BIOSYNTHESIS OF UREA

II. ARGININE SYNTHESIS FROM CITRULLINE IN LIVER HOMOGENATES*

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A preceding paper described the characteristics of the isolated enzyme system, prepared from ox liver acetone powder, which catalyzes the conversion of citrulline and aspartic acid to arginine and malic acid. It was shown that the fundamental requirements for arginine synthesis from citrulline are aspartic acid, Mg++, and adenosine triphosphate (ATP), the latter as a reactant in substrate concentrations (1). At the optimum enzymatic conditions established, a large proportion of the arginine-synthesizing activity of the tissue was found in the acetone powder extract.

Aspartic acid was shown to be the specific —NH₂ donor. Glutamic acid was unreactive but the combination of glutamic and oxalacetic acids could replace aspartic acid in proportion to the glutamic-aspartic transaminase activity of the enzyme preparation. The relative activity of these two amino acids is reversed in liver homogenates. Cohen and Hayano (2), corroborated by Krebs and Eggleston (3), have found that glutamic acid is about 4 times as effective as aspartic acid. Since these observations raise the question as to whether the enzyme system studied by us is the same as the one concerned with arginine synthesis in homogenates and slices, studies of liver homogenates bearing on this point were carried out. The experimental observations presented here show that the same enzymatic system is involved in all cases. It has been possible to explain conflicting observations by a uniform mechanism and to indicate the physiological pathway of amino nitrogen from amino acids to urea.

In order to relate the behavior of liver homogenates in oxygen to the behavior and requirements of the isolated system, certain general properties of homogenates, affecting arginine synthesis, should be mentioned. The adenosinetriphosphatase (ATPase) activity is much greater than that of acetone powder extracts; hence rapid generation of ~ph is necessary. In addition to the enzyme system catalyzing the citrulline to arginine re-

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action, homogenates contain appreciable concentrations of several enzymes associated with glutamic acid metabolism; glutamic-aspartic transaminase, glutamic-alanine transaminase, and glutamic dehydrogenase. Associated with homogenate particles are all the respiratory enzymes and cofactors which, except for the additional requirement of adenylie acid or ATP and Mg++, catalyze the coupled oxidative phosphorylation of respiratory substrates via the tricarboxylic cycle. The particles also contain the various factors involved in the transport of hydrogen to oxygen through pyridine nucleotides and the cytochrome system. Pyridine nucleotides and cytochrome need not be added, but if the latter becomes limiting, the whole tricarboxylic cycle shows cytochrome dependence. These are complexities contributed by the use of homogenates and are not necessarily inherent in the mechanism of arginine synthesis. Oxalacetic acid can be supplied by a rapidly respiring homogenate, since it lies in the pathway of the tricarboxylic cycle.

*Generation of High Energy Phosphate*—Under the conditions of Cohen and Hayano (2), ATP is supplied only in catalytic amounts, and no respiratory substrate other than the —NH₂ donor is added. It may be anticipated then that the ATP requirement must be met by oxidative phosphorylation and that the activity of the —NH₂ donor being tested would be limited by its capacity to act as a respiratory substrate. That such is actually the case may be shown by estimating the oxygen consumption of rat liver homogenates during arginine synthesis. L-Aspartic acid is very poorly, or not at all, oxidized by homogenates. As seen in Table I, in the presence of citrulline and aspartic acid, under aerobic conditions, the average arginine formation was 3.5 μM and the average oxygen consumption was 14.9 μM, a value approximately the same as the endogenous respiration of homogenates alone. The results were quite variable within these low limits and undoubtedly reflect the concentration of endogenous respiratory substrates at the time the tissue was obtained. With glutamic acid in place of aspartic acid, an average of 9.6 μM of arginine was formed and the oxygen consumption averaged 30.5 μM.

However, when a respiratory substrate such as pyruvic acid was added to aspartic acid, arginine was increased to 12.8 μM and the average oxygen uptake amounted to 29.4 μM. Similar accelerating effects on both arginine formation and oxygen uptake were observed when either phosphoglycerate, fumarate, oxalacetate, or α-ketoglutarate was added to aspartate (Table I). In homogenates under aerobic conditions, phosphoglycerate apparently behaved primarily as a source of pyruvate. This may be inferred from the fact that the average oxygen consumption was increased almost as much by the addition of phosphoglycerate as by that of pyruvate. The contribution of ~ph from phosphopyruvate was probably of
minor significance as compared to the amount generated by pyruvate oxidation. The acceleration of arginine formation from aspartic acid and citruline by addition of the various substrates shown in Table I demonstrates that when aspartic acid is supplemented with a source of ATP, supplied by oxidative phosphorylation, arginine synthesis increases with the rate.

