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ON THE EQUILIBRIUM AND MECHANISM OF ADENYLOSUCCINIC ACID SYNTHESIS*

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CARTER AND COHEN reported the isolation of adenylosuccinate** from yeast and demonstrated its enzymic synthesis from A5P and fumarate[1]. Studies of the yeast system indicated the equilibrium-favored AMP-S cleavage[2]. More recently, the existence of AMP-S has been demonstrated in mammalian tissue[3]. LIEBERMAN[4,5] reported that a highly purified enzyme preparation (adenylosuccinate synthetase) from *Escherichia coli*, strain B, was capable of converting I5P, GTP and L-aspartate to AMP-S, GDP and P_i. When 6-^{18}O-I5P was employed as a substrate, the isotope appeared exclusively in the P_i, suggesting the formation of a phosphorylated intermediate[5]. Although a number of mechanisms are in harmony with this observation a choice between them could not be made from these data alone. In the present paper evidence will be presented which may permit such a choice to be made from among those mechanisms consistent with available data.

MATERIAL AND METHODS

Materials

All nucleotides were commercial preparations. I5P, GTP and GDP were obtained as the crystalline sodium salts and further purified by anion-exchange chromatography. Nucleotide concentrations were determined from their spectral properties and ribose: phosphate ratios. GTP and GDP were further characterised by their labile phosphorous. AMP-S was synthesized enzymically from A5P and fumarate and isolated according to the suggestion of COHEN AND CARTER[2]. L-aspartate-^{14}C was a product of Schwartz Laboratories.

Preparation and assay of adenylosuccinate synthetase

The enzyme was obtained from *E. coli*, strain B, by the method of LIEBERMAN[5]. Enzymic activity was assayed by measuring the increase in optical density at 280 mμ with the Beckman DU Spectrophotometer in reaction mixtures containing 200 μmoles glycine buffer (pH 8.0), 4 μmoles $MgSO_4$, 0.02 μmole GTP, 0.30 μmole I5P, 1.0 μmole L-aspartate, 0.84 mg streptomycin sulfate

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** The following abbreviations are used; adenylosuccinic acid, AMP-S; inosine-5'-phosphate, I5P; guanosine triphosphate, GTP; guanosine diphosphate, GDP; adenosine-5'-phosphate, A5P; inorganic orthophosphate, P_i; perchloric acid, PCA; adenosine triphosphate, ATP.

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and the enzyme in a volume of 1.4 ml. Incubation was at 37° for 30 min and the reaction was terminated with 1.0 ml 7% PCA. Aspartate was added to control mixtures after the addition of PCA. A unit of enzyme solution was considered to be the amount necessary to cause an optical density increase of 0.100 at 280 mμ. Protein was determined turbidimetrically[6]. All preparations of adenylosuccinate synthetase were stored at 3° and maintained their activity for at least 1 month.

Isolation of nucleotides

Reaction mixtures were terminated either by the addition of 7% PCA or by boiling for 2 min at 100° when samples were to be analyzed by chromatography. Aliquots were chromatographed at 3° on Dowex-1 Cl^- columns (3.0 cm high, 1.0 cm diameter, 2% cross-linkage, 200–400 mesh). Aspartate and I5P were eluted with 0.01 *N* HCl, AMP-S with 0.01 *N* HCl–0.015 *N* KCl and GDP and GTP with 0.01 *N* HCl–0.035 *N* KCl. AMP-S-^{14}C and aspartate-^{14}C were separated at 3° on Dowex-1 Cl^- columns (2.5 cm high, 1.0 cm diameter, 2% cross-linkage, 200–400 mesh) with 100 ml 0.01 *N* HCl. AMP-S-^{14}C was eluted from the columns with 5 ml of 1 *N* HCl. Radioactivity, corrected for self-absorption, was determined with a Geiger-Müller tube (^{32}P) and a Tracerlab windowless Flow Counter (^{14}C).

