BIOSYNTHESIS OF UREA

I. ENZYMATIC MECHANISM OF ARGinine SYNTHESIS FROM CITRULLINE*

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(Received for publication, February 4, 1949)

In urea synthesis, according to the Krebs-Henseleit ornithine cycle (1), the transfer of nitrogen to citrulline to form arginine (Step II) was thought to occur from NH3. It was not until Cohen and Hayano (2) succeeded in demonstrating rapid arginine synthesis in liver homogenates from glutamic acid and citrulline that the transfer from an amino acid rather than from NH3 was recognized as a main pathway in urea formation. Under their conditions, arginine was formed in the presence of oxygen, Mg++, and catalytic amounts of adenosine triphosphate (ATP). This was the same reaction observed earlier by Borsook and Dubnoff (3) in kidney slices and considered at the time to be a transamination with simultaneous oxidative removal of 2 H atoms (transimination). An α-keto acid, supposedly the second product of the reaction, would of course escape detection in respiring preparations. Whereas in the kidney slice experiments of Borsook and Dubnoff, both aspartic and glutamic acid were equally effective as amino group donors, Cohen and Hayano found in liver homogenates that glutamic acid was about 4 times as effective as aspartic acid. They concluded therefore that glutamic acid was the specific donor in the Borsook-Dubnoff reaction, aspartic acid being utilized only to the extent that it could be converted to glutamic acid.

This investigation was undertaken to elucidate further some fundamental aspects of the enzymatic mechanism involved.

As briefly reported earlier, it has been possible to obtain arginine synthesis with a partially purified enzyme system prepared by alcohol fractionation of acetone powder extracts of mammalian liver (4). In the isolated system, aspartic acid and citrulline are converted to arginine and malic acid in the presence of Mg++. ATP participates directly as a reactant and the reaction proceeds anaerobically without involving the transport of H atoms. In view of the evidence presented below, the transfer

* Aided by grants from the Williams-Waterman Fund of the Research Corporation, the American Cancer Society (recommended by the Committee on Growth of the National Research Council), the Office of Naval Research, and the United States Public Health Service.
The ability of ADP to act as the $\sim$ph donor in this reaction is not yet known.
of nitrogen is formulated as an exchange between the OH group of the isourea form of citrulline and the amino group of aspartic acid through formation of an intermediary condensation product, as shown in Reaction 1.

Intermediary Condensation Product—That malic acid formation is actually associated with arginine synthesis is shown by the following evidence: Neither product of the reaction appears unless both citrulline and aspartic acid are present simultaneously; malic acid always appears in amounts equivalent to the arginine formed; malic acid cannot be formed from aspartic acid independently. It may be seen from Table I that with the complete system (citrulline, aspartic acid, ATP, generated from phosphoglyceric acid, and Mg++) an equal amount of arginine and malic acid was formed in the 20 minute period, while in the absence of either aspartic acid or citrulline no reaction occurred. It is especially significant that, when citrulline was omitted (Table I, Line 2), malate was not formed from aspartic acid. The possibility that malic acid might be formed by a separate reaction was tested further. When an alcohol-fractionation and thoroughly dialyzed preparation was used as the enzyme source, such as that used to obtain the data shown in Table I, neither malic acid nor arginine was formed when aspartic acid was replaced by NH₃ or by the combination of NH₃ and oxalacetic acid. This may be taken not only as evidence that NH₃ is unreactive in this system, but also that malic acid cannot be formed by reduction from oxalacetate. Further proof is afforded by evidence that any pyridine nucleotides which might participate in hydrogen transport have been removed. The preparation employed contains some malic dehydrogenase and glutamic dehydrogenase. When oxalacetic acid and glutamic acid were present together, in the absence of citrulline, no malic acid was formed (Table I, Line 6). These dehydrogenases are known (5) to be capable of catalyzing the following dismutation: glutamate + oxalacetate ⇌ α-ketoglutarate + NH₃ + malate. Under such conditions malate should have appeared if diphosphopyridine nucleotide (DPN) had been present in sufficient concentration. It is therefore evident that under our experimental conditions the system is incapable of forming malic acid by reactions other than that involving interaction between citrulline and aspartic acid, even when oxalacetate and a source of H atoms are supplied.

