Purification of Adenylosuccinate Synthetase from Rabbit Skeletal Muscle*

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SUMMARY

Adenylosuccinate synthetase has been purified more than 250-fold from extracts of rabbit muscle acetone powder by heating to 60°, ammonium sulfate fractionation, phosphocellulose and hydroxylapatite chromatography, and Sephadex G-150 gel filtration. Crystals have been obtained by dialysis against 60 to 87% ammonium sulfate. Enzyme activity is stable for about 2 weeks in phosphate buffer containing 1 mM dithiothreitol; for longer periods, dialysis against saturated ammonium sulfate is preferable. The enzyme is quite basic, has a molecular weight of approximately 54,000, and does not appear to dissociate into subunits upon treatment with sodium dodecyl sulfate or mercaptoethanol. A broad maximum in enzyme activity occurs at pH ~ 6.6, and the kinetic parameters of the enzyme differ significantly from those of the Escherichia coli or Ehrlich ascites-tumor cell enzymes. $K_m$ values for IMP, GTP, and L-aspartate are $2 \times 10^{-4} M$, $1 \times 10^{-3} M$, and $3 \times 10^{-4} M$, respectively, for the muscle enzyme. GDP, AMP, adenylosuccinate, argininosuccinate, phosphate, arsenate, and sulfate inhibit enzyme activity. Enzyme activity is unaffected by incubation for 24 hours with EDTA, azide, and phenylmethylsulfonyl fluoride, but is completely destroyed by freezing or by 18-hour incubation with 5,5'-dithiobis(2-nitrobenzoic acid). Adenylosuccinate synthetase activity has also been found in crude extracts of acetone powders of rabbit heart, liver, kidney, brain, and lung.

Adenylosuccinate synthetase (IMP-L-aspartate ligase (GDP), EC 6.3.4.4) catalyzes the following reaction in the de novo biosynthesis of purines (Equation 1). Adenylosuccinate was shown to be an intermediate in the formation of AMP from IMP and aspartate (1, 2), and various kinetic and mechanistic studies were done with partially purified enzyme from Escherichia coli (3–8). Rudolph (9) obtained a more homogeneous preparation of the E. coli enzyme using ammonium sulfate fractionation and DEAE-cellulose and Sephadex G-100 chromatography. Davey (10) found adenylosuccinate synthetase activity in water extracts of rabbit liver and skeletal muscle, but none in extracts of heart, lung, and kidney. Atkinson and co-workers (11) studied the effect of thio-IMP on the kinetic parameters of partially purified adenylosuccinate synthetase from Ehrlich ascites-tumor cells. Because of its position in the pathways for recycling AMP by hypoxanthine-inosine salvage (12) or the purine nucleotide cycle (13) and as a branch point in de novo purine biosynthesis, the kinetics and regulation of adenylosuccinate synthetase are of potentially great interest.

$$\text{IMP} + \text{L-aspartate} + \text{GTP} \xrightarrow{\text{Mg}^{2+}} \text{Adenylosuccinate}$$

(1)

However, the purification of the enzyme from normal mammalian tissues necessary for such studies has not previously been carried out. This paper describes the preparation of nearly homogeneous adenylosuccinate synthetase from rabbit muscle.

EXPERIMENTAL PROCEDURE

Materials

Dithiothreitol (Cleland's reagent), D- and L-aspartic acid, L-glutamic acid, L-glutamine, L-malic acid, cellulose phosphate, imidazole, inosine, hypoxanthine, and the sodium salts of XMP, IMP, IDP, ITP, GTP, dGTP, GDP, GMP, CTP, UTP, and