RESULTS AND DISCUSSION

On the basis of those data obtained from studies with 6-^{18}O-I5P at least four mechanisms for the synthesis of AMP-S are possible: The phosphorylation of I5P by GTP (nucleophilic attack on carbon 6 [7]) to form 6-phosphoryl-I5P which then reacts with aspartate to form AMP-S; reaction of GTP and aspartate to yield N-phosphoaspartate followed by a simultaneous double-displacement reaction[7] with I5P to produce AMP-S; phosphorylation of the enzyme by GTP, reaction of the phosphoryl enzyme with I5P producing the E-I5P intermediate, followed by enzyme displacement by aspartate in the synthesis of AMP-S; and the addition of aspartate amino nitrogen to the 6-carbonyl carbon of I5P, phosphorylation of the intermediate carbon 6 hydroxyl by GTP and subsequent P_i elimination[10].

Equilibrium studies

It has been suggested that the mechanism of three substrate systems may be elucidated from equilibrium-exchange studies[10]. For example, if the following mechanism were operating and the system allowed to come to equilibrium, the reaction mechanism could be investigated.

$$\text{I5P} + \text{GTP} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \text{6-phosphoryl-I5P} + \text{GDP} \qquad (1)$$

$$\text{6-phosphoryl-I5P} + \text{L-aspartate} \underset{k_4}{\overset{k_3}{\rightleftarrows}} \text{AMP-S} + P_i \qquad (2)$$

The maximal possible rate of L-aspartate $\longrightarrow$ AMP-S is equal to $-k_3$ (6-phosphoryl-I5P) (L-aspartate) $+k_4$ (AMP-S) (P_i). The maximal possible rate of $P_i \longrightarrow$ GTP is equal to $-k_4$ (AMP-S) (P_i) $+k_3$ (6-phosphoryl-I5P) (L-aspartate) or $-k_2$ (6-phosphoryl-I5P) (GDP) $+k_1$ (I5P) (GTP) depending upon which step is rate limiting. Thus L-aspartate $\longrightarrow$ AMP-S $= P_i \longrightarrow$ GTP if the last step in the sequence was rate limiting while L-aspartate $\longrightarrow$ AMP-S $> P_i \longrightarrow$ GTP if step (1) was rate limiting. Under no conditions could $P_i \longrightarrow$ GTP $>$ L-aspartate $\longrightarrow$ AMP-S.

Before attempting the mechanistic analysis described above, it was necessary to establish the equilibrium constant for the reaction. Repeated attempts to attain equilibrium in either direction with large amounts of enzyme were unsuccessful, initially. Furthermore, neither of the equilibria could be shifted by the addition of

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substrate or product. It was noted that the enzyme preparations used[5] contained appreciable quantities of adenylosuccinate-cleavage enzyme[1,2] activity. Repeated attempts to remove the contaminant according to the procedure of LIEBERMAN[5] proved unsuccessful. Preparations o adenylosuccinate synthetase which were devoid of, or contained trace amounts of AMP-S hydrolytic activity were consistently prepared in the following manner: The enzyme solution from the "low-pH fraction I"[5] was diluted with water to a protein concentration of 1.75 mg/ml at room temperature. 0.70 ml calcium phosphate gel[11] (23 mg solids/ml) was added per 5 ml diluted solution and the pH adjusted to 6.0. The suspension was stirred and immediately centrifuged at 2000 × *g* at room temperature. The supernatant solution was further purified with $(NH_4)_2SO_4$ and low pH fractionation[5]. The specific activity and yield of the modified preparation was found to be comparable to those used by other investigators[5].

In Table I and Figs. 1 and 2 are presented data on determinations of the apparent equilibrium constant (K_{eq}) in the forward direction. The average value obtained under the conditions described at pH 8.0 and 37° with varying substrate concentrations was 2.9 in the forward direction. It was found that the adenylosuccinate-cleavage enzyme activity was present in System 3 while a small amount was present in System 5. This is readily seen from the figures and Table I. The results obtained with System 3 suggest an attainment of equilibrium, *i.e.*, degradation of a product with a compensatory shift in the equilibrium. The data shown in Fig. 2 indicate that System 5 could be driven to the right by the addition of enzyme after 65 min. Adding enzyme after 120 min did not cause the synthesis of additional AMP-S. It might be well to mention that the stoichiometery of the reaction is well established[4,5]. It was also noted that all preparations of the enzyme contained some "GTP'ase" activity, most of which was removed by "low-pH treatment II"[5]. By compensating for this activity in complete systems minus L-aspartate it was possible to determine GDP

