Biosynthesis of Urea

VII. REVERSIBLE FORMATION OF ARGININOSUCCINIC ACID*

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Previous investigations on the enzymatic mechanism of arginine synthesis have established that citrulline condenses with aspartic acid to form an intermediate compound, argininosuccinic acid, which subsequently undergoes conversion to arginine. The formation of argininosuccinic acid is accompanied by the utilization of ATP and the release of Pi.¹ The presence of myokinase in the enzyme preparations had prevented any conclusion as to whether ADP or AMP was formed in the reaction (1, 2). It has also been shown previously that two separable enzymes are involved in the reaction mechanism. Each of these has been purified, one from mammalian liver, the other from yeast. The yeast enzyme, which was inactive alone, caused a proportionate increase in activity when added to a fixed excess of the liver enzyme (3).

The present studies are concerned with the respective functions of the participating enzymes, and with establishing the cleavage products of ATP. Some of the earlier results showed that the yeast enzyme also influenced the release of Pi.¹ Supplementation of the liver enzyme with an excess of the yeast enzyme resulted in a 5-fold stimulation of argininosuccinate formation and a 10-fold increase in Pi liberation. Expressed stochiometrically, the stimulation was such that the ratio of Pi released to argininosuccinate formed or citrulline was changed from a value of approximately 1 to a value of almost 2.

The possibility was considered that the cleavage of ATP might take place so as to liberate P-P, a type of cleavage originally disclosed by Kornberg (4) in the reversible synthesis of DPN and subsequently found in a number of ATP-dependent reactions associated with the activation of acetate (5), amino acids (6), and sulfate (7, 8), as well as in the synthesis of pantothentic acid (9). As the results of the present investigation show, P-P and AMP are the products of the cleavage and the ratio of 2, mentioned above, can be accounted for by hydrolysis of P-P by the yeast enzyme simultaneously with the main reaction.

**RESULTS**

Identification of Yeast Enzyme as Inorganic Pyrophosphatase—Preliminary experiments designed to ascertain whether highly purified pyrophosphatase would substitute for the yeast enzyme were carried out. In two parallel experiments, the mammalian enzyme was supplemented either with yeast enzyme or with pyrophosphatase that had been recrystallized five times. It can be seen from the results shown in Table I that the low rate of condensation catalyzed by the mammalian enzyme was stimulated to approximately the same extent by crystalline pyrophosphatase as by the yeast enzyme. In both cases also the ratio of Pi released to argininosuccinate formed or citrulline utilized (Δ Pi to Δ citrulline) was increased from a value of approximately 0.7 to 2.0.

For the present studies, purified mammalian enzyme was obtained from kidney rather than from liver. To obtain the data presented in Table I, the ATP was supplied in substrate amounts, for simplicity, although the condensation rate is thereby reduced to about one-half the usual rate given with catalytic amounts of ATP, phosphoglyceric acid, and an ATP-generating system (2).

Further corroboration of the identity of the yeast enzyme with pyrophosphatase was obtained by determining the ratio of pyrophosphatase activity to argininosuccinate-promoting activity during purification of pyrophosphatase. A summary of the results, given in Table II, shows that there was no separation of the two activities over a 100-fold purification range.² The crystals were then dried by exposure to acetone with some resulting inactivation. Both activities were lost to the same extent.

Accumulation of P-P—More direct evidence for the position of cleavage of ATP was obtained from the finding that P-P is a primary product of the reaction. For this purpose the condensation was carried out with relatively large amounts of the kidney enzyme in the presence of fluoride ion to inhibit the traces of pyrophosphatase known to be present as a contaminant. P-P was estimated enzymatically in a second incubation with highly purified pyrophosphatase. These conditions, as shown in Table III, almost completely repressed the liberation of P₁ and led to the accumulation of P-P in amounts equivalent to the utilization of citrulline. In the absence of fluoride, the sum of P₁, found as P₁, and as P-P was twice the citrulline value. It is evident therefore that the P₁, which appears in the absence of fluoride does not originate directly from the terminal position of ATP but is derived secondarily from P-P. It should be mentioned in this connection that argininosuccinic acid formation is not inhibited by the fluoride concentration used (0.01 M).