**Table I**

**Synthesis of Arginine from Citrulline in Rat Liver Homogenates under Aerobic Conditions in Absence and Presence of Malonate**

In addition each vessel contained 20 µM of L-citrulline, 5 µM of ATP, 10 µM of MgSO₄, 0.3 ml. of 0.25 M potassium phosphate, pH 7.5, and 0.5 ml. of 25 per cent homogenate. Final volume 3.0 ml.; 38°; time 40 minutes. The values are given in micromoles.

<table>
<thead>
<tr>
<th>Substrate added, 20 µM</th>
<th>With L-aspartate</th>
<th>With L-glutamate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arginine</td>
<td>O₂ uptake</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>A.D.*</td>
</tr>
<tr>
<td>None</td>
<td>3.5</td>
<td>±0.8</td>
</tr>
<tr>
<td>Pyruvate†</td>
<td>12.8</td>
<td>±0.7</td>
</tr>
<tr>
<td>3-Phosphoglycerate</td>
<td>12.9</td>
<td>±0.9</td>
</tr>
<tr>
<td>Oxalacetate</td>
<td>14.4</td>
<td>±0.4</td>
</tr>
<tr>
<td>Fumarate</td>
<td>13.4</td>
<td>±1.0</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>11.1</td>
<td>±0.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>With 60 µM malonate per vessel</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
</tr>
<tr>
<td>Pyruvate</td>
</tr>
<tr>
<td>3-Phosphoglycerate</td>
</tr>
<tr>
<td>Oxalacetate</td>
</tr>
<tr>
<td>Fumarate</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
</tr>
</tbody>
</table>

* Average deviation.
† 2.5 µM of fumaric acid were added as a primer. Good respiration was often obtained without it, but the addition insured uniformly high values. Fumarate was not added to pyruvate when glutamate was employed; nor was it added in any malonate experiment.

Multiple Function of Glutamic Acid—When the same substrates were added to glutamic acid, different effects were observed, as shown in Table I. The addition of either pyruvate or phosphoglycerate caused a 40 per
cent inhibition of arginine synthesis and a variable decrease in oxygen consumption. The addition of oxalacetate or of fumarate caused a slight increase in arginine synthesis without appreciably affecting the average oxygen consumption, while the addition of α-ketoglutarate caused a decrease in both arginine formation and oxygen uptake.

The high activity of glutamic dehydrogenase in liver homogenates accounts for the fact that glutamic acid is as good a respiratory substrate as α-ketoglutaric acid (4). Furthermore only a fraction of the glutamic acid present need be oxidized to furnish a large supply of ATP, for, as shown by Ochoa, the oxidation of α-ketoglutarate to succinate generates three ~-ph bonds (5) and further oxidation to fumarate generates one such bond, while the further complete oxidation of pyruvate is associated with the generation of fifteen ~-ph bonds (6). The dependence of α-ketoglutarate oxidation upon cocarboxylase (5–7) is therefore related to the decreased arginine synthesis observed in livers of vitamin B1-deficient rats (8) by von Fahrlander, Nielsen, and Leuthardt.

If the point of view is taken that glutamic acid is active in so far as it can be converted to aspartic acid, once the ~-ph requirement is satisfied, then the acceleration of arginine synthesis by addition of oxalacetate to glutamate\(^1\) may be explained as being primarily due to the appearance of extra aspartic acid formed by transamination with glutamic acid. Fumarate, being an immediate precursor of oxalacetate, had the same effect. Malate would be expected to act similarly. In the absence of added oxalacetate, aspartate can arise from glutamate by transamination with the oxalacetate formed during oxidation of glutamate via α-ketoglutarate and the Krebs tricarboxylic cycle. Oxalacetate will also be formed by a one-step oxidation of the malic acid appearing as the second product in arginine synthesis.