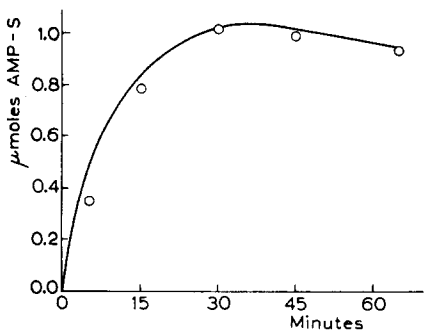

Fig. 1. Synthesis of AMP-S with time; equilibrium approached from the forward direction. The contents of the reaction mixture are described in Table I–System 3. AMP-S was estimated spectrophotometrically (E_m 290 mμ at pH 1 = $3.75 \cdot 10^3$). AMP-S was isolated by chromatography after 60 min and estimated spectrophotometrically (E_m 267 mμ at pH 1 = $16.9 \cdot 10^3$).

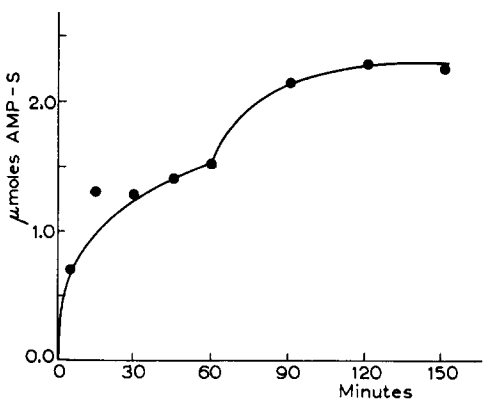

Fig. 2. Synthesis of AMP-S with time; equilibrium approached from the forward direction. The contents of the reaction mixture are described in Table I–System 5. AMP-S was estimated spectrophotometrically (E_m 290 mμ at pH 1 = $3.75 \cdot 10^3$). 260 units enzyme added at 65 and again at 120 min after initiation of reaction.

The falling off of the curve after 30 min is believed to be due to adenylosuccinate-cleavage activity[1] in the enzyme preparation.

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TABLE I

EQUILIBRIUM STARTING WITH GTP, I5P AND L-ASPARTATE*

System	Added initially			Equilibrium values						Time of incubation min	Enzyme units	K_{eq}
	GTP μmoles	I5P μmoles	L-Aspartate μmoles	GTP[4] μmoles	I5P[5] μmoles	L-Aspartate[5] μmoles	AMP-S[1] μmoles	GDP[2] μmoles	P_i[3] μmoles			
1	2.13	0.93	2.00	1.22	0.22	1.29	0.68	0.91	1.09	180	93	2.0
2	1.42	1.39	2.00	0.33	0.58	1.19	0.75	1.09	1.19	240	78	4.3
3	3.30	1.89	2.00	1.95	0.63[6]	0.79	0.94	1.35	1.59	65	124	2.1
4	4.40	0.95	2.00	3.43	0.07	1.12	0.66	0.97	1.22	60	124	2.9
5**	4.35	3.78	4.00	1.30	2.36	2.36	2.00	3.10	3.53	60	130	3.0
										90	260	

* In addition the systems contained 100 μmoles glycine buffer (pH 8.0), 0.9 mg streptomycin sulfate, 25 μmoles $MgSO_4$. L-Aspartate was omitted in the blank. Incubation was carried out at 37° for the period indicated in Table 1.

[1] AMP-S was determined chromatographically assuming E_m 267 mμ at pH 1 = $16.9 \cdot 10^3$ [1]. Values within 5% of those indicated were obtained by A_{290} measurements at pH 1 by assuming E_m 290 mμ = $3.75 \cdot 10^3$.

[2] GDP was estimated by subtracting P_i from GTP present initially.

[3] P_i was determined by the procedure of TAUSSKY AND SHORR[8].

[4] Residual GTP was calculated as follows: initial GTP — Final P_i + initial P_i.

[5] I5P and L-aspartate were estimated as follows: Final P_i — Final blank P_i.

[6] Isolated and found to be 0.62 μmole.

