THE ENZYMATIC STEPS IN UREA SYNTHESIS*

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Investigations on the synthesis of arginine from citrulline (Step II of the Krebs-Henseleit urea cycle) and citrulline from ornithine (Step I) by rat liver homogenates have been reported previously (1-3). The study of the latter reaction was made possible by the apparently critical magnesium ion concentration required for the function of the liver transimination (citrulline to arginine) system. An attempt to isolate these reactions further has led to the use of differential centrifugation methods. By this means a separation of the two reactions has now been effected.

The system for the synthesis of citrulline was found to be associated with the insoluble residues of the rat liver cell, while the system involved in the synthesis of arginine required two fractions, a soluble protein component plus the insoluble residue.

The use of these tissue fractions has led to more detailed information regarding the optimum requirements for Steps I and II. It has now been shown that the citrulline to arginine reaction involves the transfer of hydrogen through the cytochrome system. Furthermore, the step ornithine to citrulline is shown to require the presence of magnesium ions and high concentrations of either ATP (adenosine triphosphate) or AMP (adenylic acid), as well as ornithine, glutamic acid, ammonium, potassium, phosphate, and bicarbonate ions, carbon dioxide, and oxygen at the concentrations used previously with whole homogenate.

It is the purpose of the present paper to describe in detail the experiments leading to the above findings.

Procedures

Preparation of Homogenates—A 20 per cent homogenate in isotonic KCl, prepared as described previously (1), is centrifuged 10 minutes at 2000g in a cold room kept at 1°. The resulting cloudy supernatant fluid, used as such or in diluted form, is designated in this study as the supernatant. The sediment is washed in a volume of isotonic KCl equal to that of the supernatant, poured off, and centrifuged for another 10 minute period at the same speed. The washed sediment is taken up in the same volume of isotonic KCl and used as such. This preparation is referred to as the supernatant.

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residue in this paper. 0.5 ml. of residue suspension contained from 1.0 to 1.3 mg. of tissue N.

Homogenates of heart muscle and pigeon breast muscle were prepared in the following manner. The fresh tissue was chilled, cut into small pieces, homogenized in a volume of isotonic NaCl sufficient to make a 25 to 30 per cent homogenate in the Potter and Elvehjem apparatus (4), and filtered through four layers of gauze before use. A volume of 0.5 ml. was used per flask.

Other Tissue Preparations—For freezing treatment, preparations prepared as described above were stoppered tightly and left in a deep freeze compartment overnight. Prior to using they were thawed out at room temperature.

Boiled homogenate preparations were made by heating the residue or supernatant in a steam bath for 10 minutes and filtering off the coagulated proteins. The light greenish yellow filtrate was then used. 0.5 ml. contained from 0.3 to 0.4 mg. of N.

Preparations stored overnight were kept in stoppered tubes at 1°.

An acetone powder of the supernatant was prepared by adding 2 volumes of ice-cold acetone to the supernatant, followed by collection of the precipitate by suction filtration. The residue was dried in vacuo over H₂SO₄ and ground in a mortar with isotonic KCl before use.

An acetone powder of whole homogenate was prepared by adding 5 volumes of acetone to the homogenate, followed by centrifugation and desiccation of the residue in vacuo over H₂SO₄. The powder was taken up in isotonic KCl before use.

Incubation—All incubations were carried out in Warburg flasks at 38° for 1 hour unless otherwise specified. 1.0 to 1.5 mg. of residue N was used per flask for studies of Step I; 0.5 to 0.8 mg. each of supernatant and residue N for Step II. The total incubation volume was 3.0 ml.

Anaerobic experiments were carried out as described before (3).

Analytical—Methods and procedures for the analyses of urea and citrulline have been described before (1, 3). Urea was measured as the end-product of the citrulline to arginine reaction, since the activity of liver arginase in our preparations was always such as to insure the complete hydrolysis of arginine.

Preparations Used—Sources of the preparations used have been listed previously (1, 3).

Results

Synthesis of Arginine from Citrulline (Step II)

Differential Centrifugation—The results obtained with the use of homogenate fractions prepared by centrifugation are shown in Table I. Neither
the residue nor supernatant was active alone, even in the presence of added calf liver arginase. Recombination of the two fractions, however, resulted in the regeneration of almost the total activity of the original whole homogenate. Heating of either fraction resulted in the loss of activity. Freezing destroyed only the residue activity. Dialysis against isotonic NaCl was without influence. Acetone powders of the supernatant and whole homogenate showed slight activity when fortified with fresh residue. Both fractions were relatively stable to storage, in contrast to the instability of whole homogenate, which lost two-thirds of its activity on storage for 24 hours at 1°.