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AMP were supplied by Sigma Chemical Co. (St. Louis, Mo.). N-2-Hydroxyethylphipera-zine-N'-2-ethanesulfonic acid (HEPES buffer) and the sodium salt of UMP were purchased from Calbiochem (Los Angeles, Calif.). L-Asparagine was obtained from K & K Laboratories (Plainview, N.Y.) and 5,5'-dithiobis(2-nitrobenzoic acid) from Aldrich Chemical Co. (Milwaukee, Wis.). Enzyme grade ammonium sulfate and phenylmethanesulfonyl fluoride were purchased from Schwarz-Mann (Orangeburg, N.J.). DEAE-cellulose (gel and paper) and carboxymethyl-cellulose were supplied by Reeve Angel (Clifton, N.J.), DEAE Sephadex and Sephadex G-150 by Pharmacia Fine Chemicals (Piscataway, N.J.), and Bio-Gel TFF (hydroxyapatite) by Bio-Rad (Los Angeles, Calif.). Sodium mono- and dihydrogen phosphate, magnesium chloride, manganese chloride, zinc chloride, calcium chloride, hydroxyamine hydrochloride, EDTA, sodium bicarbonate, sucrose, and sodium succinate were purchased from Baker (Phillipsburg, N.J.). Cellophane dialysis tubing was purchased from Fisher (Pittsburg, Pa.) and boiled in an EDTA-sodium bicarbonate solution before use. Ceptrilo membrane cones were purchased from Amicon (Lexington, Mass.). Muscle from young rabbits was obtained from Pel-Freeze (Rogers, Ark.) and stored at -20°.

**Methods**

**Assay and Definition of Unit—**A spectrophotometric assay similar to that of Rudolf (9) was used. The absorbance increase at 280 nm associated with adenylosuccinate formation was followed at room temperature (21–22°C) on a Gilford model 240 spectrophotometer equipped with a 50 mv Honeywell recorder. The reaction was carried out in a quartz cuvette with a 1-cm light path. To 2.0 ml of 50 mM pH 7.0 HEPES1 buffer were added 10 μl of 50 mM IMP, 10 μl of 25 mM GTP, 20 μl of 0.8 mM MgCl2, and an appropriate amount of enzyme, usually 5 to 100 μl. This was mixed by covering with Parafilm and inverting several times, and its absorbance offset to zero on the recorder. Then 20 μl of 0.40% L-aspartate (pH 7) were mixed with the solution in the cuvette, the cuvette replaced in the light path, and the change in absorbance at 280 nm followed for 3 to 6 min (chart speed ¼ inch per min, full scale absorbance 0.250). The reaction was linear over this period of time; changes in absorbance from 0.010 to 0.100 absorbance units could be reproducibly observed, and typical rates were on the order of 0.0020 to 0.0300 Å per min. No reaction occurred if any one of the above reagents was missing. One unit of activity was defined as the amount of enzyme necessary to give an increase in absorbance at 280 nm of 1.0 absorbance unit per min. Specific activity was then defined as units of activity per mg of protein. Protein concentrations were estimated as milligrams of protein per ml = 1.55 × Abs0 - 0.76 × Abs1 (14). Concentrations of the nucleotide stock solutions were determined from the absorbances measured at their characteristic absorption maximum (15).

**Preparation of Rabbit Muscle Acetone Powder—**Two pounds of rabbit muscle were thawed, cut in small pieces, and as much connective tissue as possible removed, all at 0°. The muscle was then blended with 4 to 5 volumes of cold (-20°) reagent grade acetone at high speed for 1 to 2 min in a commercial Waring Blender. The resulting slurry was rapidly filtered on a Buchner funnel and the filtrate discarded. The cake was again blended with 4 to 5 volumes of cold acetone and filtered. The second cake was spread on Whatman No. 3MM chromatography paper and allowed to air dry, then stored at -20° until use. Typically about 150 to 200 g of dry acetone powder were obtained. The adenylosuccinate synthetase appeared quite stable to storage in this form. Initial experiments carried out with a powder 18 months old gave total activities ½ to ¾ those obtained with powders used within a month after preparation.

**Polyacrylamide Gel Electrophoresis—**Throughout the purification, polyacrylamide gel electrophoresis was used as a sensitive gauge of the extent of purification. Initially, the procedure used was that of Ornstein and Davis (16), with 7% polyacrylamide gels, pH 8.3 Tris-glycine buffer and 20 to 100 μg of protein. Enzyme activity was found to be stable for at least 72 hours in the running buffer. However, after the fourth step in the purification, all the protein appeared to migrate as a single broad band when this method was used, remaining very close to the top of the gel. Since column chromatography clearly indicated that the protein mixture was not homogeneous at this stage, sodium dodecyl sulfate gel electrophoresis (17) was substituted for the Ornstein-Davis procedure. Samples of 1 to 50 μg of protein were digested with 1% sodium dodecyl sulfate and 1% β-mercaptoethanol for 1 hour at 45° in 10 mM pH 7 phosphate buffer. The digested sample was then layered on the gel and run in 50 mM pH 7 phosphate buffer containing 0.5% sodium dodecyl sulfate. The gels were stained with 0.25% Coomassie brilliant blue, destained in methanol-acetic acid, and stored in 7% acetic acid. Using this method, several bands could be distinguished on samples where only a single broad band had appeared with the Ornstein-Davis procedure.