** System 5 incubated 60 min with 130 units enzyme and incubation continued for 90 min with additional 260 units of enzyme. All components except aspartate were determined.

and residual GTP with certainty. Neither the specificity nor the properties of the phosphatase were investigated. However, the possibility that this activity is not a property of adenylosuccinate synthetase cannot be ruled out.

The K_{eq} in the reverse direction was found to be 10.0 as shown in Table II. Certain technical difficulties which were encountered in this determination require that more credence be placed on the constants determined in the forward direction.

TABLE II

EQUILIBRIUM STARTING WITH AMP-S, GDP AND P_i*

*Equilibrium values***						
AMP-S[1] *μmoles*	*GTP*[4] *μmoles*	*L-Aspartate*[2] *μmoles*	*I5P*[2,6] *μmoles*	*GDP*[5] *μmoles*	P_i[3] *μmoles*	K_{eq}
1.87	1.02	0.47	0.47	0.50	2.40	10.0

* The reaction mixture also contained 250 μmoles glycine buffer (pH 8.0), 25 μmoles $MgSO_4$, 0.9 mg streptomycin sulfate, $^{32}P_i$ ($5.15 \cdot 10^5$ counts/min), 78 units enzyme (spec. act. 250), 2.40 μmoles AMP-S, 0.78 μmole GDP, 1.75 μmoles P_i and 0.73 μmole GTP. AMP-S was omitted in the blank. The system was incubated for 90 min at 37°. An additional 91 units of enzyme were added and incubation continued for 75 min. Final total volume 2.10 ml.

[1] AMP-S was determined chromatographically assuming E_m 267 mμ at pH 2 = $16.9 \cdot 10^3$ (ref. [1]).

[2] Estimated from the incorporation of $^{32}P_i$ into GTP.

[3] P_i was determined by the procedure of TAUSSKY AND SHORR[8].

[4] Determined as follows: initial GTP + (GTP-^{32}P)-P_i in blank.

[5] Estimated as follows: initial GDP — (GTP-^{32}P) + GDP in blank.

** (GTP-^{32}P) was calculated as Norit-adsorbable ^{32}P after subtracting blank activity[9].

[6] Determined after chromatography to be 0.50 μmole assuming E_m 248 mμ at pH 3 = $12.2 \cdot 10^3$.

It was necessary to include GTP in this system as the phosphatase activity, although small, was found to markedly interfere with the incorporation of $^{32}P_i$ into GTP at low concentrations of GTP. In preliminary experiments where additional enzyme was not added (see Table II) the rate of P_i-GTP exchange was slight. Equilibrium attainment in the reverse direction was also found to be slower than for the forward direction. By assuming the K_{eq} to be 5.0 it was possible to demonstrate a shift in the equilibrium by the addition of either L-aspartate or P_i as indicated by the change in optical density at 280 mμ.

If one assumes the free energy of hydrolysis of GTP to be similar to that of ATP, it is possible to determine the free energy of AMP-S hydrolysis. A value of —9.3 kilocalories/mole at pH 8.0 and 30° and excess magnesium was recently reported[12]. Thus in the following equations,

$$\text{GTP} + \text{I5P} + \text{L-aspartate} \rightarrow \text{AMP-S} + \text{GDP} + P_i; \quad \Delta F_1^\circ = -0.7 \text{ kcal/mole}$$
$$\text{GDP} + P_i \rightarrow \text{GTP} + H_2O; \quad \Delta F_2^\circ = +9.3 \text{ kcal/mole}$$
$$\text{I5P} + \text{L-aspartate} \rightarrow \text{AMP-S} + H_2O; \quad \Delta F^\circ = +8.6 \text{ kcal/mole}$$

the free energy of AMP-S hydrolysis to I5P and aspartate is —8.6 kcal/mole*. These calculations suggest that AMP-S may be considered a "high-energy compound". This conclusion is supported by the observation that I5P, a reaction product, can tautomerize while AMP-S cannot.

* In this treatment it is further assumed that the measured equilibrium constant is not appreciably altered at 30°.

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It should be noted that streptomycin sulfate was contained in all systems investigated. The inclusion of this material had a twofold purpose; (a) it inhibited the action of polynucleotide phosphorylase[13], present as a trace contaminant, and (b) it markedly activated adenylosuccinate synthetase. A 20–25 % increase in enzymic activity was observed with a streptomycin-sulfate concentration of 0.6 mg/ml test mixture.