1 The abbreviations used are: P-P, inorganic pyrophosphate; P₁, orthophosphate; 3-PGA, 3-phosphoglyceric acid; and Tris, tris(hydroxymethyl)aminomethane.

2 Previously reported as a brief communication (10).
Effect of inorganic pyrophosphatase on condensation rate and Pi formation

The reaction mixtures contained 400 pmoles of Tris buffer, pH 7.5; 26.6 pmoles of MgSO4; 15 pmoles of ATP; 20 pmoles each of L-citrulline and L-aspartate; 0.7 or 0.8 mg. of kidney condensing enzyme, specific activity 50; and, where indicated, 90 units of yeast enzyme of specific activity 1100 or pyrophosphatase recrystallized five times, in a final volume of 4 ml. The incubation period was 20 minutes at 38°.

A summary of inorganic pyrophosphate formation in condensation reaction

\[
\text{Citrulline + aspartate + ATP} \rightarrow \text{argininosuccinate + AMP + P-P} \quad (1)
\]

\[
P-P + \text{H}_{2}O \rightarrow 2 \text{Pi} \quad (2)
\]

\[
\text{Citrulline + aspartate + ATP + H}_2\text{O} \rightarrow \text{argininosuccinate + AMP + 2 Pi} \quad (3)
\]

Reaction 3 (the sum of Reactions 1 and 2) represents the stoichiometry of the condensation at maximal velocities, i.e. in the presence of an excess of pyrophosphatase.

Role of Pyrophosphatase—Quite clearly the acceleration of the condensation rate is brought on by the removal of P-P. Moreover, the ratio of the two activities, given in the last column of Table II, shows that under selected conditions, i.e. in the presence of an excess of the mammalian enzyme, the velocity of condensation depends directly on the velocity of P-P hydrolysis. In fact this dependence constitutes the basis of the assay of the yeast enzyme (3). The value for the ratio was found to be almost 3. If, to facilitate comparison, the pyrophosphatase activity is expressed as μmoles of P-P removed (rather than μmoles of P1 formed), then the pyrophosphatase activity is actually only 1.5 times the condensation-promoting activity. Considering that in the condensation-promoting assay, the hydrolysis of P-P arising from ATP cleavage probably takes place at P-P concentrations which are below pyrophosphatase saturation, the two activities are seen to be quite comparable. The condensation-promoting activity of pyrophosphatase can therefore be ascribed entirely to the pyrophosphatase action.

Reversal of Condensation and Specificity for AMP—The formulation of the mechanism given in Reaction 1 prompted a renewed examination of reversibility. When large amounts of the condensing enzyme were used, it was found that the cleavage of argininosuccinic acid, as measured by the appearance of citrulline, takes place in the presence of Mg++, AMP, and P-P. However, the same amount of citrulline was formed when ADP was substituted for AMP. In order to obtain unequivocal proof of nucleotide specificity, it was necessary to remove the interfering myokinase activity. Treatment of a small portion of the enzyme with the anionic exchange agent, DEAE-cellulose (11), as described under “Experimental” removed most of the myokinase activity and resulted in some further purification of the condensing

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Additions</th>
<th>Δ Phosphate</th>
<th>Δ Citrulline</th>
<th>Ratio of Δ phosphate to Δ citrulline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast enzyme</td>
<td>mg.</td>
<td>mg.</td>
<td>μmoles</td>
<td>μmoles</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>+1.16</td>
<td>−1.60</td>
</tr>
<tr>
<td>0.08</td>
<td>0</td>
<td>+15.2</td>
<td>−8.0</td>
<td>1.91</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>+1.38</td>
<td>−2.20</td>
</tr>
<tr>
<td>0.020</td>
<td>+23.1</td>
<td>−10.9</td>
<td>1.85</td>
<td></td>
</tr>
</tbody>
</table>

* Inoubulated controls from which citrulline and aspartate were omitted contained approximately 2 μmoles of Pi, most of which was present at zero time. All the phosphate values have been corrected by this amount.