**RESULTS**

**Purification**

All procedures, except as otherwise stated, were carried out at 0–4°. Buffer pH values were measured at room temperature. Deionized water was used for all solutions. Centrifugations, unless otherwise specified, were done at approximately 25,000 × g in a Sorvall RC2-B refrigerated centrifuge (15,000 rpm with SS-34 head or 12,000 rpm in GSA head, depending on the volume of material). Enzyme grade ammonium sulfate was used throughout. Enzyme activity was found to be relatively stable at 0° for up to several weeks in 5 mM pH 7 sodium phosphate buffer containing 1 mM dithiothreitol; "buffer," unless otherwise stated, refers to this system.

A typical preparation of adenylosuccinate synthetase from rabbit muscle is described in detail below and summarized in Table 1. Similar results have been obtained several times with different acetone powder preparations.

**Step 1: Extraction of Acetone Powder—**Rabbit muscle acetone powder (66 g) was stirred gently for 2½ hours (using a magnetic stirrer) with 660 ml of a solution 50 mM in KCl and 1 mM in dithiothreitol. The resulting slurry was centrifuged for 20 min and the pellet discarded. The supernatant volume was 536 ml. Estimation of protein concentrated by ultraviolet absorption was inaccurate at this stage because of the large absorption occurring at 260 nm, presumably due to free nucleotides in the extract. Dialysis of a small amount of supernatant against 50 mM KCl11 mM dithiothreitol removed most of these (18) and allowed a more accurate determination of the protein concentration. Loss of activity on dialysis was quite small since the solution was very concentrated at this point. Assay for the enzyme adenylosuccinate lyase (which converts adenylosuccinate to AMP) showed almost no adenylosuccinate lyase activity present in this preparation. This could have been due to inactivation of the lyase during preparation of the acetone powder, or possibly the

1The abbreviation used is: HEPES, N-2-hydroxyethylpipera-zine-N'-2-ethanesulfonic acid.
muscle lase is strongly inhibited by Hepes buffer as is the lase from human red blood cells.2

Step 2: Removal of Inactive Protein by Heat. The acetone powder supernatant from Step 1 was divided into two approximately equal batches, and each batch heated separately to an internal temperature of 60-61°C for 1 min. The solution was quickly cooled to 5°C in an ice bath, centrifuged, and the resulting pellet discarded. The volume of supernatant was 510 ml. Dialysis against 50 mM KCl-1 mM dithiothreitol was necessary before ultraviolet absorption could be used for an accurate estimation of protein concentration.

Step 3: Fractionation with Ammonium Sulfate. The heated supernatant from Step 2 was stirred in an ice bath as a total of 114 g of powdered (NH₄)₂SO₄ was slowly added (22.6 g of (NH₄)₂SO₄ per 100 ml of heat step supernatant). The resulting cloudy solution was centrifuged for 20 min and the pellet discarded. The supernatant was again stirred in an ice bath as a total of 2.7 g (5.8 g/100 ml of enzyme solution) of powdered ammonium sulfate was slowly added. The resulting very cloudy solution was centrifuged for 20 min. The supernatant was discarded and the pellets redissolved in a minimum volume of buffer. The redissolved pellets were then dialyzed overnight against two changes of 10 volumes of buffer. The resulting dialysate had a volume of 28.0 ml. The absorption at 290 nm was about half that at 280 nm and protein concentration was estimated without further dialysis against KCl. This was also true for all the following steps.

These first three steps were generally all carried out the same day, but the enzyme activity appeared reasonably stable in 50 mM KCl-1 mM dithiothreitol for at least 2 days.