Equilibrium-exchange reactions

As already mentioned, it might be possible to gain some insight into the mechanism of three substrate reactions through analysis of substrate–product exchange at equilibrium. Systems approximating equilibrium conditions were prepared containing large amounts of enzyme. Equilibration was continued by incubating the test systems at 37° for 20 min, after which time tracer amounts of L-aspartate-^{14}C and $^{32}P_i$ were added. A calculated amount of non-radioactive P_i was added along with the L-aspartate-^{14}C in order to preclude equilibria shifts. Similar adjustments were made when $^{32}P_i$ was added. Two such systems along with the results obtained in these studies are presented in Tables III and IV. It should be noted that in both experiments

TABLE III

EQUILIBRIUM EXCHANGE REACTIONS–LOW AMP-S AND L-ASPARTATE*

$^{32}P_i$ counts/min/μmole	GTP-^{32}P[1] counts/min/μmole	$P_i \rightarrow$ GTP % incorporation	L-Aspartate-^{14}C counts/min/μmole	AMP-S-^{14}C[2] counts/min/μmole	L-Aspartate $\rightarrow$ AMP-S % incorporation
202,000	1560	0.77	13,650	583	4.27

* Reaction mixture contained 1.1 μmoles AMP-S, 0.90 μmole GDP, 1.0 μmole P_i, 2.0 μmoles L-aspartate, 0.047 μmole I5P, 1.16 μmoles GTP, 200 μmoles glycine buffer (pH 8.0), 1.2 mg streptomycin sulfate, 40 μmoles $MgSO_4$, 125 units enzyme (spec. act. 200) in a total volume of 3.0 ml. Incubation was for 20 min at 37°. $K_{eq} = 8.9$. 0.25 μmole L-aspartate-^{14}C (30,700 counts/min) and 0.10 μmole $^{32}P_i$ (222,000 counts/min) added and incubation continued for 2.5 h.

[1] GTP-^{32}P determined on Norit[9] after termination of reaction with 7 % PCA. Blank contained all components except AMP-S, aspartate and I5P. Norit-adsorbable ^{32}P was subtracted from value of complete system.

[2] AMP-S-^{14}C activity was determined by chromatography after boiling sample at 100° for 2 min.

TABLE IV

EQUILIBRIUM EXCHANGE REACTIONS-HIGH AMP-S AND L-ASPARTATE*

$^{32}P_i$ counts/min/μmole	GTP-^{32}P[1] counts/min/μmole	$P_i \rightarrow$ GTP % incorporation	L-Aspartate-^{14}C counts/min/μmole	AMP-S-^{14}C[2] counts/min/μmole	L-Aspartate $\rightarrow$ AMP-S % incorporation
294,000	495	0.17	20,800	332	1.60

* Reaction mixture contained 2.5 μmoles AMP-S, 0.90 μmole GDP, 1.0 μmole P_i, 5.0 μmoles L-aspartate, 0.047 μmole I5P, 1.16 μmoles GTP, 200 μmoles glycine buffer (pH 8.0), 1.2 mg streptomycin sulfate, 50 μmoles $MgSO_4$, 98 units enzyme (spec. act. 230) in a total volume of 3.0 ml. Incubation was for 20 min at 37°. $K_{eq} = 8.3$. 0.37 μmole L-aspartate-^{14}C (104,000 counts/min) and 0.16 μmole $^{32}P_i$ (294,000 counts/min) added and incubation continued for 2.5 h.

[1] GTP-^{32}P determined on Norit[9] after termination of reaction with 7 % PCA. Blank contained all components except AMP-S, aspartate, and I5P. Norit-adsorbable ^{32}P was subtracted from value of complete system.

[2] AMP-S-^{14}C activity was determined by chromatography after boiling sample at 100° for 2 min.

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the concentration of I5P was maintained low, *i.e.*, one-twentieth the level of other substrates and products. This adjustment was necessitated in order to minimize equilibria shifts caused by degradation of AMP-S and GTP. Even in the presence of alterations in the concentrations of AMP-S and GTP the overall equilibrium of the system would not be affected, thus permitting valid equilibrium-exchange measurements to be made.