The fact that arginine and malic acid are formed simultaneously from the interaction of the two substrates strongly suggests that the mechanism of nitrogen transfer involves a preliminary condensation of the amino group of aspartic acid with the ureido C of citrulline to form a C—NH—C

See the last section of Paper II concerning the DPN dependence of this reaction.
linkage, followed by cleavage on the second side of the nitrogen, as shown in Reaction 1.

The question arises as to whether Reaction 1 is mediated by a single enzyme, with transient formation of the intermediate such as is postulated in transamination between keto and amino acids (6, 7), or whether it proceeds in a stepwise manner. Very recently two enzymes have been separated by repeated ammonium sulfate fractionation of acetone powder extracts of ox liver (8). One of them catalyzed Reaction 1, a, as shown by the disappearance of citrulline and the simultaneous appearance of inorganic phosphate. When the resulting deproteinized reaction mixture

<table>
<thead>
<tr>
<th>Substrate added</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Aspartate, 20 μM</td>
<td>Arginine</td>
</tr>
<tr>
<td>L-Glutamate, 20 μM</td>
<td>μM</td>
</tr>
<tr>
<td>Oxaacetate, 30 μM</td>
<td>14.4</td>
</tr>
<tr>
<td>L-Citrulline, 20 μM</td>
<td>0.0</td>
</tr>
<tr>
<td>3-Phosphoglycerate, 50 μM</td>
<td>0.9</td>
</tr>
<tr>
<td>+</td>
<td>7.0</td>
</tr>
<tr>
<td>+</td>
<td>0.0</td>
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<tr>
<td>+</td>
<td>0.0</td>
</tr>
<tr>
<td>+</td>
<td>0.0</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

was incubated with the second enzyme, arginine and malic acid were formed simultaneously as shown in Reaction 1, b, by what appears to be a purely hydrolytic step. A detailed study of the two enzymes is now in progress and will be reported at a later date. The preliminary results indicate, however, that, under suitable conditions, accumulation of the postulated intermediate can be demonstrated, and that the transfer of the -NH₂ group proceeds by at least two enzymatic steps.

Role of ATP—Unlike keto-amino transamination, high energy phosphate (\(\sim\text{ph}\))³ is utilized in Reaction 1 and the phosphate transfer occurs specifically from ATP. For reasons which are not at present understood, ATP causes inhibition in concentrations above \(5 \times 10^{-3}\) M, but at this

³ This symbol as a designation of the energy-rich phosphate bond follows the usage introduced by Lipmann (9).
level 3 to 4 $\mu$M of arginine are formed in a 20 minute period from 10 $\mu$M of ATP as the only source of $\sim$ph, under conditions otherwise similar to those employed for obtaining the data given in Table I.

Competition with contaminating adenosinetriphosphatase (ATPase), which was appreciable even in alcohol-fractionated preparations, introduced uncertainties in the investigation of the stoichiometric participation of ATP. However, recent evidence obtained from a preliminary study of Reaction 1, $a$, in which ammonium sulfate-fractionated enzyme was employed, indicates that inorganic phosphate and an intermediate product are formed in equivalent amounts. Whether phosphorylation precedes condensation or occurs simultaneously with it remains to be investigated.

**Nature of Condensation**—Sufficient data are not available to permit the calculation of the energy change in each step of the ornithine cycle, but it is reasonable to assume, in view of the endergonic nature of urea synthesis from NH$_3$ and CO$_2$, that citrulline and arginine formation are each endergonic, and that, as far as arginine synthesis is concerned, it is in the condensation Reaction 1, $a$, that energy, derived from ATP, is actually utilized.

The condensation product is a substituted guanidine, whereas a Schiff base type of compound is formulated as an intermediate in keto-amino transamination. It seems more probable that aspartic acid will condense with the isourea rather than the urea form of citrulline, since O-methyl and S-methyl isourea readily form guanidines with amines, chemically, whereas urea does not. Although information regarding the phosphorylation step is still lacking, phosphorylation of isocitrulline would appear to be more likely than aspartic acid phosphorylation, for it provides a means of shifting the citrulline equilibrium toward the isourea form. Once the intermediate is formed, hydrolytic cleavage of the C—N bond of the aspartic acid moiety would presumably be favored by the formation of the highly resonating monosubstituted guanidine group of arginine as compared to the more restricted resonance of the $N, N'$-disubstituted guanidine group of the intermediate (10).