The inhibition previously observed with crude enzyme preparations (2) was caused by inhibition of pyrophosphatase. Inhibition of condensation does occur, however, with fluoride concentration above 0.01 M.

Manner of ATP Cleavage Associated with Formation of Argininosuccinic Acid—The observations described thus far suggest a formulation involving two successive enzymatic steps represented by Reactions 1 and 2 below. In the first step, the condensation catalyzed by the kidney (or liver) enzyme is accompanied by a cleavage of ATP at the β-pyrophosphoryl bond. This is followed by a second step, catalyzed by pyrophosphatase, in which the pyrophosphate is removed by hydrolysis.

Condensation-promoting activity of yeast inorganic pyrophosphatase

Conditions of fractionation and enzymatic assay were as given in the “Experimental” section.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Yeast pyrophosphatase</th>
<th>Condensation-promoting</th>
<th>Ratio of pyrophosphatase to condensation-promoting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autolysate.........</td>
<td>408</td>
<td>164</td>
<td>2.46</td>
</tr>
<tr>
<td>Fractionation with (NH4)2SO4</td>
<td>883</td>
<td>278</td>
<td>3.20</td>
</tr>
<tr>
<td>Ethanol fractionation...</td>
<td>19,300</td>
<td>6,160</td>
<td>3.12</td>
</tr>
<tr>
<td>Elution from alumina gel.....</td>
<td>37,100</td>
<td>11,900</td>
<td>3.12</td>
</tr>
<tr>
<td>Crystallization.........</td>
<td>53,200</td>
<td>16,700</td>
<td>3.19</td>
</tr>
<tr>
<td>Acetone drying........</td>
<td>35,000</td>
<td>11,500</td>
<td>3.04</td>
</tr>
</tbody>
</table>

* Pyrophosphatase activity is expressed as μmoles of Pi liberated per mg. of protein per hour at 37°.
† Condensation-promoting activity is expressed as μmoles of citrulline utilized per mg. of yeast protein per hour beyond that consumed with the condensing enzyme alone.

Inorganic pyrophosphate formation in condensation reaction

Each tube contained in a final volume of 4 ml., 30 μmoles each of L-citrulline and L-aspartate; 400 pmoles of Tris buffer, pH 7.5; 5 μmoles of ATP; 26.6 μmoles of MgSO4; 50 μmoles of phosphoenolpyruvate; 0.3 mg. of purified pyruvic kinase; 1.0, 0.9, or 11.8 mg. of kidney condensing enzyme (specific activity, 34), and fluoride in the final molarity indicated. The tubes were incubated for 20 minutes at 38°.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Conditions</th>
<th>Citrulline uptake</th>
<th>Products Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast condensing enzyme</td>
<td>Fluoride</td>
<td>μmoles</td>
<td>μmoles</td>
</tr>
<tr>
<td>1</td>
<td>50</td>
<td>0.0</td>
<td>1.7</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>0.01</td>
<td>1.4</td>
</tr>
<tr>
<td>200</td>
<td>0.01</td>
<td>8.0</td>
<td>11.0</td>
</tr>
<tr>
<td>200</td>
<td>0.01</td>
<td>5.8</td>
<td>0.0</td>
</tr>
<tr>
<td>400</td>
<td>0.0</td>
<td>15.5</td>
<td>20.2</td>
</tr>
<tr>
<td>400</td>
<td>0.01</td>
<td>11.7</td>
<td>0.09</td>
</tr>
</tbody>
</table>

* P-P is expressed as P1 found after enzymatic hydrolysis.
Comparison of AMP and ADP as substrates for cleavage of argininosuccinic acid

Each tube contained 20 μmoles of l-argininosuccinate; 12 μmoles of sodium-P-P; 400 μmoles of potassium maleate, pH 6.05; 28 μmoles of MgCl₂; AMP or ADP as indicated, and 3.08 mg. of kidney condensing enzyme, specific activity 107 (390 units) in a final volume of 4.0 ml. The tubes were incubated for 10 minutes at 38°.

