UREA SYNTHESIS BY LIVER HOMOGENATES*

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The Krebs-Henseleit theory (1) of urea synthesis (see the modified scheme below) has received support from the later work of Krebs (2, 3), of Gornall and Hunter (4), and from isotope studies (5–8).

\[
\text{Ornithine} + \text{CO}_2 + \text{NH}_3 \rightarrow \text{citrulline} \quad (I)
\]

\[
\text{Urea} \quad (II) \quad \text{glutamic acid} \quad \text{NH}_3
\]

\[
\text{citrulline} \quad (III) \quad + \text{HOH} \rightarrow \text{arginine}
\]

The synthesis of citrulline from ornithine and arginine from citrulline has heretofore been associated only with intact liver cells. In the course of studies on the mechanism of the conversion of citrulline to arginine, a highly active system was found in liver homogenate capable of forming urea in the presence of glutamic acid, adenosine triphosphate (ATP), cytochrome c, an oxygen atmosphere, and magnesium ions.

The obvious similarity of the substrates, citrulline and glutamic acid, to that required for the transimination system found by Borsook and Dubnoff (9) in kidney slices led us to a general study of this reaction in rat tissue slices and homogenates (10). The rôle of glutamic acid as the imino group donor to arginine was shown to be very specific, not replaceable by other similar compounds (except glutamine) or by ammonium chloride. In previous work (11), it was reported that liver slices synthesized urea to a limited extent only from citrulline and glutamic acid, more readily from ammonium chloride, and to a considerable extent when both ammonium chloride and glutamic acid were provided. In the present paper, data will be presented which demonstrate in liver the presence of a potent transimination system which in all probability is the mechanism by which citrulline is converted to arginine in the urea cycle.

Procedures

Incubation of Liver Slices and Homogenates—The detailed procedures followed for experiments with liver slices and homogenates have been pre-

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Previously described (10). Minor changes were made in the incubation systems employing slices. Warburg flasks with side arms were used for both incubation and urea determination. Each flask contained glucose, dl-citrulline, and Krebs-Ringer-phosphate buffer, pH 7.4, in the main compartment. The side arm contained either ammonium chloride or glutamic acid or both. The final volume in the flasks was either 3.5 or 3.9 ml. as indicated. 20 per cent aqueous potassium hydroxide was used in the center well.

Oxygen Uptake Determinations—For oxygen uptake determinations, the incubation mixtures were prepared as described. All flasks were charged with pure tank oxygen prior to introduction into the bath. 20 per cent aqueous potassium hydroxide was used in the center wells.

Determination of Urea—Urea was determined manometrically with a purified urease preparation by the method of Krebs and Henseleit (1).

Determination of Citrulline—Citrulline was determined in aliquots after urea decomposition with a purified urease prepared according to the method of Archibald and Hamilton (12). After the analysis for urea, the contents of the flasks were deproteinized with 1 ml. of 10 per cent trichloroacetic acid, centrifuged, and an aliquot of the clear supernatant taken for the analysis of citrulline by the colorimetric method of Archibald (13). It was found that these aliquots contained very little chromogenic material other than citrulline, and consequently the Amberlite blank was omitted.

Determination of Glutamic Acid—Glutamic acid was determined manometrically on an aliquot of the incubated sample brought to pH 5.0 with 3 M acetate buffer, with a lyophilized preparation of Escherichia coli. The authors are indebted to Dr. R. H. Burris for a generous supply of this preparation.

Determination of α-Ketoglutaric Acid—α-Ketoglutaric acid was determined according to the method of Krebs (14).

Results

Urea Synthesis by Liver Homogenate—The formation of urea from citrulline through arginine by transimination with glutamic acid was shown to occur at an unusually high rate as compared with slices (10). Comparative rates of urea synthesis from glutamic acid, ammonium chloride, or both are shown in Fig. 1. The amount of urea formed in the presence of glutamic acid by homogenate exceeded that formed from other substrate combinations with both homogenates and slices. The maximum amount of urea formed with glutamic acid occurred after 60 minutes. The failure of homogenate after 1 hour may have been due to a depletion of ATP or the coenzymes. With ammonium chloride and glutamic acid, the urea formed in the first 20 to 30 minutes was almost equal to that formed by glutamic
acid alone; however, after this time the rate was not maintained. In all probability, this effect is related to the toxic effect of ammonium ions, which would accumulate during the early incubation period. This system is apparently sensitive not only to increases in ammonium ions but also to \( \alpha \)-ketoglutaric acid (Table I).