**Properties of Enzyme System**

*Phosphoglyceric Acid As Generator of $\sim$ph*—Owing to competition with ATPase, the rate of the over-all reaction is low to a misleading degree, when ATP is used directly. In order to approach maximum rates, and at the same time to avoid the complications of ATP inhibition, it was found more feasible to employ ATP as the catalyst and phosphoglyceric acid as the source of $\sim$ph. In making use of phosphoglyceric acid as a source of $\sim$ph, advantage has been taken of the presence of phosphoglyceromutase.
(11), enolase (12), and phosphoenol transphosphorylase in acetone powder extracts of liver. These enzymes are likely to be limiting in the crude extract and to become increasingly more so upon fractionation. They were therefore supplied in excess by addition of an ammonium sulfate fraction prepared from rabbit muscle extract. This muscle fraction in-

![Graph](https://example.com/graph.png)

**Fig. 1.** Dependence of the rate of arginine synthesis on the concentration of L-aspartate, L-citrulline, magnesium sulfate, ATP (scale 0 to 5 μM), and 3-phosphoglycerate. In A, B, C, and D, Curve 1 represents acetone powder extract, 38 mg. of protein per tube; Curve 2, as in Curve 1, supplemented with muscle extract. The conditions are otherwise as in Table I.

increased the rate of arginine synthesis, without itself possessing arginine-synthesizing activity.

The phosphoglyceric acid dependence curves (Fig. 1, C) show that a large excess was required in order to achieve maximum rates and that the addition of muscle extract caused a 35 per cent stimulation. The ATP curves in Fig. 1, C show that under these conditions ATP functioned catalytically; maximum rates were reached with 1.6 μM.

**Optical Specificity**—The enzyme system reacts only with the natural
isomers of citrulline and aspartic acid. Optical specificity was investigated on a rate basis, in each case the activity of the DL isomer being compared with that of the L isomer at two concentrations. As shown in Table II, the rate of arginine synthesis is the same with 20 μM of DL-aspartic acid as with 10 μM of the L form and similarly with citrulline.

Identification of Products of Reaction—Although little doubt now remains that urea is formed exclusively from arginine, alternative pathways (see for example Bach (13)) have occasionally been proposed. Arginase was present in excess in all the enzyme preparations employed and arginine was therefore estimated as urea by the colorimetric method of Archibald (14). In order to establish with certainty that arginine was the primary product, ornithine was isolated from a large scale enzymatic run and identified as dibenzoyl-L-ornithine (see "Experimental"). L-Malic acid was identified enzymatically by means of a highly purified preparation of the "malic" enzyme of Ochoa, Mehler, and Kornberg (15), which acts only on the L form, and by isolation as cinchonine L-malate.

Substrate Specificity—Thus far no amino acid other than aspartic acid has been found to react with citrulline. The amino acids tried were DL-serine, DL-lysine, DL-isoleucine, L-leucine, L-proline, DL-alanine, DL-amino-adipic acid, DL-aminopimelic acid, L-tyrosine, L-histidine, DL-tryptophan, DL-ornithine, DL-valine, L-cysteine, glycine, DL-methionine, DL-threonine, DL-phenylalanine, and L-glutamic acid. They were all tested both with and without oxalacetic acid, at the concentration known to be optimum for aspartic acid, with a 40 minute incubation period and twice the amount of acetone powder extract ordinarily used.

Aspartic acid may be replaced by a combination of glutamic acid and oxalacetic acid, but not by glutamic acid alone (Table I, Lines 4 and 5).

### Table II

**Optical Specificity of Aspartic Acid and Citrulline in Arginine Synthesis**

The conditions are as in Table I, but without muscle extract.