<table>
<thead>
<tr>
<th>Amount of nucleotide added</th>
<th>Citrulline formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmoles</td>
<td>With AMP</td>
</tr>
<tr>
<td>----------------</td>
<td>----------</td>
</tr>
<tr>
<td>1.91</td>
<td>1.84</td>
</tr>
<tr>
<td>3.81</td>
<td>2.38</td>
</tr>
<tr>
<td>5.72</td>
<td>2.33</td>
</tr>
<tr>
<td>7.62</td>
<td>2.28</td>
</tr>
</tbody>
</table>

Stoichiometry of argininosuccinic acid cleavage coupled to generation of ATP

Each tube contained 50 μmoles of glucose; 30 μmoles of potassium fluoride; 10 μmoles of AMP; 15 μmoles of MgCl₂; 250 μmoles of potassium maleate, pH 6.2; argininosuccinic acid and P-P as indicated; 0.32 mg. of purified yeast hexokinase (678 units); 0.3 mg. of purified muscle myokinase (4900 units); and 4 mg. of kidney condensing enzyme (120 units) in a final volume of 3.0 ml. The tubes were incubated at 38° for 60 minutes (Experiment 1) or 30 minutes (Experiment 2).

Characteristics of Reverse Reaction

Effect of pH—The velocity of the backward reaction shows a marked dependence on pH (Fig. 1) and displays an optimum at pH 6.7 with acetate or Tris-maleate buffer. The optimum in the acidic region contrasts with the alkaline pH maximum shown in the forward reaction (pH 8.7) under conditions of pyrophosphatase excess (3), where, among other complex changes, the cleavage of ATP to 2 moles of P; would be expected to release approximately 2 H⁺.

It was of interest to explore the pH dependence of the condensation reaction in the forward direction in the absence of pyrophosphatase (Reaction 1) since the pyrophosphoryl cleavage of ATP would then be associated with the release of less H⁺. These experiments were carried out under conditions similar to those used to obtain the data of Table III, that is, with large amounts of the kidney enzyme in the presence of fluoride. The optimum was found to be approximately pH 8.5.

Effect of Time and Enzyme Concentration—The velocity of the backward reaction falls off rapidly with time and with enzyme concentration (Figs. 2 and 3). The reasons for the departure from linearity are not fully understood. Since the course of the time curve suggests approaching equilibrium, an attempt was made to measure the extent of reaction. Aspartate formation in relation to citrulline was investigated in separate experiments, the results of which are given in Table VI. Aspartic acid was estimated spectrophotometrically, at the end of the reaction, from the oxidation of DPNH produced by coupling glutamic-aspartic transaminase with malic dehydrogenase, a procedure suggested by Pfleiderer et al. (12). Aspartic acid and citrulline were liberated in equivalent amounts.

The nucleotide specificity seems to be restricted to ATP. The triphosphates of other nucleotides, such as inosinic, guanylic, and uridylic acids were found to be incapable of replacing ATP when tested in the forward reaction.

Stoichiometry of Argininosuccinic Acid Cleavage—The specificity of the requirements for reaction reversal, as well as the stoichiometric data given below, show that the cleavage of argininosuccinic acid to citrulline and aspartic acid represents a true reversal of Reaction 1. The relationship among argininosuccinic acid disappearance, P-P utilization, and citrulline production was found to have the expected molar ratio of 1:1:1 (Table V). Since myokinase contaminated the enzyme, ATP formation was estimated indirectly as glucose-6-P, according to the procedure of Kornberg (4). The incubations were carried out in the presence of glucose and an established excess of both hexokinase and myokinase to insure the formation of 2 moles of glucose-6-P for each mole of ATP originally formed. Since the incubations were somewhat prolonged, fluoride was also added to prevent removal of P-P by contaminating pyrophosphatase.

The amount of glucose-6-P found (2 moles) corresponds to the formation of 1 mole of ATP with the cleavage of 1 mole of argininosuccinic acid.

Aspartic acid formation in relation to citrulline was investigated in separate experiments, the results of which are given in Table VI. Aspartic acid was estimated spectrophotometrically, at the end of the reaction, from the oxidation of DPNH produced by coupling glutamic-aspartic transaminase with malic dehydrogenase, a procedure suggested by Pfleiderer et al. (12). Aspartic acid and citrulline were liberated in equivalent amounts.