**Urea Synthesis by Liver Slices**—Krebs (1) found that urea was formed more rapidly when glutamic acid or lactic acid and ammonium chloride, rather than ammonium chloride alone, was present in the incubation mix-

**Fig. 1.** Relative rates of urea synthesis with different substrates by liver homogenate and slices. Substrate and cofactor final concentration are as follows: Slices, glucose, ammonium chloride, glutamic acid, each at \( 1.54 \times 10^{-2} \text{ M} \), \( dl \)-citrulline \( 3.08 \times 10^{-3} \text{ M} \); tissue dry weights 12 to 17 mg. Homogenates, \( dl \)-citrulline and glutamic acid, each at \( 5.72 \times 10^{-3} \text{ M} \), cytochrome c \( 4.72 \times 10^{-4} \text{ M} \), adenosine triphosphate \( 8.57 \times 10^{-4} \text{ M} \); tissue concentration per flask \( 1.46 \text{ mg. of N} \). Final flask volumes as follows: slices \( 3.9 \text{ ml.} \), homogenates \( 3.5 \text{ ml.} \). \( \Delta \) represents \( \text{NH}_4\text{Cl} \), and glutamic acid; \( \square \) represents \( \text{NH}_4\text{Cl} \); and \( \bigcirc \) glutamic acid.

ture. This observation has been confirmed in the present study as regards glutamic acid (Fig. 1). Urea synthesis from citrulline proceeded more rapidly when both glutamic acid and ammonium chloride were present in the medium. Ammonium chloride alone yielded only about 50 per cent as much urea at the end of a 2 hour incubation period, while glutamic acid alone gave rise to only 15 per cent.

**Comparison of Optimum Systems of Slices and Homogenates**—On a mg. of tissue N basis with optimum systems of slices and homogenates, the urea
formed by homogenate from glutamic acid and citrulline was more than twice that of the optimum system (citrulline plus glutamic acid plus ammonium chloride) for slices (Fig. 1). It was found that by increasing the tissue concentration in homogenate studies to 2.5 to 3.0 mg. of N per flask the reaction would go to completion within 2 hours. Tissue slices at the rate shown on Fig. 1 would take more than 5 hours.

Comparison of Utilization of Substrates by Homogenates and Slices—Since measurements of the disappearance of glutamic acid in experiments with slices revealed that only 1 per cent disappeared in 1 hour, one-half of which was accounted for as urea, it would indicate that slices are unable to utilize glutamic acid in the system investigated to any appreciable extent. In all probability, the limiting factor is the inability of glutamic acid to diffuse through the liver cell wall at a sufficiently rapid rate. In order to evaluate this possibility, experiments were set up in which the oxygen uptake of liver slices and homogenates were compared in the presence of the same substrates in concentrations found to be optimum for urea synthesis. Liver has been known to contain the most active glutamic acid dehydrogenase system of all the tissues studied (15). The relative rates of oxidation of glutamic acid and related substrates in experiments with slices and homogenates would be expected to be a measure of the diffusibility of the glutamic acid through the cell wall of the slices. Homogenates were fortified with all the known necessary cofactors. Since the oxygen uptakes are expressed on a tissue nitrogen basis, it would be assumed that the potential enzyme activity of the two preparations on this basis would be equal.

### Table I

**Inhibition of Urea Synthesis in Homogenates by Ammonium Chloride and α-Ketoglutaric Acid**

<table>
<thead>
<tr>
<th>Group</th>
<th>Inhibitor added, final molarity</th>
<th>Urea formed per mg. tissue N (micros)</th>
</tr>
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<tbody>
<tr>
<td>A. Ammonium chloride</td>
<td>0.0</td>
<td>37.45</td>
</tr>
<tr>
<td></td>
<td>0.00715</td>
<td>35.90</td>
</tr>
<tr>
<td></td>
<td>0.0143</td>
<td>33.30</td>
</tr>
<tr>
<td></td>
<td>0.0286</td>
<td>23.68</td>
</tr>
<tr>
<td>B. α-Ketoglutaric acid</td>
<td>0.0</td>
<td>42.40</td>
</tr>
<tr>
<td></td>
<td>0.01142</td>
<td>13.31</td>
</tr>
</tbody>
</table>
Any differences in the activity of the two systems would, therefore, in all probability be due to either the limiting rate of diffusion into the liver slices or the inadequate fortification of the homogenate. As seen from Figs. 2 and 3, the rate of oxygen consumption of the homogenate systems is of the order of 6 times greater in the case of glutamic acid than for slices. It should also be noted that the slices had a final glutamic acid concentration 2.5 times greater than that used with homogenates. These results can mean only that the inability of liver slices to utilize glutamic acid for either oxidation or transimination is due to the relatively poor diffusibility of this compound. The somewhat greater urea-synthesizing activity of the combination of glutamic acid plus ammonia with slices than the additive activities of the two compounds separately may be due to either (1) an effect of the ammonium ion on the diffusibility of glutamic acid into the cell or (2) the probability that ammonia is utilized in Step I by a reaction not involving glutamic acid as an intermediate. The experiments on oxygen
consumption of slices and homogenates do not support the first suggestion but neither do they exclude this possibility. The second suggestion could explain why the combination of glutamic acid plus ammonia, or glutamine, would provide optimum amounts of substrate for Steps I and II. Glutamic acid alone diffuses into the liver slice too slowly to provide an adequate concentration of either ammonia or glutamic acid for Steps I and II respectively, and thus yields little urea. On the other hand, ammonium chloride would be expected to provide an adequate concentration of ammonium ions for synthesis of intermediates for Step I and also to give rise to glutamic acid through the glutamic dehydrogenase system. Findings of experiments in which isotopic ammonia was used lend support to the latter possibility (8). The high activity of glutamine in urea synthesis with slices is in all probability due to its hydrolysis to glutamic acid and ammonia, the optimum substrate combination for slices. The fact that glutamine is more active in liver slices from fasted animals than ammonium chloride suggests that fasted liver lacks the necessary substrates for the sufficiently rapid synthesis of glutamic acid from α-ketoglutaric acid and added ammonia, and thus Step II of the cycle is the limiting reaction.