Although the synthesis of aspartic acid has not been shown directly in homogenates when glutamic acid is the substrate offered, much indirect evidence indicates that such must the case. No satisfactory explanation has yet been offered of the inhibition, reported from other laboratories, of arginine synthesis by pyruvate (2, 8, 9), by α-ketoglutarate (3, 8, 9), and malonate (2, 3, 10) when glutamate is the \(\text{--NH}_2\) donor. These inhibitions may all be explained as being due to an interference either with the formation of or with the further reaction of aspartic acid.

Inhibition by α-Ketoglutarate—Cohen has reported the various transaminase activities of rat liver. From his data (11) and from what is now known of the relative activities of glutamic-aspartic transaminase and of

\(^1\) When the rate of arginine synthesis is decreased by reducing the ATP concentration, a 30 to 40 per cent stimulation by oxalacetate can be demonstrated.
glutamic-alanine transaminase (12, 13), the rates of the three transamination reactions listed in descending order of activity are glutamate-oxalacetate, glutamate-pyruvate, and aspartate-pyruvate, the first being by far the most rapid and the third rather slow, since it is the combined result of the first two reactions (12, 14).

Inhibition of arginine synthesis by α-ketoglutaric acid when glutamate is the source of –NH₂ varies from about 40 to 60 per cent, depending on the amount of keto acid added (3, 9, 15). As shown in Table I, with 20 μM each of α-ketoglutarate and glutamate, a 54 per cent inhibition was found. This inhibition may be attributed to reversal of the transamination between glutamate and oxalacetate; i.e., glutamate + oxalacetate ⇄ α-ketoglutarate + aspartate. Such inhibition would not be expected if glutamic acid were the specific –NH₂ donor. The inhibition by α-ketoglutarate would furthermore tend to be maintained by slow oxidative removal from a reaction mixture already containing enough keto acid (derived from the glutamic acid) to saturate the tricarboxylic cycle.

On the other hand, with 20 μM each of α-ketoglutarate and aspartate, a stimulation of arginine synthesis was observed almost as great as that produced by the addition of other respiratory substrates. Here the aspartate concentration is initially in excess as far as enzyme saturation is concerned. The addition of an equal amount of α-ketoglutarate might at most reduce the aspartate to 15 μM by transamination, but even at this level the rate of arginine synthesis would be expected to be quite rapid providing ATP were being supplied, as it is in this case, by α-ketoglutarate oxidation. Under these conditions the stimulation caused by supplying ATP is the main effect observed.

Von Fahrlander, Nielsen, and Leuthardt (8) have shown that the α-ketoglutarate inhibition can be overcome by added NH₃. The observation is consistent with the explanation given above, for the addition of NH₃ to α-ketoglutaric acid would remove some α-ketoglutaric acid by reductive amination to glutamic acid.

Inhibition by Pyruvic Acid—As shown in Table I, pyruvate stimulates arginine formation when aspartate is the source of –NH₂, but causes a 40 per cent inhibition when glutamate is the –NH₂ donor. This inhibition can also be explained as an effect on the formation of aspartic acid caused by the removal of some glutamic acid from the reaction mixture by transamination, even though glutamic-pyruvic transamination is relatively slow in liver. The appearance of α-ketoglutaric acid, formed by the transamination of glutamate with pyruvic acid, would augment the inhibition, as explained in the preceding section.

As with α-ketoglutarate, the pyruvate inhibition is also relieved by
added \textit{NH}_3 \textit{(8)}, an effect which may be explained in the same way. The similar results obtained with phosphoglyceric acid (Table I) are in accord with the rapid conversion of the latter to pyruvic acid.

Krebs and Eggleston (3) reported an acceleration of the synthesis of arginine by citrate and succinate when aspartate is the source of \textit{NH}_2. This is understandable in so far as oxidation of these substrates through the tricarboxylic cycle generates the necessary \textit{pH}. Inhibition by citrate when glutamate is the source of \textit{NH}_2 (3) may be explained by oxidation of citrate to \textit{a}-ketoglutarate. The inhibition by succinate (3) observed under these conditions, however, is not easy to understand unless it is assumed that succinate interferes with the oxidation of \textit{a}-ketoglutarate derived from glutamate.