<table>
<thead>
<tr>
<th>Amino acid varied</th>
<th>Added</th>
<th>20 min.</th>
<th>40 min.</th>
<th>60 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>μM</td>
<td>μM</td>
<td>μM</td>
</tr>
<tr>
<td>L-Citrulline</td>
<td>20</td>
<td>11.3</td>
<td>15.4</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7.6</td>
<td>7.5</td>
<td>7.7</td>
</tr>
<tr>
<td>DL-Citrulline</td>
<td>20</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>L-Aspartate</td>
<td>20</td>
<td>12.0</td>
<td>15.4</td>
<td>15.8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7.6</td>
<td>8.4</td>
<td>8.0</td>
</tr>
<tr>
<td>DL-Aspartate</td>
<td>20</td>
<td>7.6</td>
<td>8.2</td>
<td>8.0</td>
</tr>
</tbody>
</table>
The activity of this combination can be readily explained by the formation of aspartic acid by transamination. This has been corroborated by the assay of various preparations for glutamic-aspartic transaminase activity. Although the rate of aspartic acid appearance under the experimental conditions employed for arginine synthesis cannot be calculated from the assay data, the relationship can be shown by comparing, in several preparations, the rate at which arginine is formed by the combination of glutamic and oxalacetic acid, or aspartic acid, with the transaminase concentration of the preparation. For example, in 20 minutes, 0.5 ml. of a crude extract of ox liver acetone powder, containing 30 units of transaminase (see "Experimental"), catalyzed the formation of 8.1 μM of arginine with aspartic acid, and 7.2 μM with glutamic and oxalacetic acids, while 0.4 ml. of the alcohol-fractionated preparation used to obtain the data in Table I, containing 12 units of transaminase, catalyzed the formation of 14.4 μM of arginine with aspartic acid, and 7.0 μM with glutamic and oxalacetic acids, in the same period of time. In other words, the difference between the two rates is small when transaminase is present in excess, but very large when the transaminase concentration is limiting. Acetone powder extracts of rat liver were found to behave in an entirely similar way toward aspartic acid, toward glutamic acid, and toward the combination of glutamic and oxalacetic acids. Presumably the same may be said of mammalian liver in general.

If glutamic-alanine transaminase were also present in acetone powder extracts, the combination of alanine, oxalacetic acid, and a catalytic amount of α-ketoglutaric acid would be expected to form aspartic acid, as suggested by Green, Leloir, and Nocito (16) and demonstrated by O'Kane and Gunsalus (17). This combination, tested in acetone powder extracts, did not give rise to aspartic acid (as measured by arginine appearance) for the reason that glutamic-alanine transaminase, being low in liver tissue to start with, probably fails to survive acetone treatment.

The formation of arginine from α-aminoadipic acid and citrulline in kidney slices, reported by Dubnoff and Borsook (18), might be interpreted, in view of Braunstein's observation (19) that α-aminoadipic acid can transaminate with pyruvic acid, by the presence in slices of enzymes capable of transferring the amino group of α-aminoadipic acid to oxalacetate by transamination reactions. Acetone powder extracts were tested with α-aminoadipic acid and various combinations of oxalacetic acid, pyruvic acid, and α-ketoglutaric acid in the hope ofcoupling the two transaminating systems. The results were negative, as with alanine.

Substrate Dependence and Other Properties—The enzyme system, which is quite soluble, can be readily extracted from acetone powders of mammalian liver (rat, pig, ox) with water or dilute phosphate buffer, and is
fairly stable to low temperature fractionation with ethyl alcohol or ammonium sulfate. The pH optimum of the over-all reaction lies between pH 7.4 and 7.5 in agreement with that found by Cohen and Hayano (20) for liver homogenates.

The requirement for Mg++ is rather high, as shown in Fig. 1, D, half saturation occurs with 3.8 \( \mu M \) of \( MgSO_4 \) with and without muscle extract, corresponding to \( 0.95 \times 10^{-3} M \). In addition to the known dependence upon Mg++ of \( \sim \)ph transfer from phosphopyruvic to adenosine diphosphate, Mg++ is specifically required in the condensation Reaction 1, a. When ATP was the only \( \sim \)ph donor, the reaction did not proceed in the absence of Mg++ and, in the presence of the latter, 0.01 \( M \) fluoride caused approximately 50 per cent inhibition. Studied separately, the condensing enzyme showed, in common with a number of other phosphate-transferring enzymes, the same specific requirement for Mg++ and inhibition by fluoride as the over-all system. These observations indicate in part the basis of the fluoride inhibition found in homogenates by Cohen and Hayano (2).

Citrulline and aspartic acid concentration dependence curves are shown in Fig. 1, B and 1, A. Half saturation of the enzyme system was reached in both cases with 4.8 \( \mu M \) (1.2 \( \times 10^{-3} M \)) with and without added muscle extract.