Since the function of the insoluble residue could be demonstrated in other tissues such as heart and pigeon breast muscle homogenates, incapable of synthesizing arginine per se, it was believed that the residue contained the system for the transport of hydrogen. In addition, the necessity for cytochrome c, present in bound form in the residue, has been shown. Since the supernatant from liver is not replaceable by that from other tissues, with the exception of kidney, it is believed to contain the enzyme catalyzing the synthesis. Evidence that this enzyme is present in solution has been obtained in experiments in which a supernatant, prepared by centrifuging a rabbit liver homogenate at 40,000g for 2 hours, was found to be active.

Fumarate was included in the incubation medium, since it was found to stimulate the synthesis some 25 per cent. Oxalacetate was equally effective. The function of these oxidizable substrates may be associated with the regeneration of ATP.

While Step I was shown to be sensitive to potassium ions, this is not the case with Step II in which sodium or potassium ions were equally effective.

Cytochrome c—The rôle of cytochrome c in the transamination reaction was first suggested by Borsook and Dubnoff. Our early studies with whole liver homogenate showed its effect to be inconstant. The present resolution of homogenate into two parts has now made a study of this question possible. Potter observed that the dissipation of cytochrome c took place to a greater extent when water instead of saline was used to homogenize liver. An experiment was devised whereby the sediment from a centrifugation was taken up in water, stored for 10 minutes, and restored to isotonicity. This residue plus the supernatant was pipetted into flasks containing increments of cytochrome c. Fig. 1 illustrates the results of this experiment. The optimum cytochrome c concentration was about $3 \times 10^{-5}$ M. A control carried out at the same time with normally prepared residue, with no added cytochrome c, gave a value of 70.3 micro-liters. This would indicate that the normal concentration of cytochrome
c bound in the residue is of the order of $1.6 \times 10^{-5}$ M. Supplementation of the supernatant with cytochrome $c$ showed no synthesis of arginine from citrulline.

**Synthesis of Citrulline from Ornithine (Step I)**

*Diff erential Centrifugation*—The activity of the system catalyzing this reaction remains in the sediment on centrifugation. Table I shows the relative activities of whole homogenate, regenerated homogenate, and the residue. Although the total yield of citrulline obtained with the residue was low, it was 24 per cent more active than whole homogenate on the basis of mg. of N. The addition of boiled supernatant increased the overall yield, but not the yield per mg. of N. Additional supplementation of the medium with magnesium ions and adenine nucleotides increased the rate of synthesis as much as 460 per cent at 0.003 M AMP-ATP.

The stability of the enzyme system to repeated washings was investigated (Table II). The highest activity per mg. of residue N was attained with the first washing. Residue washed two and three times was still somewhat more active than the non-washed preparation. Further washings resulted in considerable loss of activity; thus after six washings, the
Table I

Activity of Homogenate Fractions in Steps I and II

Final substrate concentrations for Step I, dl-ornithine hydrochloride 0.0033 M, L-glutamic acid 0.033 M, ammonium ions 0.0067 M, AMP 0.0005 M, ATP 0.0005 M, phosphate at pH 7.15, 0.017 M, bicarbonate ions 0.0077 M, and potassium ions to bring the medium to isotonicity. The supplement for whole homogenate contained no magnesium ions; the supplement for the residue contained 0.01 M magnesium ions. Gas phase, 5 per cent CO₂-95 per cent O₂. Step II, dl-citrulline 0.0033 M, L-glutamic acid 0.0066 M, ATP 0.001 M, fumarate 0.0066 M, phosphate at pH 7.5, 0.017 M, magnesium ions 0.0033 M, and potassium ions to bring the medium to isotonicity. Gas phase, O₂. 20 per cent KOH in the center wells.

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<td>Per cent activity per mg. N</td>
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| Whole homogenate (0 time) | 100.0
| “ “ (30 min. storage) | 74.0
| Residue + supernatant (30 min. manipulation time) | 72.3
| “ + supplement optimum for whole homogenate | 123.8
| “ + boiled supernatant | 157.2
| “ + supplement optimum for residue | 201.1
| Supernatant + supplement optimum for whole homogenate | 1.0
| “ + heart muscle homogenate | 65.5
| “ + pigeon breast muscle homogenate | 45.0

Table II

Effect of Repeated Washings on Residue

Final substrate concentrations, dl-ornithine hydrochloride 0.0033 M, L-glutamic acid 0.033 M, ammonium ions, 0.0067 M, AMP 0.0005 M, ATP 0.0005 M, phosphate at pH 7.15, 0.017 M, bicarbonate ions 0.0077 M, magnesium ions 0.01 M, and potassium ions to bring the medium to isotonicity. Gas phase, 5 per cent CO₂-95 per cent O₂. Each washing resulted in the loss of about 10 per cent residue N.