\textbf{Inhibition by Malonic Acid—}The inhibition by malonate of arginine formation when glutamate is the source of \textit{NH}_2 has been reported by Cohen and Hayano (2) and confirmed in the laboratories of Leuthardt (10) and of Krebs (3). The latter two groups have also shown that fumarate overcomes the inhibition. A comparison of the data obtained without malonate with corresponding values obtained in the presence of malonate (Table I) again confirms these observations. This comparison shows further that the inhibition is not exerted on arginine synthesis \textit{per se}. When aspartate was added to homogenates under optimum conditions, \textit{i.e.} in the presence of a source of ATP, no malonate effect was observed. A satisfactory explanation of all these observations can be offered in terms of the specificity for aspartic acid in the conversion of citrulline to arginine and of the well known inhibition of succinic dehydrogenase by malonate (16). When oxidation of \textit{a}-ketoglutarate is blocked at the succinate stage, oxalacetate will not be available for the synthesis of aspartic acid.

The main inhibitory effect on glutamate is clearly not associated with interference of ATP generation, for the addition of malonate to aspartate plus pyruvate only decreased arginine formation from an average of 12.8 \textit{\mu}M to an average of 11.4, while lowering the oxygen uptake from an average of 29.4 \textit{\mu}M to an average of 19.9. In the absence of malonate, respiration and ATP generation were presumably excessive, so that the appreciable reduction of oxygen consumption caused by malonate resulted only in a small decrease in arginine synthesis. Similarly with aspartic acid as the source of \textit{NH}_2, in the presence of either phosphoglycerate, oxalacetate, fumarate, or \textit{a}-ketoglutarate, malonate caused but a small reduction of arginine synthesis along with an appreciable reduction in oxygen consumption. The oxidation of these substrates is of course curtailed at the succinate stage. The oxidation step, \textit{a}-ketoglutarate to succinate, as mentioned above, yields three \textit{pH} bonds per atom of oxygen. The high ratio explains why an adequate supply of ATP can be maintained in the presence
of malonate. A high final concentration of malonate was employed (0.02 M) in order to assure a maximum inhibition of succinate oxidation.

In contrast to the results with aspartate, malonate caused a large decrease in arginine synthesis when glutamate was the source of –NH₂. Oxygen consumption was appreciably reduced, as with aspartate. Thus, the oxygen fell from an average of 30.5 μM to 18.9, while the synthesis of arginine decreased from an average of 9.6 to 2.6 μM. As shown in Table I, oxalacetate overcomes the malonate inhibition. Fumarate had a similar effect, obviously due to its conversion to oxalacetate by oxidation. Malate would be expected to have the same effect. Pyruvate, phosphoglycerate, and α-ketoglutarate, which in the presence of malonate continued to produce a stimulation of arginine synthesis, with aspartate as the source of –NH₂, had no effect in overcoming malonate inhibition of glutamate, since they cannot supply oxalacetate in the presence of malonate and in addition are inhibitory by themselves.

Anaerobic Synthesis of Arginine in Homogenates—Once the requirements of the system are known, it becomes possible to obtain arginine formation in homogenates under anaerobic conditions by utilizing glycolytic reactions to generate ~ph. Table II shows the amount of arginine formed in a representative experiment, in the presence of citrulline, aspartic acid, and phosphoglyceric acid or hexose diphosphate.² None was formed in

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**Table II**

**Synthesis of Arginine from Citrulline in Rat Liver Homogenates under Anaerobic Conditions**

Further additions and other conditions are as in Table I, except that N₂ replaced O₂ in the gas space and 4 mg. of DPN were added to each vessel containing hexose diphosphate. The dry weight of 0.5 ml. of homogenate and 0.4 ml. of supernatant respectively was 34.7 mg. and 17.1 mg. The values are given in micromoles.

<table>
<thead>
<tr>
<th>Substrate added</th>
<th>Arginine found</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole homogenate</td>
</tr>
<tr>
<td>L-Aspartate, 20 μM</td>
<td>L-Glutamate, 20 μM</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td></td>
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<td>+</td>
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</tr>
</tbody>
</table>

*DPN was included with hexose diphosphate, since the anaerobic inactivation of DPN is very rapid. A dismutation between pyruvic acid and triose phosphate was undoubtedly responsible for the availability of ~ph.
the absence of a supply of ~ph. The addition of phosphoglyceric acid or hexose diphosphate caused no arginine formation when glutamic acid was substituted for aspartic acid, unless oxalacetic acid was also added. These results are similar to those obtained with acetone powder extracts. No further efforts were made to increase the rate of synthesis by improved conditions.