Estimation of Enzyme Activity—In order to estimate the specific activity (units per mg. of protein) of various fractions, as purification proceeds, a unit has been chosen as the amount of enzyme which will catalyze the formation of 1 \( \mu M \) of arginine per hour under standardized test conditions, these being a 20 minute incubation period during which time no more than 10 \( \mu M \) of arginine have been formed from the 20 \( \mu M \) of citrulline and aspartic acid added. Under these conditions the rate of arginine formation is linear from 10 to 25 minutes, as shown in Fig. 2, A. An induction period may be noted in the first 10 minutes, undoubtedly due to the time lag necessary to reach an optimum concentration of intermediate, as might be expected in a stepwise reaction. It was not abolished by the addition of muscle extract and is therefore not likely to be associated with a limit in ATP formation. Above 25 minutes, the rate falls off, owing to declining substrate concentrations.

Regardless of the specific activity of the preparation, enzyme concentration dependence studies reveal a disproportionate increase in activity with increasing enzyme concentration. Fig. 2, B shows the rates obtained with three different preparations. Since muscle extract was added in excess, and separate experiments indicated that arginase was not a limiting factor, the exponential effect may be ascribed to the presence of the two enzyme components of Reaction 1, mentioned above.
The choice of conditions for estimating specific activity must be somewhat arbitrary under such circumstances. In actual practice during fractionation, the conditions were sufficiently reliable to permit recovery of most of the initial activity. Muscle extract was routinely added in excess and, when necessary, 1 mg. of a partially purified arginase preparation, having a specific activity of 34 units per mg., was also added (see "Experimental").

Data for the specific activity of several alcohol-precipitated fractions, as compared with the initial extract, are given in Table III. In the particular preparation recorded, after one fractionation procedure, the activity was found to concentrate in Fractions 3 and 4, the latter having the highest activity and a 3-fold purification over the original extract. Difficulty was occasionally encountered in reproducing these values because of the two-component nature of the system.

In the fifth column the activity is expressed in terms of the $Q_{\text{urea}}$ value of Krebs to facilitate comparison of liver acetone powder extracts with homogenates under the conditions of Cohen and Hayano (2) and with slices under the original conditions of Krebs and Henseleit (1); i.e., ornithine, $\text{NH}_3$, and lactate. The latter comparison is perhaps justified, since Gornall and Hunter (21) have shown that the conversion of citrulline to arginine is the rate-limiting step of the ornithine cycle.

Arginine Synthesis in Homogenates and Slices—On comparing the total activity of acetone powder extract of rat liver (calculated from the specific
activity) to that of the tissue homogenate or slice, it appears that about 50 per cent of the original activity can immediately be accounted for. If allowance is made for some destruction by acetone treatment and for the fact that a single extraction might not be exhaustive, the result indicates that a large amount of the arginine-synthesizing activity is to be found in the isolated system. Although a discrepancy appears to exist between homogenates and the isolated system with respect to the specificity

<table>
<thead>
<tr>
<th>Species</th>
<th>Preparation</th>
<th>Conditions</th>
<th>Specific Activity</th>
<th>( Q_{\text{urea}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Slice</td>
<td>( \text{NH}_2, \text{lactate}^* )</td>
<td>18</td>
<td>1.50 33.0</td>
</tr>
<tr>
<td></td>
<td>Homogenate</td>
<td>Glutamate†</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acetone powder extract</td>
<td>Aspartate, pyruvate‡</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ox</td>
<td>( ^{\sim} \text{ph} ) + muscle extract</td>
<td>0.84 18.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EthOH Fraction 3</td>
<td>( ^{\sim} \text{ph} ) + muscle extract</td>
<td>0.90 20.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&quot; 3</td>
<td>&quot; + muscle extract</td>
<td>0.33 7.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&quot; 4</td>
<td>&quot; + muscle extract</td>
<td>0.50 11.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&quot; 4</td>
<td>&quot; + muscle extract</td>
<td>0.35 7.8</td>
<td></td>
</tr>
</tbody>
</table>

* \( Q_{\text{urea}} \) (microliters per mg. of protein per hour) recalculated from the data of Krebs and Henseleit, assuming 85 per cent of the dry weight to be protein.
† Calculated from the rate of arginine synthesis per mg. of N given by Cohen and Hayano.
‡ Calculated from the data given in Table I of Paper II, assuming 85 per cent of the dry weight to be protein.

