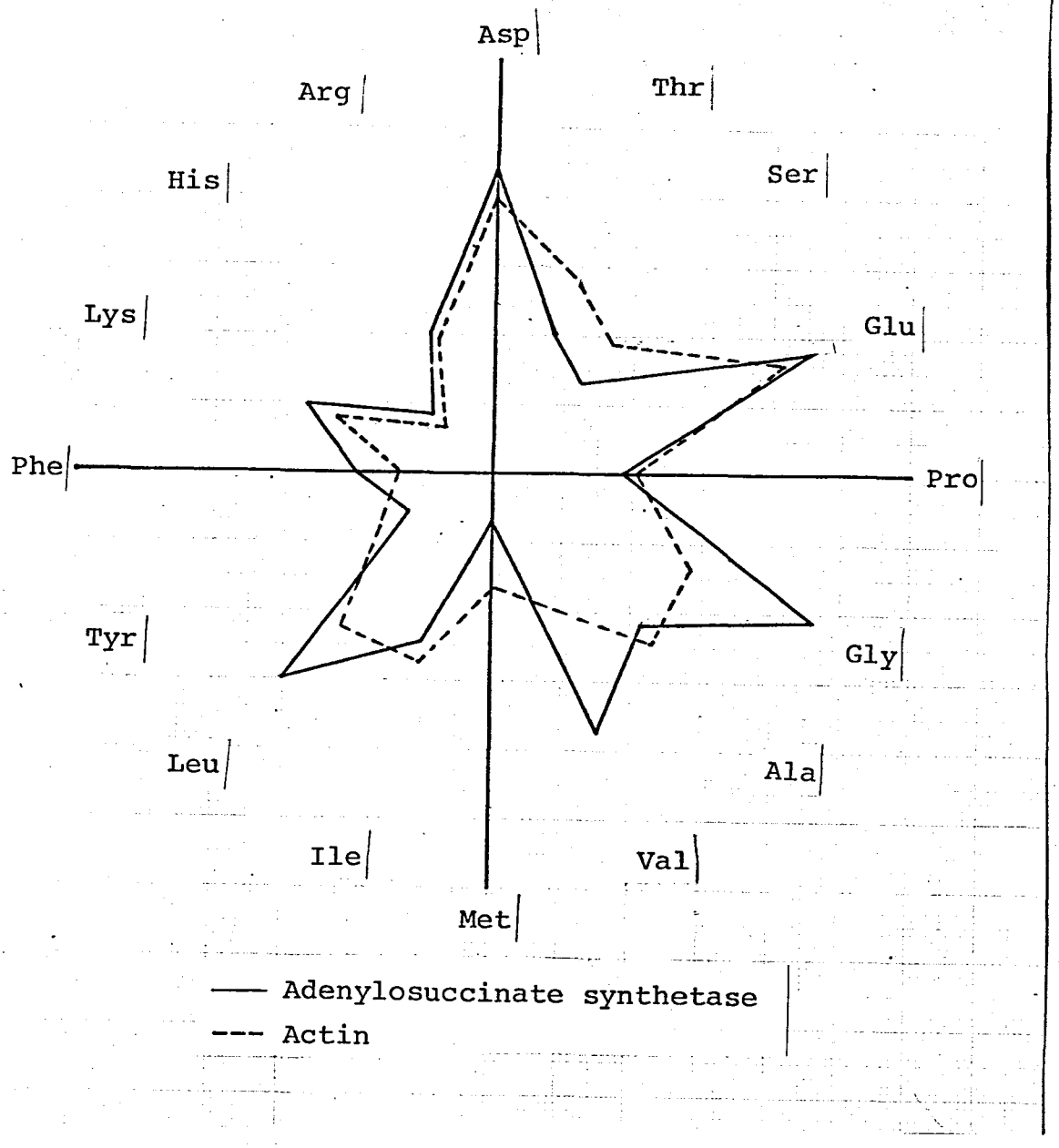

Diagram 1. Comparison of star-diagrams of amino acid compositions of adenylosuccinate | synthetase and actin. The star-diagram of amino acid composition of actin was prepared according to the data by Elzinga (Elzinga, M., Biochemistry, 9, 1365 (1970)).

論文内容の要旨

博士論文題名 STUDIES ON ENZYMES INVOLVED IN THE PURINE NUCLEOTIDE CYCLE IN RAT

(プリンヌクレオチドサイクルに関与する酵素の研究)

学位申請者 小川宏文

新しいアンモニア産生機構であるプリンヌクレオチドサイクル(略、サイクル)の生体内に於る代謝調節機構を明らかにすることを目的とした。即ちこのサイクルを構成する三つの酵素を単離し、その性質を検討することによって生体内での調節機構を考察した。

①.アデニロサクシネートシンテターゼ(AdSS)をラットの骨格筋から抽出し、熱処理、塩析、イオン交換及びゲルクロマトグラフィーによって100倍精製し、ダイヤ型の結晶化に成功した。この標品は物理化学的手法により、分子

量5万のサブユニット2個からなる解離会合型の蛋白であることが推定された。この酵素は種種のヌクレオシド三燐酸では影響を受けず、種種の二燐酸及び一燐酸で阻害される傾向をもつが、プリン代謝の分岐点に存在するにも拘らず、特異的な調節因子は見当らなかった。

②. AdSSは解糖系の中間体であるフルクトース二燐酸で著しく阻害を受けた。この非拮抗的阻害は解糖系のオシレーションと、サイクルのそれの同調性をもたらすものと解釈された。③. 骨格筋及び心筋のAdSSの等電点は8.8，肝臓及び癌細胞のそれは6.0の二つのアイソザイムの存在を明らかにした。④. アデニロサクシナーゼ(AdSase)は非常に不安定な酵素であるが、熱処理やグリセロール等の添加で精製を可能にした。⑤. 各組織のAdSaseの等電点はほぼ中性であり、しばしば酸性側にマイナーピークがみられた。いずれもKm値は他種のそれより100倍程大きかった。⑥ AdSaseは重金属イオン(Cd^{++}, Hg^{++}, Cu^{++}など)により著しく阻害

され、SH剤やEDTAの添加で活性が回復したので、この酵素反応にはSH基が重要な役割をもつことが示唆され、又この性質はサイクルを任意に抑制することを可能にした。⑦. 筋ホモジネート残渣から、新たに多量のAdssが抽出された。残渣中のアクチンに結合していたのである。そのモル比は塩のない条件で 1:6 (Adss:アクチン)に結合することが分った。肝臓のAdssは結合しなかった。⑧. Adssのアミノ酸組成はアクチンのそれと似ていた。特にLys, His, Arg, Asp, Glu, Pro, Ala, Ile含量は一致していた。⑨. AMPデアミナーゼも筋肉残渣より抽出され、ミオシンとの相互関係が示唆された。

以上、筋肉内でこのサイクルは、運動(筋収縮)に呼応して、そのエネルギーを供給する解糖系と連関しながら(オシレーション)、収縮後の酸性に傾いたpHを中和するために積極的にアンモニアを放出する機構である、という仮説を提出したい。