The results of experiments carried out with this preparation are given in Table IV. AMP was found to be considerably more active than ADP. The small amount of citrulline found in the presence of ADP may be attributed to hexokinase and myokinase contamination of the ADP by AMP.

The nucleotide specificity seems to be restricted to AMP. The triphosphates of other nucleotides, such as inosinic, guanylic, and uridylic acids were found to be incapable of replacing ATP when tested in the forward reaction.

<table>
<thead>
<tr>
<th>Substrates or Products</th>
<th>Amount utilized or formed</th>
<th>Initial</th>
<th>Final</th>
<th>Net change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles</td>
<td>μmoles</td>
<td>μmoles</td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Argininosuccinic acid</td>
<td>13.1</td>
<td>10.8</td>
<td>-2.3</td>
<td></td>
</tr>
<tr>
<td>P-P</td>
<td>14.4</td>
<td>12.1</td>
<td>-2.3</td>
<td></td>
</tr>
<tr>
<td>F₁</td>
<td>4.0</td>
<td>4.6</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Citrulline</td>
<td>0.0</td>
<td>2.34</td>
<td>+2.34</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-P</td>
<td>0.0</td>
<td>4.44</td>
<td>+4.44</td>
<td></td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Argininosuccinic acid</td>
<td>9.0</td>
<td>7.9</td>
<td>-1.1</td>
<td></td>
</tr>
<tr>
<td>P-P</td>
<td>9.8</td>
<td>8.7</td>
<td>+1.1</td>
<td></td>
</tr>
<tr>
<td>F₁</td>
<td>3.07</td>
<td>3.67</td>
<td>+0.60</td>
<td></td>
</tr>
<tr>
<td>Citrulline</td>
<td>0.0</td>
<td>1.20</td>
<td>+1.20</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-P</td>
<td>0.0</td>
<td>2.11</td>
<td>+2.11</td>
<td></td>
</tr>
</tbody>
</table>
made to stimulate the rate by adding an excess of potato apyrase in order to remove ATP and regenerate AMP. No stimulation was found.

In the absence of any assurance that even the 10-minute incubation period represents initial velocities, no attempt was made to pursue the kinetics for enzyme-substrate affinity measurements. However, provisional values for the optimal substrate concentrations were obtained from 10-minute velocity measurements, varying one substrate at a time. The process was repeated until the optimum for each substrate was determined in the presence of optimal levels of the other substrates. The substrate concentrations found to be optimum were the ones used to observe the time course of the reaction and the dependence on enzyme concentration (Figs. 2 and 3). Exhaustion of one of the substrates seems therefore to be excluded as an explanation of the rapid decline in rates.

\[ P_i \text{ Liberation Utilizing a Generating System for ATP—As mentioned above, a } \Delta P_i \text{ to } \Delta \text{ citrulline ratio of } 2 \text{ was found in previous experiments with purified ox liver enzyme. This ratio was observed only when the ATP was supplied directly in substrate amounts. With a generating system which consisted of catalytic amounts of ATP, substrate amounts of 3-PGA, and a crude rabbit muscle fraction as the source of glycolytic enzymes, the ratio found was usually approximately } 1.6 (2, 3). \]

Similar observations (Experiment 1, Table VII) have also been obtained in the present studies with condensing enzyme prepared from hog kidney. Since a ratio appreciably less than 2 is incompatible with the stoichiometry of Reaction 3, it was of interest to resolve the discrepancy.

