

Enzymic Synthesis of Carbamylaspartate from Citrulline in Extracts from Rat Liver Mitochondria

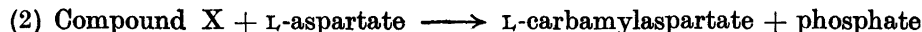
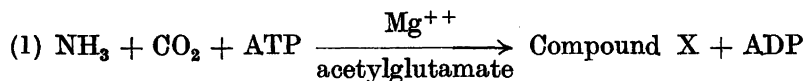
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The formation of carbamylaspartate from citrulline and aspartate has been demonstrated to proceed in at least two steps. First, carbamylphosphate was formed from the carbamyl group of citrulline and ATP. Secondly, carbamylphosphate and aspartate formed carbamylaspartate. The formation of carbamylphosphate from citrulline required ATP, acetylglutamate and Mg^{++} . Ornithine was identified as the second product of the reaction. The reaction was very strongly inhibited by ornithine.

The carbamyl group of citrulline was found to be utilized for the biosynthesis of polynucleotide pyrimidines in pigeon liver by Schulmann and Badger¹. These authors could not demonstrate the specific incorporation of this group into polynucleotide pyrimidines of the rat. Later work indicated, however, that rat liver slices formed orotic acid-¹⁴C from carbamyl labeled citrulline². Since orotic acid is formed from aspartic acid *via* CA-aspartate* in rat liver slices⁴, it seemed likely that the utilization of citrulline took place by a transfer of its carbamyl moiety to aspartic acid. The possibility of such a mechanism has been considered earlier and argininosuccinate^{4, 5} was suggested as an intermediate in the formation of CA-aspartate from citrulline and aspartate.

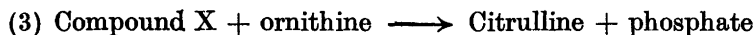
The enzymic formation of CA-aspartate in rat liver has recently been shown to proceed in at least two steps⁶⁻⁸:



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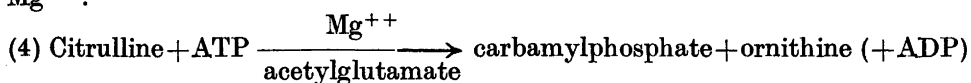
** The following abbreviations are used in this paper: CA = carbamyl; CAP = carbamylphosphate; ATP, ADP and AMP = adenosine tri-, di- and monophosphate, respectively; PGA = 3-phosphoglyceric acid; MP = muscle protein fraction for regeneration of ATP from ADP + PGA¹⁵; PCA = perchloric acid; P_i = inorg. phosphate.

Reaction (1) has been described earlier by Grisolia and Cohen⁹ as the first step in citrulline synthesis, the second being:



The recent work of Jones *et al.*¹⁰ has demonstrated the identity of compound X with carbamylphosphate in reaction (3).

The investigations described here were carried out to determine if CA-aspartate could be formed from citrulline through these known enzymic mechanisms. Using preparations from rat liver mitochondria, evidence has been obtained for a reaction by which carbamylphosphate (CAP) and ornithine are formed from citrulline and ATP in the presence of acetylglutamate and Mg^{++} :



When aspartate was included during the reaction the formation of CA-aspartate could be demonstrated. Some of these experimental results have been briefly published earlier^{11,12}.

MATERIALS AND METHODS

L-Citrulline-carbamyl-¹⁴C was synthesized according to Smith¹³. CAP was prepared according to Jones *et al.*¹⁰ and *L-acetylglutamate* according to Karrer *et al.*¹⁴ ATP, ADP, AMP and PGA were commercial products (Schwartz and Sigma). A muscle protein fraction¹⁵ was prepared for the regeneration of ATP from ADP and PGA. ATP-³²P (labeled in the two terminal phosphate groups) was prepared from AMP through oxidative phosphorylation¹⁶.