Step 4: Phosphocellulase Chromatography. The dialysate from Step 3 was layered on a phosphocellulose column (1.5 cm × 26 cm), bed volume 45 ml) which had previously been equilibrated with 5 mM buffer. The column was washed with approximately 90 ml of the same buffer, during which time a large amount of inactive yellowish protein was eluted. Elution was continued with a gradient consisting of 200 ml of 5 mM NaP-1 mM dithiothreitol pH 7.0 buffer and 200 ml of 102.5 mM NaP-1 mM dithiothreitol pH 7.0 buffer at a flow rate of 50 ml per hour. Enzyme activity was eluted at approximately 62.5 mM buffer as a shoulder on the high concentration side of a large inactive protein peak. The total volume of the combined active fractions was 340 ml. The total activity recovered was only 42% of the total activity initially put on the column, but no further activity was recovered even on elution with up to 0.5 M buffer.

Step 5: Hydroxylapatite Chromatography. Approximately 4 g of dry Bio-Gel HTP powder were equilibrated with 70 mM buffer and the fines decanted. A slurry of the pre-equilibrated hydroxylapatite in a minimum amount of 70 mM buffer was poured directly into the combined active fractions from Step 4 and let stand for 90 min, swirling gently several times. After the gel was allowed to settle, the supernatant liquid showed no activity. The supernatant was then decanted into a 1.5 cm column which had a small (~ 0.5 ml) layer of hydroxylapatite over a 3/4 inch layer of well packed glass wool. Finally, the hydroxylapatite-enzyme slurry was rinsed into the column and the column (15 ml bed volume, flow rate ~ 16 ml per hour) was rinsed with about 15 ml of 70 mM buffer. The column was then eluted with a linear gradient consisting of 100 ml of 70 mM buffer and 100 ml of 0.50 M KCl 1 mM dithiothreitol pH 7 buffer. Enzyme activity was eluted at approximately 125 mM buffer on the high concentration side of a larger inactive protein peak. The total volume of combined active fractions was 45.5 ml. Approximately 86% of the total activity put on the hydroxylapatite column could be recovered even after several days on the column.

Step 6: Concentration by (NH₄)₂SO₄ Precipitation. The combined active fractions from Step 5 were stirred gently at 0°C as 27 g (60 g/100 ml of enzyme solution) of powdered ammonium sulfate were slowly added. The suspension was let stand for 90 min after addition was completed and then centrifuged for 20 min. The supernatant was discarded and the pellet redissolved in a minimum amount of 5 mM buffer. The dissolved enzyme was centrifuged 15 min and the supernatant used in the next step.

Step 7: G-150 Gel Filtration. The clarified redissolved ammonium sulfate pellet from Step 6 was layered on a Sephadex G-150 column (2 × 56 cm) which had been previously equilibrated with 5 mM buffer. The enzyme was then eluted with the same buffer at a flow rate of ~ 7 ml per hour. Enzyme activity emerged coincident with a well defined protein peak at 92 ml. The elution volume was found to be 1.8 × V₀ in general, even with different columns. The elution profile is shown in Fig. 1. The combined active fractions had a volume of 15.75 ml and contained 67% of the activity put on the column. Sodium dodecyl sulfate gel electrophoresis showed a single heavy band which ran slightly ahead of a bovine serum albumin standard (mol wt 68,000) and two barely visible slower bands. Enzyme activity was found to be stable for at least 8 weeks of storage as a precipitate under (NH₄)₂SO₄, after dialysis against a saturated ammonium sulfate solution.

Crystallization of Enzyme. About 27 ml of G-150 active fractions (from a different preparation than the above, approximately 14 mg of protein) were dialyzed for 24 hours against about 200 ml of 60% saturated ammonium sulfate. At approximately 24-hour intervals, 10 ml increments of saturated ammonium sulfate were added until the dialysis solution was 65 to 67% saturated. At this point, small diamond-shaped crystals of enzyme were present. These were centrifuged, redissolved in 4 to 5 ml of buffer, and recrystallized twice in the same manner. More than 90% of the initial activity was present at a specific activity of 13.8 units per mg, representing an increase of about 65% over the original G-150 active fractions, even after storage.
under 67% ammonium sulfate for 2 months. A photomicrograph of the crystals is shown in Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the twice recrystallized enzyme showed a single band.