The rates of arginine synthesis, anaerobically, were much lower, however, than those obtained with an equal amount of homogenate under aerobic conditions. Owing to the fact that the efficiency of anaerobic phosphorylation is lower than that of aerobic phosphorylation, competition with ATPase is less successful anaerobically. A considerable portion of the ATPase present is associated with the suspended particles of tissue homogenates and can be removed by high speed centrifugation. The last column of Table II gives the amounts of arginine synthesized by an equivalent amount of supernatant after removing the particles. Arginine, when formed, was found to be higher than in the whole homogenate in each case.

As a result of aerobic experiments with glutamic acid in which the supernatant and centrifuged particles of homogenates were studied separately, Cohen and Hayano (17) came to the conclusion that both the particles and the supernatant were essential for arginine synthesis. From the anaerobic data of Table II, it may be seen that both the arginine-synthesizing system and transaminase are present in the supernatant. It is clear from the foregoing discussion that the particles were required for the generation of ATP and of oxalacetic acid.

Since the behavior of liver homogenates, under a variety of experimental conditions, is in accord with predictions based upon the mechanism and requirements of the isolated system, there appears to be no need for considering that more than one enzyme system is concerned with the conversion of citrulline to arginine in mammalian liver. The evidence indicates that in homogenates glutamic acid must function as a nitrogen carrier, as ATP generator, and as a source of oxalacetic acid.

**Physiological Pathway of Amino Nitrogen Transfer**

The individual steps in the transfer of nitrogen from amino acids to form urea and their relationship to the tricarboxylic cycle are summarized in Fig. 1. In homogenates, when glutamic acid is the substrate offered, the transfer would start at Step B (transaminase) as soon as an adequate concentration of oxalacetic acid accumulates. In slices, when NH₃ and lactate are the substrates offered, nitrogen transfer to citrulline would start at Step A (glutamic dehydrogenase) with α-ketoglutaric acid made available from endogenous sources or from lactic acid oxidation. Hydrogen
transport for Step A might come from malate oxidation or from other dismutations. The transfer of nitrogen to ornithine (Step E) is provisionally shown as occurring through NH$_3$. This step has been studied by Cohen and his collaborators. According to their most recent report (18) carbamylglutamic acid will replace the CO$_2$ but not the NH$_3$ requirement in citrulline formation from ornithine. In the intact animal NH$_3$, arising from oxidative deamination of amino acids (19–21), would enter, as with slices, at Steps A and E. The scheme represents an expansion of the ornithine cycle originally proposed by Krebs and Henseleit (22).

The interrelationships with the tricarboxylic cycle occur through several additional connecting cycles. The transfer of NH$_3$ to oxalacetic acid, through Steps A and B, is a cyclic process in which α-ketoglutaric acid, supplied from the tricarboxylic cycle, is utilized catalytically. Another cycle is created by the turnover of oxalacetic acid through Steps B, C (arginine synthesis), and D (malic dehydrogenase). At the same time phos-

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**Fig. 1.** Pathway of amino nitrogen transfer to citrulline in urea synthesis and the relationships of the ornithine cycle to the tricarboxylic cycle.
phorylations, coupled with the tricarboxylic cycle, supply ATP for both citrulline and arginine synthesis.

Transfer of Amino Nitrogen to Oxalacetic Acid—The assumption of an obligatory incorporation of NH₃ in aspartic acid by transamination between glutamate and oxalacetate prior to urea formation (Steps A and B) appears to be justified for a number of reasons. Evidence is lacking for the existence in mammalian tissues of an aspartase or of an aspartic dehydrogenase analogous to glutamic dehydrogenase with respect to activity and reversibility. Also there is the observation that liver homogenates are incapable of oxidizing L-aspartic acid at an appreciable rate. In addition liver homogenates behave as though aspartic acid were an intermediate in arginine synthesis under a wide variety of conditions, as shown above. It may be pointed out that investigations of intact animals with N¹⁵-labeled amino acids and NH₃ show that aspartic acid as well as glutamic acid have the highest rate of turnover (20). Finally, Steps A, B, C, and D can be carried out anaerobically in crude extracts of ox liver acetone powder which contain these enzymes, through the coupling of several oxidation-reductions with transamination.