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<td>No. of residue washings</td>
<td>Per cent activity remaining per mg. residue N</td>
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<td>5</td>
<td>56.6</td>
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activity was 17.5 per cent that of the original. The addition of vacuum-concentrated washings and an extract of boiled fresh homogenate (8) did not reactivate the residue. Fortification with cytochrome c, biotin, glutathione, coenzyme I, coenzyme II, pyridoxal phosphate, pyridoxine, pyridoxamine, and thiamine pyrophosphate was also without effect.
Requirement of Magnesium Ion—In previous work with whole homogenate (3), magnesium ions were eliminated purposely to prevent the conversion into arginine and urea of the citrulline synthesized. With a lack of magnesium ions the optimum yield obtained was 40 to 50 microliters of citrulline per mg. of whole homogenate N. In the newly devised system the addition of magnesium ions increased the yield 400 per cent, the optimum concentration being $9 \times 10^{-3}$ M (Fig. 2). It is uncertain whether this effect is due to the function of the ATP regenerating system, or directly associated with the synthesis itself. Manganese ions were without effect.

Requirement of ATP and AMP—Fig. 3 illustrates the requirements of the whole homogenate and residue systems for ATP and AMP. With whole homogenate AMP is at least twice as efficient as ATP. With residue ATP and AMP are almost equally good, and considerably more efficient per unit concentration. The yield of citrulline at the 6 micromole level (0.02 M) was at least 3 times that shown with the whole homogenate system. These effects might be explained on the basis of the requirements of competitive reactions in the case of whole homogenate no longer encountered in the washed residue preparations, or on the basis of the presence of an optimum magnesium ion concentration. Numerous experiments devised to determine the specificity of the system for either ATP or AMP were unsuccessful. While high yields of citrulline could be obtained with high concentrations of these reagents, the final molarity of 0.001 M (AMP 0.0005 M + ATP 0.0005 M) used throughout the study was chosen for reasons of economy.

Effect of Incubation Time—In a previous study (3) an unexplained cessa-
tion of synthesis was noted after 45 minutes of incubation (Fig. 4). In-
vestigation of this effect with residue showed no stoppage at this time,
but instead a linear reaction extending beyond 120 minutes in a system
containing 3 micromoles of AMP-ATP. With 9 micromoles of AMP-ATP
present, synthesis proceeded very rapidly, the yield being more than twice
that obtained with 3 micromoles.

Effect of Tissue Nitrogen—An evaluation of the effect of residue concen-
tration on the synthesis was made (Fig. 5). With increasing concentrations

![Graph showing the effect of AMP, ATP, or both on the formation of citrulline by whole homogenate and residue.](http://www.jbc.org/)

of tissue the theoretical amount of citrulline was almost realized, in con-
trast to the results obtained with whole homogenate in which higher tissue
concentrations stimulated the removal of citrulline. In the presence of
9 micromoles of AMP-ATP, the initial rate of synthesis was somewhat
higher than in the case of 3 micromoles. Synthesis with the higher quan-
tity, however, abruptly stopped short of theoretical at higher tissue con-
centrations. The reason for this is not apparent. It is possible that the
higher concentrations of ATP are hydrolyzed with such rapidity as to
lower the pH of this system.
Fig. 4. Effect of incubation time on the formation of citrulline by whole homogenate and residue. Final substrate concentrations for whole homogenate, DL-ornithine hydrochloride 0.0033 M, l-glutamic acid 0.033 M, ammonium ions 0.0067 M, AMP 0.001 M, phosphate at pH 7.15, 0.017 M, bicarbonate ions 0.0077 M, and potassium ions to bring the medium to isotonicity. Final substrate concentration for residue same as above except for AMP 0.0005 to 0.0015 M, ATP 0.0005 to 0.0015 M, and magnesium ions 0.01 M. Gas phase, 5 per cent CO₂-95 per cent O₂.

Fig. 5. Effect of tissue concentration on the formation of citrulline by homogenate residue. Final substrate concentrations, DL-ornithine hydrochloride 0.0033 M, L-glutamic acid 0.033 M, ammonium ions 0.0067 M, AMP 0.0005 to 0.0015 M, ATP 0.0005 to 0.0015 M, magnesium ions 0.01 M, phosphate at pH 7.15, 0.017 M, bicarbonate ions 0.0077 M, and potassium ions to bring the medium to isotonicity. Gas phase, 5 per cent CO₂-95 per cent O₂.
**Requirement of Oxygen and Carbon Dioxide**—AMP and ATP at high concentrations were incapable of supporting the synthesis of citrulline in an anaerobic atmosphere.