The crude rabbit muscle fraction that is used for the generation of ATP from 3-PGA contains phosphoglyceric mutase, enolase, and pyruvic kinase. It also contains an ATPase-like activity. The latter is responsible for most of the \( P_i \) liberated in the incubated controls (amino acid substrates omitted from otherwise complete reaction mixtures) which were included to provide the correction blank. Studies carried out by Meyerhof and Junowicz-Kozolaty (13) and more detailed investigation by Krimsky\(^3\) indicate that the ATPase activity is largely caused by Reactions 4 and 5 as shown below.

\[
\begin{align*}
3\text{-PGA} + \text{ATP} &\rightleftharpoons 1,3\text{-diphosphoglyceric acid} + \text{ADP} \\
1,3\text{-diphosphoglyceric acid} + \text{H}_2\text{O} &\rightleftharpoons 3\text{-PGA} + \text{P}_i.
\end{align*}
\]

The equilibrium for Reaction 4, catalyzed by phosphoglyceric kinase, (present also in the crude muscle fraction) is far to the left, but displacement in the direction of the formation of 1,3-diphosphoglyceric acid may readily be brought about by the rephosphorylation of ADP which occurs in the presence of 3-PGA and the generating system\(^3\). The unstable 1,3-diphosphoglyceric acid so formed then undergoes dephosphorylation (Reaction 5) which results in a liberation of \( P_i \) from ATP and ultimately from 3-PGA. The conditions for promotion of Reactions 4 and 5 were most favorable in the incubated controls where argininosuccinic acid formation and concomitant ATP utilization were absent. The blank value so obtained for \( P_i \) released by these side reactions will therefore be appreciably higher than that which occurs in the presence of the condensation reaction which successfully competes with phosphoglyceric kinase for ATP.

The validity of this explanation is borne out by the results obtained with two generating systems which contained purified enzymes. When phosphoenolpyruvate and highly purified pyruvic kinase were used (Experiment 2, Table VII), a \( \Delta P_i \) to \( \Delta \text{ citrulline ratio of } 2.02 \) was obtained. With the use of a

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\( ^3 \) Personal communication from I. Krimsky (to be published).
Effect of ATP-generating system on release of Pi in presence and absence of ATP utilization

Each tube contained 30 μmoles each of L-citrulline and L-aspartate; 400 μmoles of Tris buffer, pH 7.5; 5 μmoles of ATP; 20.6 μmoles of MgSO₄; 1.47 mg. of kidney condensing enzyme; specific activity 30 (50 units); 0.018 mg. of pyrophosphatase; and in addition, for Experiment 1, 50 μmoles of 3-PGA and 3 mg. of lyophilized rabbit muscle fraction; for Experiment 2, 50 μmoles of phosphoenol pyruvate and 0.3 mg. of purified pyruvic kinase; and, for Experiment 3, 50 μmoles of 3-PGA, 0.33 mg. of purified PGA mutase, 0.4 mg. of purified enolase, and 0.3 mg. of purified pyruvic kinase in a final volume of 4 ml. All tubes were incubated for 20 minutes at 38°.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Δ Pᵢ (μmoles)</th>
<th>Δ Pᵢ (μmoles)</th>
<th>Δ Pᵢ (μmoles)</th>
<th>Δ Pᵢ (μmoles)</th>
<th>Ratio of Δ Pᵢ to L-citrulline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-PGA and crude muscle fraction</td>
<td>0.2</td>
<td>20.7</td>
<td>21.5</td>
<td>13.4</td>
<td>1.60</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphoenol pyruvate and pyruvic</td>
<td>+2.0</td>
<td>+27.4</td>
<td>+25.2</td>
<td>-12.6</td>
<td>2.02</td>
</tr>
<tr>
<td>kinase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-PGA, PGA mutase, enolase, pyruvic</td>
<td>+2.4</td>
<td>+34.8</td>
<td>+32.4</td>
<td>-15.7</td>
<td>1.90</td>
</tr>
</tbody>
</table>

The reversibility of the reaction is of interest in that it permits the eventual evaluation of the "energy level" of C—N bonds of this type. The cleavage of this bond is associated with the generation of ATP. The equilibrium position of the reaction has not yet been estimated, but the relative ease of reversibility suggests that the "energy level" may prove to be closer to ATP than that of the amide bond of glutamine (14).

Since the cleavage of argininosuccinic acid to arginine and fumaric acid is a reversible reaction which involves a ΔF° of +2778 calories (15, 16), it follows as a consequence of the present findings that the over-all conversion of citrulline to arginine should be readily reversible in the absence of pyrophosphatase.