Ion exchange chromatography of CAP was carried out by gradient elution at 5°. Two columns of Dowex-2-formate (0.8 cm² × 10 cm) were connected with a 1 000 ml mixing flask, containing water, which received 2.25 M ammonium formate, pH 5.0, from a reservoir flask. P_i (10 column volumes), CAP (15 column volumes), ADP (34 column volumes) and ATP (45 column volumes) were completely separated (*cf.* also Ref.⁹).

Preparation of enzymes. The enzymes carrying out the overall synthesis of CA-aspartate from citrulline and aspartate were prepared by freezing and thawing rat liver mitochondria in 0.1 M NaHCO₃⁷.

The enzyme preparation which was used for the study of reaction (4) was made in a slightly different manner. Mitochondria from *ca.* 40 g rat liver were prepared in 0.25 M sucrose-0.01 M versene (*cf.* Ref.⁹) suspended in 50 ml of distilled water, and quickly frozen in dry ice. The frozen mass was lyophilized directly, and the dry powder stored in a desiccator at 0°. Immediately before use the powder was extracted with 0.02 M glycylglycine buffer, pH 8.0, for 5 minutes in an ice bath and centrifuged at 20 000 g for 15 minutes at 0°. The supernatant was used for the experiments. The lyophilized powder could be stored for several days with a slow decrease of activity. When in solution the enzyme deteriorated in a few hours, even at 0° and despite the addition of glutathione.

Assay of enzyme activity. The *over all reaction* (formation of CA-aspartate) was determined by the isotope dilution method as described earlier^{6,8}.

Reaction (4). A convenient determination of CAP formation was based on its decomposition to release free CO₂ in the presence of a strong mineral acid. The amount of CAP formed during an experiment was calculated from the total radioactivity of the liberated ¹⁴CO₂ and the specific activity of the precursor citrulline-carbamyl-¹⁴C. Incubations were carried out in stoppered 25 ml Warburg vessels, containing 0.5 ml of 12 N H₂SO₄ in the side arm, and 0.5 ml of 6 N NaOH in the center well. At the end of the experiment 100 μmoles of NaHCO₃ were added to the incubation mixture, the flask was

stoppered again, and the sulfuric acid added from the side arm. The flask was then shaken at 37° for 30 minutes, during which time all of the CAP was decomposed and the liberated CO₂ was collected in the center well. The completeness of CO₂ collection under these conditions was established in model experiments with radioactive CAP.

The carbonate in the center well was precipitated with saturated Ba(OH)₂, washed twice with hot water, filtered on to a paper disk of standard size, and counted as an infinitely thick sample in an end window Geiger-Müller counter. The number of μ moles ¹⁴CO₂ liberated could then be calculated from an experimentally determined conversion factor.

In this way all CAP formed during the experiment (including CAP broken down to CO₂ during the incubation) was determined. The assay method, of course, also determined any ¹⁴CO₂ formed from citrulline without the intermediate formation of CAP. Under the proper conditions this "background formation" of CO₂, however, did not amount to more than 10–15 % of the total amount of CO₂ formed from citrulline (cf. Fig. 1).

RESULTS

Over-all formation of CA-aspartate from citrulline

The over-all reaction was first demonstrated in rat *liver slices*. The conditions were the same as those described for orotic acid synthesis³ except for the use of 200 μ moles of citrulline-carbamyl-¹⁴C and a carrier of 100 μ moles of non-labeled CA-aspartate. From the isotope content of the reisolated CA-aspartate it was calculated that approximately two μ moles of CA-aspartate had been formed during the 4-hour incubation period.

In liver homogenates CA-aspartate synthesis from citrulline could also be demonstrated, though only to a small and varying extent. The homogenates were centrifuged at 100 g for 10 minutes at 0° to remove most of the cell nuclei and then for 15 minutes at 15 000 g (cf. Ref. 6). Activity for CA-aspartate formation from citrulline per mg nitrogen was roughly five times larger in the sediment (mitochondria) than in the supernatant (cell sap + microsomes). All subsequent experiments were carried out with mitochondrial preparations.