of the \( -\text{NH}_2 \) donor, the data in Table III leave little doubt that the same enzyme system is involved, regardless of the substrates offered or of other variations in the experimental conditions. The fact that in extracts aspartic acid can be replaced, through transamination, by the combination of glutamate and oxalacetate suggests that in homogenates glutamic acid functions in arginine synthesis indirectly, by transfer of the \( -\text{NH}_2 \) group to oxalacetate, rather than by an interaction with citrulline catalyzed by a second arginine-synthesizing system exhibiting glutamate specificity. Additional evidence for this and for the apparently poor activity of aspartic acid in liver homogenates will be discussed in Paper II.

In both liver slices and homogenates, urea synthesis has been reported to be completely dependent upon a supply of oxygen. In Step II of the
ornithine cycle, any direct oxidation-reduction mechanism has been excluded, but the participation of \( \sim \)nph has been shown to be essential for arginine synthesis. The oxygen dependence in respiring preparations must then be associated with the generation of ATP by oxidation of respiratory substrates. In slices, added lactate, and in homogenates some of the glutamic acid serves in this capacity. The coupling of phosphorylation with the oxidative steps of the tricarboxylic cycle has been observed repeatedly in tissue preparations (22).

**EXPERIMENTAL**

*Methods*—The enzymatic reaction was carried out in small test-tubes kept at 0° prior to incubation. All additions were made with chilled solutions of substrates, previously adjusted to pH 7.5 with dilute KOH; the cold enzyme solution was added last. The tubes were then transferred to a water bath at 38° for the stated time interval, allowing 2 minutes for temperature equilibration, and the reaction was stopped with 2 ml. of 15 per cent metaphosphoric acid per 4 ml. of reaction mixture. Water was then added to a volume of 10 ml., the mixture filtered, and 0.5 ml. aliquots of the filtrate used for the estimation of urea by the method of Archibald (14) modified slightly. When more precise temperature control was necessary, all the additions except ATP were made at 0°. The tubes were then set in the water bath and the ATP added after temperature equilibration. This procedure gave slightly lower values. The complete system, as used routinely for measurements of enzyme activity, contained, in addition to the enzyme in 0.05 M potassium phosphate buffer at pH 7.5, 0.25 M potassium phosphate buffer, 0.4 ml.; 0.1 M L-aspartate, 0.2 ml.; 0.1 M L-citrulline, 0.2 ml.; 0.033 M MgSO\(_4\), 0.4 ml.; 0.05 M ATP, 0.08 ml.; 0.1 M 3-phosphoglyceric acid, 0.5 ml.; when indicated, 8.8 mg. of muscle fraction in water; and water to make a final volume of 4 ml. To conserve materials, reactions were frequently carried out on half the scale and the analytical data doubled to corresponding full scale values. The amount of arginine formed was calculated from the urea estimation, since arginase was always present in excess.

When malic acid was also estimated, 0.25 M glycylglycine buffer replaced phosphate. The reaction was stopped by heating the tubes in a boiling water bath for 10 minutes after bringing the mixture to pH 6.0 with 0.02 to 0.04 ml. of 1 \( \times \) sulfuric acid, the tubes were cooled to room temperature, water was added to a volume of 10 ml., and the mixture filtered. Malate was estimated spectrophotometrically in suitable aliquots of the filtrate by means of the "malic" enzyme of pigeon liver, as described by Ochoa, Mehler, and Kornberg (15).

Transaminase activity was estimated by the method of Green, Leloir,
and Nocito (16) and is expressed in their units, one unit being that amount of enzyme which forms oxalacetic acid equivalent to 100 μl. of CO₂ in 10 minutes at 38° under given conditions.

Protein was estimated spectrophotometrically at 280 μμ, corrected for nucleic acid impurities according to Warburg and Christian (12).