Amino Nitrogen Transfer Anaerobically—In the first dismutation, the following reactions occur:

1. Malic acid + DPN₉⁺ ⇌ oxalacetic acid + DPNₖ₉⁻
2. NH₃ + α-ketoglutaric acid + DPNₖ₉⁻ ⇌ glutamic acid + DPN₉⁺
3. Glutamic acid + oxalacetic acid ⇌ α-ketoglutaric acid + aspartic acid
4. Malic acid + NH₃ ⇌ aspartic acid

The dismutation, catalyzed by malic and glutamic dehydrogenases in the presence of transaminase (Reactions 1, 2, and 3), resulted in the formation of aspartic acid (Reaction 4) starting with malate, NH₃, and α-ketoglutarate in the presence of catalytic amounts of diphosphopyridine nucleotide (DPN). Citrulline, ATP, Mg²⁺, and phosphoglyceric acid were also added, and the appearance of aspartic acid was measured by arginine synthesis, as shown in Fig. 2, Curve 1. Curve 1a represents blank values when either DPN, NH₃, or malic acid was omitted.

In the second dismutation, triose phosphate dehydrogenase (Reaction 5) replaced malic dehydrogenase.

5. Triose phosphate + H₃PO₄ + DPN₉⁺ ⇌ diphosphoglyceric acid + DPNₖ₉⁻
6. NH₃ + α-ketoglutaric acid + DPNₖ₉⁻ ⇌ glutamic acid + DPN₉⁺
7. Glutamic acid + oxalacetic acid ⇌ α-ketoglutaric acid + aspartic acid
8. Triose phosphate + H₃PO₄ + NH₃ + oxalacetic acid →
   diphosphoglyceric acid + aspartic acid
Fig. 2. Transfer of NH₃ to citrulline through aspartic acid by anaerobic dismutation and transamination in acetone powder extracts of ox liver. Curve 1, malate, α-ketoglutarate dismutation; Curve 1a, the same as Curve 1 in the absence of either DPN, NH₃, or malate; Curve 2, triose phosphate, α-ketoglutarate dismutation; Curve 2a, the same as Curve 2 in the absence of either DPN, NH₃, or oxalacetate; Curve 3, arginine formation with aspartate in the same amount of extract. All the tubes contained 4 μM of ATP, 20 μM of L-citrulline, 13 μM of MgSO₄, 0.4 ml. of 0.25 M potassium phosphate, pH 7.5, and 1.0 ml. acetone powder extract in a final volume of 4 ml.; temperature 38°. Further additions were Curve 1, 20 μM each of NH₃Cl and l-malate, 10 μM of α-ketoglutarate, 50 μM of d-3-phosphoglyceric acid, and 2 mg. of DPN. Curve 2, 20 μM of NH₃Cl, 10 μM of α-ketoglutarate, 30 μM of oxalacetate, 25 μM of hexose diphosphate, and 2 mg. of DPN. Curve 3, 20 μM of l-aspartate, 50 μM of 3-phosphoglycerate.

Here aspartic acid, as measured by arginine synthesis, was formed (Reaction 6) when oxalacetic acid, NH₃, hexose diphosphate, DPN, α-ketoglutarate, and aspartic acid were present.

*The extract contains aldolase.*
tartaric acid, ATP, Mg++, and citrulline were added. This is shown in Curve 2. The diphosphoglyceric acid formed supplied ϕ-ph. Curve 2a represents blank values when either DPN, NH₃, or oxalacetate was omitted. Curve 3 represents the rate of arginine synthesis in the same amount of extract starting with aspartic acid, ATP, Mg++, and phosphoglyceric acid.

One or more of the enzymes catalyzing Reactions 1, 2, 3, and 5 were probably present in limiting concentrations. Because of this and of the relatively high Michaelis constant of the arginine-synthesizing system with respect to aspartic acid \((1.2 \times 10^{-3} \text{ M})\), the rate of arginine synthesis was lower in Curves 1 and 2 than in Curve 3. On the other hand, because of the rather large Michaelis constant, the rate of aspartic acid synthesis in Curves 1 and 2 was doubtless somewhat higher than that reflected by the arginine values.