Properties

Identification of Adenylosuccinate as Reaction Product—A reaction mixture containing 250 \( \mu \text{M} \) IMP, 125 \( \mu \text{M} \) GTP, 8 \( \text{mM} \) MgCl\(_2\), 4 \( \text{mM} \) L-aspartate and approximately 40 \( \mu \text{g} \) of enzyme in 50 \( \text{mM} \) pH 6.6 HEPES was allowed to go to about 50% completion, as determined by \( \Delta A_{280} \). A difference spectrum using a blank containing no L-aspartate showed a maximum at 270 nm (\( \lambda_{\text{max}} = 288 \text{ nm} \) for adenylosuccinate at pH 7; \( \lambda_{\text{max}} = 284 \text{ nm} \) for IMP at pH 6; \( \lambda_{\text{max}} = 252 \text{ nm} \) for GTP and GDP at pH 7). In addition, a reaction mixture containing 2 \( \text{mM} \) IMP and GTP, 8 \( \text{mM} \) MgCl\(_2\) and L-aspartate, and 200 \( \mu \text{g} \) of enzyme gave an ultraviolet absorbing spot with the same \( R_T \) as an adenylosuccinate standard when chromatographed on Whatman No. 1 paper in a 1:4:10 mixture of acetic acid-water-acetone (19). A reference mixture containing no L-aspartate showed no spot for adenylosuccinate. This system separates IMP, adenylosuccinate and GTP, and GDP cochromatographs with GTP.

Molecular Weight—The molecular weight of the enzyme was estimated by use of a Sephadex G-150 column (2 \( \times \) 58 cm) according to Andrews (20). The G-150 column was calibrated after Step 7 of the purification procedure as follows. One milliliter of a solution containing 3 \( \text{mg} \) per ml of each of aldolase (mol wt 158,000), ovalbumin (mol wt 43,000), and RNase (mol wt 13,700) in 5 \( \text{mM} \) buffer (9 mg of protein total) was layered on the column and the protein eluted with the same buffer. The elution volume of each protein peak was determined by following the absorbance at 280 nm. A plot of the elution volume versus the log of the molecular weight gave an approximate molecular weight of 54,000 for adenylosuccinate synthetase. The molecular weight was also determined by a method similar to that of Weber and Osborn (17), using sodium dodecyl sulfate polyacrylamide gel electrophoresis with bovine serum albumin (mol wt 68,000), ovalbumin (mol wt 43,000), and chymotrypsin (mol wt 25,000) as standards. A molecular weight of 54,000 to 56,000 was obtained by this method for the twice recrystallized enzyme, both in the presence and absence of mercaptoethanol.

pH Optimum—Plots of activity of the G-150 active fractions versus pH showed a rather broad maximum at pH 6.6 (half-maximal activities at pH 5.4 and pH 7.7) in both 50 \( \text{mM} \) HEPES and 50 \( \text{mM} \) imidazole buffer, although the actual activities were about 25% lower in imidazole than in HEPES. Liebermann (3) found the pH optimum for the E. coli enzyme to be 7.3 to 7.5 in glycine buffer, and Davey (10) found the pH optimum for the crude rabbit muscle enzyme to be 6.9 to 7.5 in imidazole buffer.

Enzyme Inactivation—Preliminary experiments showed that incubation with 2 \( \text{mM} \) EDTA for 24 hours did not affect enzyme activity. Incubation of about 75 \( \mu \text{g} \) of enzyme in 5 \( \text{mM} \) buffer with 90 \( \mu \text{g} \) phenylmethanesulfonyl fluoride, 0.2% sodium azide, or 0.9 \( \text{mM} \) hydroxylamine hydrochloride also caused no deterioration in enzyme activity over a 4-day period at 4°C, although the hydroxylamine hydrochloride possibly caused a very slight inhibition. However, incubation under the same conditions for 18 hours with 2.2 \( \mu \text{M} \) DTT caused complete loss of enzyme activity. Heating at temperatures above 60°C for any length of time, freezing, dialysis of dilute solutions, or use of membrane-type ultrafilters caused almost complete inactivation. Nearly complete loss of activity also occurred within 6 to 8 days upon standing in dilute solution or within 2 days upon omission of dithiothreitol from the buffer.