Preparation and Fractionation of Enzyme System—Acetone powder was prepared in a cold room at 2° from fresh ox liver chilled at the slaughterhouse. Small trimmed pieces amounting to 115 gm. were ground for 30 seconds in the Waring blender with about 200 ml. of acetone, previously chilled to -5°. The contents were rapidly transferred to a beaker with more acetone at -5° (10 volumes in all), stirred rapidly for 10 to 15 seconds, and filtered by suction on a large Büchner funnel. Rapid stirring with 10 volumes of acetone was repeated and the mixture was again filtered rapidly with suction. The tightly packed cake was rapidly spread out at room temperature with constant mixing to facilitate rapid drying. The activity of the dry powder stored at 2° slowly falls, decreasing to about half the value in 5 to 6 weeks.

The powder was extracted with 5 volumes of 0.1 M potassium phosphate buffer, pH 7.5, with mechanical stirring at room temperature for 20 minutes, centrifuged cold at 15,000 r.p.m., and the supernatant dialyzed at 2° against 0.05 M buffer overnight to insure zero blanks. These crude extracts contain approximately 75 mg. of protein per ml. after dialysis and, when stored at 2°, maintain activity for about 2 days but fall off rapidly thereafter.

Acetone powders of rat liver were made in a similar manner and may be stored for several weeks in the cold without loss of activity, but the extracts were found to be much more unstable than ox preparations. The extraction with 5 volumes of buffer was therefore carried out at 0° as well as centrifugation, and cold dialysis was limited to a 3 hour period. With these precautions the activity falls off to about 50 per cent in 24 hours and is almost completely gone 2 days after extraction.

In a typical alcohol fractionation, 315 ml. of buffered extract prepared from 100 gm. of beef liver acetone powder were chilled to 0°, transferred to a bath kept at -5°, and absolute ethyl alcohol, kept at -50°, added very slowly with mechanical stirring. The temperature of the mixture was not allowed to rise above 0° at the beginning of the addition and thereafter was allowed to drop slowly to -5° by the time the first alcohol addition was completed. The mixture was centrifuged at -5° for 1 hour at 3000 r.p.m. and the supernatant rapidly decanted into a beaker immersed in a -10° bath. The tubes containing the precipitate were immediately packed in cracked ice, and the precipitates taken up in a total of 10 to 20 ml. of 0.1 M buffer and dialyzed in 0.05 M buffer overnight to remove traces of alcohol.
Subsequent additions of alcohol to the supernatant were made in a similar manner, except that the temperature of the extract was maintained at -10° for both the alcohol addition and centrifugation. Four fractions were thus obtained by stepwise additions of 45, 25, 35, and 30 ml. of alcohol, corresponding to 12.5, 18.2, 25, and 30 per cent alcohol for each fraction. The alcohol concentration was calculated without correcting for the volume of the precipitate removed. The specific activity was 0.0, 0.40, 0.89, and 1.50, respectively. The total activity represented a 72 per cent recovery.

Isolation and Identification of l-Malic Acid—A large enzymatic run, on 25 times the scale described above, was carried out at 38° with an excess of alcohol-fractionated enzyme containing 1505 units (1.81 gm., specific activity 0.83, without added muscle extract). After 60 minutes, 26.4 mg. of urea (440 μM) were found, corresponding to 59 mg. of malic acid and 57.2 mg. of ornithine. The reaction mixture was acidified to pH 6.0 with 3.5 ml. of 1 N sulfuric acid, deproteinized by heating in a bath at 100°, the filtrate brought to pH 1.0 with 6 ml. of 6 N sulfuric acid, and extracted with ether in a continuous extractor for 72 hours. The ether layer was then taken to dryness, dissolved in water, filtered, and brought to a volume of 10 ml. Enzymatic analysis indicated that 38.6 mg. (65 per cent of theory) were recovered. After evaporating the solution to dryness, the malic acid was then converted to the cinchonine salt by refluxing in 5 ml. of acetone with 74 mg. of d-cinchonine for 30 minutes. After standing at 0° overnight, 78 mg. of crude salt were obtained. On three recrystallizations from methyl alcohol-acetone, and one from water-acetone, the melting point was 191–192° uncorrected. The mixed melting point, with an authentic sample of melting point 192°, was 191–192° uncorrected; the reported melting point was 198° (23). The optical rotation of the cinchonine salt was

\[ \alpha_{D}^{19} = +146° \text{ (0.515% in water; } l = 2.0) \]