The data shown in Tables III and IV indicate that L-aspartate $\longrightarrow$ AMP-S $>$ $P_i \longrightarrow$ GTP at equilibrium. These data suggest that 6-phospho[illegible]-I5P may be an intermediate in the synthesis of AMP-S as mediated by adenylosuccinate synthetase. While these data are consistent with the indicated mechanism, absolute proof of its validity must await either the detection of 6-phosphoryl-I5P in such a system or its utilization with L-aspartate in the absence of GTP and I5P by adenylosuccinate synthetase. It does appear, however, that the other suggested hypotheses for AMP-S synthesis are untenable in the light of these results.

Additional substantiation of the proposed mechanism can be made by a comparison of the results from Tables III and IV. It would be expected, and indeed it was found, that as the concentration of aspartate and AMP-S were increased, their exchange rates at equilibrium relative to P_i-GTP exchange increased.

It might be well to mention the limitations inherent in analyzing reaction mechanisms using the outlined approach. Although probably valid for the analysis of non-enzymic reactions, in this treatment it must be assumed that the rate of all enzyme-substrate and product associations and dissociations exceed those reaction rates involving covalent bond formation and rupture. It is possible, for example, that GTP-^{32}P synthesized at equilibrium is formed more rapidly from $^{32}P_i$ than is AMP-S-^{14}C from aspartate-^{14}C. However, the GTP-^{32}P may not dissociate from the enzyme rapidly in its transfer and equilibration with medium GTP (non-radioactive). The enzyme-bound GTP-^{32}P could then be utilized either at its site of synthesis or at a neighboring site for $^{32}P_i$ formation. The results thus obtained may not be a valid measure of the reaction mechanism. A fuller discussion of this treatment will be presented elsewhere[14].

Exchange reactions

Further support for the postulated mechanism was sought through investigation of aspartate-^{14}C-AMP-S exchange in the following equation:

$$\text{6-phosphoryl-I5P} + \text{L-aspartate} \rightleftarrows \text{AMP-S} + P_i$$

It might be supposed that aspartate-^{14}C would exchange with AMP-S in the presence of P_i and enzyme provided any aspartate produced could exchange with the labeled aspartate in the medium. The results from one such study are presented in Table V. From these data, it appears that aspartate-^{14}C does exchange slightly with AMP-S, lending support to the mechanism above. The significance of these data are weakened, however, by two observations. First, the counting rate of AMP-S in the system lacking GDP is only twice background. Second, the system plus GDP rapidly exchanges aspartate and AMP-S even though the reaction was proceeding to the left. These data are evidence against an independent reaction as depicted above. Quite possibly the exchange observed in the absence of added GDP was caused by endogenous GDP bound to the enzyme. Results similar to those presented in Table V were obtained in an analogous reaction involving glutamine synthesis[15].

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TABLE V

L-ASPARTATE-AMP-S EXCHANGE REACTIONS*

System	*AMP-S*[1] *counts/min*	*AMP-S counts/min/μmole*	*L-Aspartate → AMP-S % incorporation*
Complete	1100	668	0.870
—GDP	33.5	20.3	0.026
—AMP-S	8.0		
—Enzyme	0		

* The complete system contained 0.44 μmole GDP, 1.65 μmoles AMP-S, 10 μmoles P_i, 0.6 mg streptomycin sulfate, 50 μmoles $MgSO_4$, 200 μmoles glycine buffer (pH 8.0), 0.58 μmole L-aspartate-^{14}C (44,500 counts/min) and 90 units enzyme (spec. act. 200). System incubated 2 h at 37°.

[1] AMP-S was isolated by chromatography after the reaction had been terminated by boiling for 2 min at 100°.

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SUMMARY

The equilibrium constant of adenylosuccinic acid synthesis from guanosine triphosphate, inosine-5′-phosphate and L-aspartate was determined to be 2.9 and 10.0 in the forward and reverse directions respectively at pH 8.0 and 37°. The ΔF° of adenylosuccinic acid hydrolysis to inosine-5′-phosphate and aspartate was calculated to be −8.6 kcal/mole. From equilibrium–exchange studies, the limitations of which are mentioned, it appears that 6-phosphoryl-inosine-5′-phosphate may be an intermediate in adenylosuccinic acid synthesis.

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