Substrate Specificity—A reaction mixture containing 0.25 mM IMP, 0.125 mM GTP, 4 \( \text{mM} \) L-aspartate, 8 \( \text{mM} \) MgCl\(_2\) and approximately 2 \( \mu \text{g} \) of enzyme in 2.00 ml of 50 \( \text{mM} \) pH 6.6 HEPES was used as a standard. No detectable reaction occurred upon replacement of IMP by 0.25 mM inosine, hypoxanthine, IDP, ATP, GDP, GMP, ITP, UTP, CTP or ATP or by 0.125 mM XMP (more could not be used because of the large \( A_{280} \)). Replacement of GTP by 0.125 mM ATP, GDP, GMP, IDP, ITP, UTP, or CTP also gave no reaction. However, 0.125 mM dITP gave a reaction rate approximately 40% that of the standard. No reaction was detectable when L-aspartate was replaced by 4 \( \text{mM} \) succinate, d-aspartate, l-glutamate, L-glutamine, L-asparagine, or L-malate. Replacement of MgCl\(_2\) by 8 \( \text{mM} \) MnCl\(_2\) or CaCl\(_2\) gave reaction rates approximately 15% and 20% the rate of the standard, respectively.

Preliminary Kinetic Experiments—\( K_m \) values for IMP, GTP, and L-aspartate in 50 \( \text{mM} \) pH 6.6 HEPES are summarized in Table II and typical double reciprocal plots are shown in Fig. 3. The plots for IMP showed definite upward curvature at high concentrations of IMP, possibly indicating substrate inhibition. It was not entirely clear whether this inhibition was GTP-dependent, although there was some indication that it was less
pronounced at lower GTP concentrations. About 25 to 50% larger values for $K_m$ were obtained in 50 mM pH 6.6 imidazole and pH 6.1 HEPES, and about 50 to 100% larger values in 90 mM pH 7.8 HEPES. $K_m$ for dGTP in pH 6.6 HEPES was about 100 times that for GTP, but the $V_{max}$ values were essentially identical.

As expected, adenylosuccinate, GDP, P_i, and AMP inhibit enzyme activity. Dixon (21) plots show $P_i$ to be a noncompetitive inhibitor for IMP, GTP, and L-aspartate with $K_i$ values of 17 mM, 12 mM, and 42 mM, respectively. Both Dixon and Lineweaver-Burk (22) plots indicate that GDP is a competitive inhibitor for GTP with $K_i = 7 \times 10^{-4}$ mM, but a noncompetitive inhibitor for IMP and L-aspartate with $K_i \approx 3 \times 10^{-3}$ mM for both. Adenylosuccinate and AMP produce significant inhibition at concentrations in the 0.1 to 1.0 mM range, while arginosuccinate and arsenate must be present at 1.0 mM concentrations or greater to cause marked inhibition. Incubation of approximately 100 µg of enzyme with 25 mM KCl, NH_4Cl, or NaAc for 1 hour followed by assay in a medium 10 mM in the same salt had no effect on enzyme activity. However, enzyme incubated and assayed with the same amounts of NaSO_4 or (NH_4)_2SO_4 had an activity 70% that of a control containing no added salt. This effect was reversible, and apparently due to sulfate inhibition since enzyme incubated with 25 mM NaSO_4 or (NH_4)_2SO_4 but assayed in 0.6 mM salt gave the same reaction rate as the control. This is consistent with a small increase in activity usually noted upon dialysis after Step 3 (ammonium sulfate fractionation); it also accounts for the apparently lower recoveries after ammonium sulfate precipitation of the hydroxylapatite active fractions since the concentrate was not dialyzed before G-150 filtration.

**TABLE II**

$K_m$ values for adenylosuccinate synthetase substrates in 50 mM pH 6.6 HEPES

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMP</td>
<td>(2 ± 1) x 10^{-4}</td>
<td>$K_m$ not dependent on [IMP] or [aspartate]</td>
</tr>
<tr>
<td>GTP</td>
<td>(1 ± 0.5) x 10^{-4}</td>
<td>$K_m$ not dependent on [GTP] or [aspartate]</td>
</tr>
<tr>
<td>dGTP</td>
<td>(3 ± 1) x 10^{-3}</td>
<td>$K_m$ not dependent on [IMP] or [aspartate]</td>
</tr>
<tr>
<td>L-Aspartate</td>
<td>(3.0 ± 1.5) x 10^{-4}</td>
<td>$K_m$ not dependent on [IMP] or [GTP]</td>
</tr>
</tbody>
</table>