The optical rotation of the uranium salt was determined under the general conditions of Dakin (23) adapted to small scale as follows: 2.5 ml. of the solution of cinchonine l-malate were quantitatively decomposed by the addition of 0.5 ml. of 0.14 N ammonia and filtered. To 2.0 ml. of the filtrate was added 0.5 ml. of 2.5 per cent uranium acetate, slightly acidified with acetic acid, and the solution was allowed to stand for 1 hour at room temperature.

\[ \alpha_{D}^{19} = -481° \text{ (0.1076%; } l = 2.0) \]

The optical rotations reported by Dakin (23) were

\[ \alpha_{D}^{19} = +146° \text{ for cinchonine l-malate} \]

\[ \alpha_{D}^{19} = -482° \text{ for the uranium salt of l-malic acid} \]
Isolation and Identification of Ornithine—The aqueous layer (about 125 ml.) obtained after ether extraction was brought to pH 9 with a few ml. of 50 per cent NaOH, and benzoylated in the usual manner with 0.35 ml. of benzoyl chloride and 6 ml. of 1 N NaOH. The mixture was filtered at pH 7, acidified, and kept at 0° overnight. The crude crystalline dibenzoyl-ornithine, obtained after filtering off the crystals and washing thoroughly with ether, weighed 133 mg. (88 per cent yield). On three recrystallizations from ethyl alcohol-acetone mixture, the compound melted at 186° uncorrected. [α]D21 = +9.3° (3.60 per cent in 1 N NaOH) nitrogen (Kjeldahl); found 8.2 per cent, calculated 8.2 per cent.

Chemical Preparations—d-3-Phosphoglyceric acid was prepared by the method of Neuberg and Lustig (24). Fleischmann’s commercial bakers’ yeast,4 dried in the laboratory, gave good yields with glucose and added DPN. ATP6 was prepared according to Lohman (25). L-Citrulline was prepared by a combination of the methods of Kurtz (26) and Gornall and Hunter (27), starting with a commercial preparation of L-arginine. We are indebted to Dr. H. B. Dunn for DL-citrulline, and to Dr. H. Waelsch for DL-α-aminoadipic acid and DL-α-aminopimelic acid. The other amino acids were commercial preparations of acceptable purity. Oxalacetic acid was prepared according to Krampitz and Werkman (28), as modified by F. Lipmann (personal communication).

Other Enzyme Preparations—Partially purified arginase was prepared from a Mn++ -activated extract of beef liver by acetone fractionation according to Van Slyke and Archibald (29).6 The arginase activity of the preparation was 34 units per mg. of protein when the unit and assay conditions of Van Slyke and Archibald (30) were employed, 1 unit being that amount which liberates 1 μM of arginine in 1 minute under given conditions.

The extract of rabbit muscle was prepared according to Racker (31). The fraction precipitating between 50 and 72 per cent ammonium sulfate saturation was dialyzed for 2 hours against running tap water and overnight against 0.02 M potassium phosphate buffer, pH 7.5, and then lyophilized. Stored at 2°, the dry powder retains activity for several months. Under the experimental conditions described, 8.8 mg. for each tube supplied an excess of the required activity. We are indebted to Dr. E. Racker for a gift of this fraction.

SUMMARY

1. An enzyme system catalyzing the conversion of citrulline to arginine has been isolated from mammalian liver and partially purified.

4 We are indebted to Mr. R. F. Light, the Fleischmann Laboratories, of Standard Brands, Incorporated, for a large gift of yeast.

6 The ATP employed was 80 to 85 per cent pure. The amounts employed are given on a 100 per cent basis.

2. Evidence is presented to show that in the reaction L-citrulline and L-aspartic acid undergo an exchange to form L-arginine and L-malic acid through an intermediary condensation which utilizes ATP and Mg++, and is presumably endergonic.

3. Two separate enzymes are involved in the over-all reaction, one catalyzing the formation of an intermediary condensation product and the second its hydrolysis.

4. Some general enzymatic properties of the system are described.

5. The mechanism is believed to represent the main physiological pathway of arginine synthesis and urea formation.

We are indebted to Mr. Morton C. Schneider for technical assistance.

BIBLIOGRAPHY

12. Warburg, O., and Christian, W., Biochem. Z., 310, 384 (1941).
BIOSYNTHESIS OF UREA: I.
ENZYMATIC MECHANISM OF
ARGININE SYNTHESIS FROM
CITRULLINE
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