Bach (9) found that formic acid could replace bicarbonate as the source of the urea carbonyl in work with tissue slices. This observation has now been confirmed with homogenates. It also appears that higher concentrations of formic acid can replace both bicarbonate and carbon dioxide.

**DISCUSSION**

It is now evident that the three steps of the Krebs-Henseleit urea cycle are not as closely related structurally in the whole cell as might be supposed. Step I, the endergonic conversion of ornithine to citrulline, and Step III, the hydrolysis of arginine to urea and ornithine, appear to be functions associated with the nuclear matter of the liver cell (10). Step II, the conversion of citrulline to arginine, also endergonic, appears to be a two-step reaction depending on a soluble cellular component, the specific synthesizing enzyme, as well as the insoluble nuclear residue containing a hydrogen transport system. The latter separation enabled the demonstration of the rôle of cytochrome c in the synthesis of arginine. While such a requirement has not as yet been shown for Step I, the possibility cannot be excluded in view of the inhibition of synthesis at low concentrations of cyanide.

A study of the over-all urea cycle with whole homogenate resulted in values never equaling those obtained with liver slices. A few of the factors known to account for the lower magnitude of homogenate results were (1) pH (pH 7.3, intermediate between the optimum pH of the two endergonic reactions (Fig. 6), was used in these studies); (2) the inhibition of the synthesis of arginine by ammonium ions (2) required for the synthesis of citrulline; and (3) the presence of 10 to 20 times the optimum concentration of glutamic acid required for Step II.

The greater effectiveness of AMP over ATP in previous work on the conversion of ornithine to citrulline (3) had led us to believe that the energy-coupling mechanism for that synthesis did not involve ATP. This point is no longer certain. The present work has shown that the synthesis can be accomplished almost equally efficiently with either AMP or ATP. The simple assumption that ATP is active by virtue of its hydrolysis to AMP is shadowed by the apparent requirement of the system for inorganic phosphate and the stimulation of activity by magnesium and fluoride ions. On the other hand, it is equally difficult to assume that the resynthesis of ATP occurs at this rapid pace. The third obvious possibility is that neither is involved as such. Clarification of this question must await further study.

It is highly probable that the conversion of ornithine to citrulline is at
least a two-step reaction (11). The centrifugation procedure utilized here did not, however, offer any evidence for the existence of more than one enzymatic system. This may or may not mean that the synthetic steps must of necessity occur simultaneously. It is, however, evident that the differentiation of these steps is requisite for the solution of the AMP-ATP and glutamic acid pictures.

**Fig. 6.** Effect of pH on Steps I and II of the urea cycle. Final substrate concentrations: **Step I**, dl-ornithine hydrochloride 0.0033 M, L-glutamic acid 0.033 M, ammonium ions 0.0067 M, AMP 0.001 M, phosphate at pH 7.15, 0.017 M, bicarbonate ions 0.0077 M, and potassium ions to bring the medium to isotonicity. Gas phase, 5 per cent CO₂-95 per cent O₂. Whole homogenate N per flask, 2.8 mg. **Step II**, dl-citrulline 0.0033 M, L-glutamic acid 0.0066 M, ATP 0.001 M, fumarate 0.0066 M, phosphate at pH 7.5, 0.017 M, magnesium ions 0.0033 M, and potassium ions to bring the medium to isotonicity. Gas phase, O₂. 20 per cent KOH in the center wells. Tissue N per flask, 1.47 mg. Since the preparation contained arginase, arginine was measured as urea.

An alternative hypothesis involving a "glutamine cycle" for the synthesis of urea has been proposed by Leuthardt and Glasson (12-14). Since no data other than those obtained with the use of liver slices were available, a few experiments with tissue homogenates were carried out. All attempts to demonstrate the synthesis of urea from glutamine were unsuccessful.

**SUMMARY**

1. Further studies in the synthesis of arginine from citrulline, and citrulline from ornithine, have been carried out with tissue homogenate fractions obtained by differential centrifugation.
2. The enzyme system catalyzing the citrulline-arginine reaction has been resolved into two parts, a soluble synthesizing enzyme fraction and an insoluble hydrogen transport fraction which appears to include the cytochrome system.

3. The enzyme system catalyzing the ornithine-citrulline reaction is associated with the insoluble residue of the liver cell. Supplementation of the residue with magnesium ions in addition to the requirements prescribed for the whole homogenate system is necessary for optimum activity. Both AMP and ATP are almost equally effective with residue.

4. The significance of these findings in relation to the Krebs-Henseleit urea cycle is discussed.

BIBLIOGRAPHY

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