DISCUSSION

The molecular weight of the adenylosuccinate synthetase from rabbit muscle appears to be very similar to that of the E. coli enzyme (9), although the pH optimum is significantly lower (3). The rabbit muscle enzyme also appears to be more basic than the E. coli enzyme since it will not stick to DEAE-cellulose, sticks quite well to phosphocellulose and carboxymethylcellulose, and migrates very slowly in the high pH buffers used for Ornstein-Davis gel electrophoresis. The $K_m$ values for GTP and aspartate are nearly the same for both enzymes, but $K_m$ for IMP is 10 times larger for the rabbit muscle enzyme than for the E. coli enzyme (7). All of the $K_m$ values for the rabbit muscle enzyme differ significantly from those reported for the Ehrlich ascites-tumor cell enzyme (11) at pH 7.5 in glycine buffer. Although the assay conditions differed somewhat, it seems likely that the enzymes from the tumor and rabbit muscle are in fact kinetically different, particularly since GDP causes no inhibition of the tumor enzyme at concentrations on the order of its $K_i$ for the muscle enzyme. There appears to be some substrate inhibition of the muscle enzyme by IMP at high concentrations. Although Lowenstein (13) has suggested that this type of inhibition is strongly GTP-dependent, we find no evidence for such an effect in our system. Since $K_m$ for IMP and $K_i$ for AMP for the rabbit muscle enzyme are on the order of concentrations found in the cell, enzyme activity may be quite dependent on changes in cellular concentrations of these two compounds, affording a very sensitive mechanism for the control of AMP synthesis.

Kinetic studies (7) on the E. coli enzyme have led to postulation of a rapid equilibrium random mechanism for that system. 300 studies have also suggested formation of phosphorylated IMP as an intermediate in the reaction (3). Both the ability of di-thiotreitol to protect enzyme activity and the ability of DTNB to cause complete loss of enzyme activity suggest that sulfhydryl groups are essential to enzyme activity. Further kinetic and mechanistic studies are currently in progress on the rabbit muscle enzyme to attempt to find direct evidence for such a phosphoryl-

![Fig. 2](http://www.jbc.org/)

*Fig. 2.* $K_m$ for IMP, GTP, and L-aspartate (200-ml reaction volume; 50 mM pH 6.6 HEPES buffer; 10 µl enzyme per assay; $1/V$ has units of $(\text{min} / \text{mM}) \times 10^{-2}$). (a) 125 µM GTP, 4 mM L-aspartate, 8 mM MgCl_2; (b) 250 µM IMP, 4 mM L-aspartate, 8 mM MgCl_2; (c) 250 µM IMP, 125 µM GTP, 8 mM MgCl_2.
ated intermediate, and to determine how adenylosuccinate, AMP, and related compounds control the relative amounts of de novo synthesis and recycling.

Although Davey (10) found no adenylosuccinate synthetase activity in water extracts of rabbit heart, lung, and kidney, we have found such activity in KCl extracts of acetone powders of rabbit brain, lung, heart, kidney, and liver. In preliminary studies, the activities in heart, kidney, and liver are about 40% those found in muscle, while the activities in brain and lung are only 10 to 20%. It should be noted that KCl extracts of fresh tissue homogenates of the above organs became turbid on addition of MgCl₂ to the enzyme in pH 6.6 HEPES and showed little or no adenylosuccinate synthetase activity. This was also true of KCl extracts of fresh muscle homogenates. Kinetic studies on liver enzyme purified through Step 6 indicate that the Kₘ values for IMP, GTP, and L-aspartate are nearly identical to those found for the muscle enzyme but very different from those reported for the enzyme from the Ehrlich mammary-de-rived tumor (11).

15N tracer experiments with 15NH₄⁺ in rats and rabbits indicate that similar amounts of 15N are incorporated into the 6-amino and ring nitrogens in liver adenine nucleotides (23), while in muscle the 15N enrichment at the 6-amino position is 70 to 200 times that of the ring nitrogens (19). These results may be indicative of significant tissue differences in the amounts of adenine nucleotides synthesized by the de novo and salvage pathways in muscle and liver and represent the strongest evidence for the in vivo functioning of the purine nucleotide cycle in muscle (24). Since adenylosuccinate synthetase is involved in both pathways, a comparison of the control mechanisms of the liver, liver tumor, and muscle enzymes would be most interesting.

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