The nature of the phosphorylation step which probably precedes the actual condensation is of considerable interest. From a chemical point of view it has been suggested that phosphoryl transfer from ATP to the ureido carbon of citrulline rather than to the amino group of aspartic acid is the most likely possibility (1-3). In view of the new findings as to the position of ATP cleavage, two possibilities must be considered: (a) adenyl transfer and (b) pyrophosphoryl transfer. Experiments are in progress to distinguish between the two. The ATP-activating systems described by other investigators involve the formation of acid anhydrides by interaction with the phosphorus of adenylic acid. Analogy with these reactions and the chemical relationship between citrulline and the disubstituted carbodiimides used in the Khorana synthesis of nucleotide triphosphates (17) favor the possibility of an adenylate of citrulline, possibly enzyme-bound, as seems to be the case with the acyl adenylate compounds (3, 6) formed in the acetate and amino acid activating systems.

Investigations of hydrolytic formation under a variety of conditions have produced negative results. The remote possibility of activation at one of the carboxyl groups seems, therefore, to be unlikely. The compound formed by adenyl attachment at the citrulline ureido carbon (Formula II) would not possess the typical acid anhydride structure.

The pyrophosphatase activity of mammalian liver and kidney is normally very high and crude preparations of the condensing enzyme obtained from these sources contain an excess of mammalian pyrophosphatase. The stimulation of 5-fold or more produced by excess pyrophosphatase is an unusually pronounced effect. Although the removal of P-P by a hydrolytic reaction seems to be wasteful, energetically, this step serves to pull the condensation reaction in the direction of synthesis. Indeed, with preparations of the condensing enzyme which are free of pyrophosphatase, the stimulation exerted by excess pyrophosphatase becomes considerably greater than 5-fold.

Whether the inhibition exerted by P-P is caused by a direct effect on the condensing enzyme or to an unfavorable equilibrium has yet to be established. There is some indication that both effects may be involved. The removal of argininosuccinic acid by an excess of the enzyme which cleaves argininosuccinate to arginine and fumarate did not result in a stimulation of the rate of condensation. On the other hand, the ease of reversibility seems to indicate that the equilibrium position of the condensation reaction is not markedly in the direction of synthesis.

Because of the low activity in the absence of pyrophosphatase, possibly tissue pyrophosphatase functions under physiological conditions in a similar capacity, as a stimulator of argininosuccinate formation. The participation of pyrophosphatase in
this capacity suggests also that arginine and urea formation might be controlled in vivo at the condensation step, possibly by influencing the level of activity of pyrophosphatase.

EXPERIMENTAL

Enzyme Preparations and Assays The condensing enzyme was prepared from an extract of acetone-dried hog kidney (18) by the fractionation procedure previously used for ox liver (3). In the alumina Cy step, higher ionic strength was required to elute the kidney enzyme. After discarding three successive eluates (0.002, 0.005, and 0.005 M potassium phosphate, pH 7.5), the activity was removed from the gel by two elutions with 0.01 M solutions of the same buffer. A second fractionation with the same gel was only partially successful in raising the specific activity. The preparation of the condensing enzyme used for most of the experiments had a specific activity of approximately 35. Activity was estimated from the rate of citrulline utilization in the presence of excess yeast enzyme (pyrophosphatase), as previously described (3). Specific activity is expressed as μmoles of citrulline utilized per mg. per hour at 38°. The preparation contained traces of pyrophosphatase (3.0 μmoles of Pi released per hour per mg.) and appreciable myokinase (0.7 μmoles of AM1 utilized per minute per mg.). Protein was estimated as before (3).

In order to remove myokinase from the condensing enzyme, an exploratory fractionation was carried out with a 5.5 × 3.2 cm. column of DEAE-cellulose (11) washed with 0.002 M potassium phosphate buffer, pH 7.5. 84 mg. of the enzyme were put on the column in 10 ml. of the same buffer and were eluted with the same buffer. The activity emerged with the first protein peak. After precipitation with ammonium sulfate and dialysis, the column treatment was repeated. The specific activity of the preparation finally obtained (9 mg.) was 112. Myokinase activity was reduced to trace amounts.