**EXPERIMENTAL**

*Procedures*—For the experiments with homogenates, the liver from young adult, well nourished rats was removed immediately after exsanguination, chilled 20 minutes in buffer, rapidly weighed, homogenized with 3 volumes of 0.1 M potassium phosphate buffer, pH 7.5, in a loose fitting Potter-Elvehjem homogenizer (23), and strained through two layers of cheese-cloth. All manipulations were carried out at 2°. Incubation was carried out at 38° in conical Warburg vessels containing a KOH solution in the center well. Prior to incubation the substrates (adjusted to pH 7.5) were added to the main compartment, the vessels immersed in an ice bath, and 0.5 ml. of the cold 25 per cent homogenate added last in a final volume of 3.0 ml. The vessels were gassed for 2½ minutes with O₂. The oxygen consumption was estimated for 40 minutes, following a 5 minute equilibration period. The amount of arginine synthesized corresponds to a slightly longer period. The reaction was stopped by adding 2 ml. of 15 per cent metaphosphoric acid. Urea estimations were carried out by the method of Archibald (24), with slight modification, and the values expressed as equivalent amounts of arginine. Arginase was present in excess. All values were corrected for the zero time urea content of the tissue and were then recalculated for a uniform dry weight of 31 mg. They are so presented in Table I. Each value, representing the average of from three to five experiments, is given with the average deviation. Estimations based on Kjeldahl nitrogen indicated that 85 per cent of the dry weight of the homogenate was protein.

The anaerobic experiments were carried out in a similar fashion, except that the vessels were gassed with N₂. When the supernatant was to be used, the particles were separated by centrifuging for 30 minutes at 15,000

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4 This value was chosen, since it represents the average dry weight of all the experimental samples.
R.P.M. at 0°. The sediment occupied 20 per cent of the total volume. No special precautions were taken to remove traces of oxygen in view of the order of magnitude involved.

The dismutation experiments were carried out with acetone powder extracts of ox liver freshly prepared as described previously (1). The specific activity, without added muscle extract, was 0.53.

Chemical Preparations—For the preparation of L-citrulline, d-3-phosphoglyceric acid, oxalacetic acid, and ATP, consult the preceding paper (1). The DPN employed was 75 per cent pure; it was prepared by a modification of the method of Williamson and Green (25). α-Ketoglutaric acid was prepared by the method of Neuberg and Ringer (26).

SUMMARY

1. The synthesis of arginine from citrulline has been studied in liver homogenates under aerobic and anaerobic conditions, comparing aspartic acid with glutamic acid as —NH₂ donors.

2. Aerobically, arginine formation proceeds more rapidly with aspartic acid as NH₂ donor than with glutamic acid, when the reaction mixture is supplemented with a respiratory substrate as a source of ~ph.

3. Evidence is presented to show that oxidation of glutamic acid, through the tricarboxylic cycle, supplies both the aspartic acid and the ~ph required for arginine synthesis. Under these conditions aspartic acid is formed by transamination of glutamic acid with the oxalacetic acid arising by glutamate oxidation. Energy-rich phosphate is generated by phosphorylations coupled with oxidation.

4. The inhibition of arginine synthesis by α-ketoglutarate and by pyruvate when glutamate is the —NH₂ donor is explained as being due to interference, at the transamination step, with the obligatory formation of aspartic acid. The inhibitions were not observed when aspartic acid was supplied directly.

5. Malonate has no effect on arginine synthesis per se; the inhibition observed when glutamate is employed is exerted through inhibition of succinic dehydrogenase, thus preventing oxalacetate formation. Oxalacetate or a closely related precursor therefore overcomes the inhibition.

6. Conditions for effecting the anaerobic synthesis of arginine in liver homogenates are described.

7. A scheme is presented for the physiological pathway of amino nitrogen transfer from amino acids to form urea, showing the interrelationships with the tricarboxylic cycle and with transamination.

We are greatly indebted to Mr. R. F. Light, the Fleischmann Laboratories of Standard Brands, Incorporated, for a large gift of yeast.
8. The anaerobic transfer of \( \text{NH}_3 \) to citrulline to form arginine has been carried out in acetone powder extracts of liver.

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