The failure of ammonia to react in the homogenate system is in all probability due to the fact that conditions for glutamic acid synthesis from ammonia were not optimum. Although the oxygen consumption experiments (Figs. 2 and 3) show a somewhat lower oxygen uptake in the case of glutamic acid plus ammonia in homogenate as compared with glutamic acid alone, this may be due to the inhibitory effect of ammonium ions (Table I) on the glutamic dehydrogenase system (15).
Balance Studies—Additional evidence that the formation of arginine from citrulline by homogenates involves the transimination reaction of Borsook and Dubnoff (9) is that the glutamic acid and citrulline disappearance can be accounted for mole for mole in the formation of urea. Thus, the ratio obtained of urea formed to glutamic acid and citrulline disappearance was 1:1.21:0.93 (Table II). The high value for glutamic acid is undoubtedly due to the fact that it is oxidized readily in the homogenate system (Fig. 2). The failure to find any accumulation of α-ketoglutaric acid in this system may be due either to its ready oxidation or to the possibility that this compound is not an end-product of the reaction.

Conversion of Ornithine to Citrulline by Homogenate—A system including ornithine plus glutamic acid or ammonium chloride, or both, in the presence of a carbon dioxide-bicarbonate buffer was investigated as to its activity in catalyzing Step I (see the accompanying scheme) of the urea cycle. No urea synthesis with this system occurred indicating that the factors required for Step II were inadequate for Step I.

<table>
<thead>
<tr>
<th>Components</th>
<th>Formation or disappearance of substrate and end-products (micromoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamic acid</td>
<td>-4.97</td>
</tr>
<tr>
<td>Citrulline</td>
<td>-3.83</td>
</tr>
<tr>
<td>Urea</td>
<td>+4.12</td>
</tr>
<tr>
<td>α-Ketoglutaric acid</td>
<td>0.00</td>
</tr>
</tbody>
</table>

DISCUSSION

With the exception of the hydrolytic conversion of arginine to urea and ornithine by arginase, our knowledge of the enzymatic steps in the urea cycle is obscure. Since the over-all process is an endergonic one, the synthetic steps in the cycle must be coupled with energy-yielding systems. The findings presented in this paper offer strong evidence in support of the coupling of one step (Step II) in this cycle with adenosine triphosphate, and thus establish at least one pathway for coupling the endergonic urea cycle with exergonic oxidation-reduction systems.

The inability of the system catalyzing Step II to convert ornithine to citrulline (Step I) under the conditions employed indicates that Step I is a more complex reaction and probably involves at least two independent
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reactions, as previously suggested by Srb and Horowitz (16) and Krebs (17). A study of this phase of the cycle is at present under investigation.

The mechanism of the transamination reaction has been considered in some detail by Borsook and Dubnoff (9) who were led by the evidence available to postulate an intermediate addition compound of citrulline plus glutamic acid with the subsequent dehydrogenation of the intermediate and the formation of arginine and α-ketoglutaric acid (or some related compound). The data from the present investigation bear directly on the mechanism of the reaction only in so far as they establish the fact that 1 mole of arginine (or urea) is formed for every mole of glutamic acid and citrulline which disappears, and further that in confirmation of Borsook and Dubnoff’s findings some suitable hydrogen acceptor must be present. The high activity of the homogenate system, however, provides a more satisfactory system for studying the transamination reaction in detail, and this is at present being done.

Finally, it should be pointed out that from the findings of the present paper it would appear that glutamic acid is an obligatory intermediate in the conversion of citrulline to arginine (Step II) in the urea cycle. This may resolve the somewhat different views held on certain aspects of the urea cycle by Leuthardt (18–20) and Bach (21).

SUMMARY

1. The conversion of citrulline to arginine by transamination with glutamic acid has been demonstrated to occur in rat liver homogenates fortified with adenosine triphosphate, cytochrome c, and magnesium ions.

2. Glutamic acid appears to be an obligatory intermediate in the introduction of ammonia at the citrulline → arginine step of the urea cycle.

3. The synthesis of urea from glutamic acid, glutamic acid plus ammonia, and ammonia by liver slices and homogenates has been studied and the findings discussed.

REFERENCES

UREA SYNTHESIS BY LIVER HOMOGENATES
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