Myokinase activity was estimated spectrophotometrically from the rate of DPNH oxidation in the presence of phosphoenol pyruvate, pyruvic kinase, lactic dehydrogenase, AhLIP, and ATP (19). Activity is expressed as μmoles of AMP utilized per minute per mg. when assayed under the conditions of Mehler et al. (24). Purified preparations of yeast hexokinase (25), muscle myokinase (26), hexose phosphate isomerase (27), muscle phosphoglyceric mutase (28), enolase (29), potato apyrase (30), and argininosuccinate-elevating enzyme (16) were prepared and assayed as described. The crude muscle fraction (52 to 72 per cent (NH₄)₂SO₄ saturation) was a lyophilized preparation (1).

Analytical Methods—The incubations were stopped with 1.5 ml. of chilled 8.3 per cent trichloroacetic acid per ml. of incubation mixture. Citrulline was estimated colorimetrically in 0.1 ml. of the filtrate as before (31). When reaction reversal was observed, a more sensitive procedure which included use of 1 ml. of the filtrate with 2 ml. of the acid mixture and 0.15 ml. of the diacetyl monoxime reagent was adopted. Pi was estimated in the filtrate by the Lohnmann and Jondrasiek procedure (32). P-P was estimated as P1 after incubation of a sample of the neutralized filtrate with 0.020 mg. of pyrophosphatase (Table III), or after heating for 10 minutes with 1 N HCl (Table V).

To obtain the stoichiometric data given in Table V, mixtures for the zero time and incubated tubes were prepared in duplicate. One of each pair was deproteinized with 3 ml. of 10 per cent trichloroacetic acid to keep the P-P in solution. Aliquots of the filtrate were used for the estimation of Pi, acid-labile phosphorus (P-P) and citrulline. The duplicates were deproteinized by heating for 2 minutes at 100° and the clarified aliquots were then taken for the estimation of argininosuccinic acid with splitting enzyme and arginase (16) and for the spectrophotometric estimation of glucose-6-P by TPN reduction in the presence of glucose-6-P dehydrogenase as described by Kornberg (4). It was necessary to add hexose phosphate isomerase to the mixture used for assay of glucose-6-P, since hexose phosphate isomerase contaminated the condensing enzyme.

Aspartic acid (Table VI) was estimated spectrophotometrically according to Pfeiderer et al. (12) by DPNH oxidation in the presence of α-ketoglutarate, transaminase, and malic dehydrogenase. Approximately 1.5 mg. of the transaminase preparation were required to convert about 0.04 μmoles of aspartic acid to malic acid in 3 minutes at room temperature in a final volume of 1.1 ml. The transaminase preparation contained sufficient malic dehydrogenase for the assay. Added aspartic acid was recovered within 5 per cent. Heat-deproteinized aliquots of the incubation mixture were used for assay.

All spectrophotometric analyses were performed at 340 μ in a Beckman model DU spectrophotometer with 1-ml. cuvettes which had a light path of 1 cm.

Substrates—the nucleotide triphosphates and DPNH were purchased from the Sigma Chemical Company. L-Citrulline, 3-PGA (1), and L-argininosuccinic acid (15) were prepared as before.
SUMMARY
1. The reversible formation of argininosuccinic acid from citrulline and aspartic acid is accompanied by the cleavage of adenosine triphosphate to adenosine monophosphate and inorganic pyrophosphate.

2. In the reverse reaction, the cleavage of argininosuccinic acid is accompanied by the formation of adenosine triphosphate and the utilization of inorganic pyrophosphate in amounts equivalent to the citrulline and aspartate formed.

3. Some characteristics of the reverse reaction are described.

4. Of the two enzymes which participate in argininosuccinate formation, one has been shown to catalyze formation of argininosuccinate. The second has been identified as inorganic pyrophosphatase.

5. Argininosuccinate-forming activity is extremely low in the absence of inorganic pyrophosphatase. The role of this enzyme in argininosuccinate formation is discussed.

REFERENCES
Biosynthesis of Urea: VII. REVERSIBLE FORMATION OF ARGININOSUCCINIC ACID
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