Non-involvement of free CO₂. It has previously been shown⁷ that the carbamyl carbon of CA-aspartate is derived from CO₂. Rat liver preparations contain a "citrullinase" which liberates CO₂ from the carbamyl group of citrulline, possibly unrelated to CAP formation¹⁷ (see also below). It was possible, therefore, that "free CO₂" released from citrulline by this enzyme was then incorporated into CA-aspartate *via* the known enzymic pathways described as reactions (1) and (2) above. Experiments were therefore designed to test whether the carbamyl carbon of citrulline equilibrated with bicarbonate in the system or was transferred to CA-aspartate over a "closed circuit". For this purpose the synthesis of ¹⁴C-labeled CA-aspartate by mitochondria was compared in tightly stoppered flasks containing either (a) citrulline-¹⁴C + non-labeled KHCO₃, or (b) KHCO₃-¹⁴C + non-labeled citrulline. At the end of the incubation CO₂ was liberated by the addition of PCA and collected and counted as BaCO₃ at infinite thickness. The amount of CA-aspartate-¹⁴C formed from the respective ¹⁴C precursors was then measured by the isotope dilution technique. The results summarized in Table I demonstrate that considerably more CA-aspartate was formed from citrulline-¹⁴C than from bicarbonate-¹⁴C, even though the specific activity of the bicarbonate at the end of the incubation in experiment 1 (citrulline-¹⁴C) was only about one tenth as great as that in

Table 1. Non involvement of "free CO₂" in CA-aspartate synthesis from citrulline-CA-¹⁴C.

Experimental conditions	+ acetylglutamate		no acetylglutamate	
	specific activity of BaCO ₃	μmoles ¹⁴ CA-aspartate formed	specific activity of BaCO ₃	μmoles ¹⁴ CA-aspartate formed
Citrulline- ¹⁴ C KHCO ₃ Phosphate buffer	13 400	0.16	7 700	0.09
Citrulline KHCO ₃ - ¹⁴ C Phosphate buffer	142 000	0.02	132 000	0.00
Citrulline- ¹⁴ C KHCO ₃ Glycylglycine buffer	2 300	0.24		

Each vessel contained 100 μmoles of citrulline, 300 μmoles of L-aspartate, 1 000 μmoles of buffer, pH 7.4, 180 μmoles of succinate, 10 μmoles of ATP, 100 μmoles of MgSO₄, 200 μmoles of KHCO₃ and KCl to give isotonicity. Where indicated the vessels contained 100 μmoles of L-acetylglutamate. Volume 16 ml. Incubation for 60 minutes at 37° with rat liver mitochondria (8.7 mg nitrogen). The carbamyl labeled citrulline contained 60 000 ct/min/μmole; KH¹⁴CO₃ contained 8 000 ct/min/μmole.

experiment 2 (bicarbonate-¹⁴C). When glycylglycine was used instead of phosphate buffer (to decrease the phosphorylation of citrulline) the specific activity of the bicarbonate at the end of the experiment was considerably decreased, but the formation of ¹⁴CA-aspartate was not materially affected. When acetylglutamate was excluded from the reaction mixture there was a decrease of ¹⁴CA-aspartate formation. This effect will be described more fully below.

The same general results were obtained when extracts of mitochondria were used rather than intact mitochondria. In these experiments the influence of increasing amounts of non-labeled ammonium carbonate on the formation of ¹⁴CA-aspartate from labeled citrulline was studied. No dilution effect was observed.

Table 2. Requirements over-all reaction.

	μmoles CA-aspartate formed
Complete	0.26
no acetylglutamate	0.07
no aspartate	0.00
no ATP, PGA and MP	0.05
no phosphate	0.23

The complete system contained 20 μmoles of citrulline-¹⁴C, 15 μmoles of L-aspartate, 10 μmoles of ATP, 60 μmoles of PGA, 2 mg MP, 40 μmoles of MgCl₂ and 100 μmoles of potassium phosphate buffer, pH 7.4. Volume = 3 ml. Incubation for 60 minutes at 37° with 40 mg of a lyophilized mitochondrial extract.

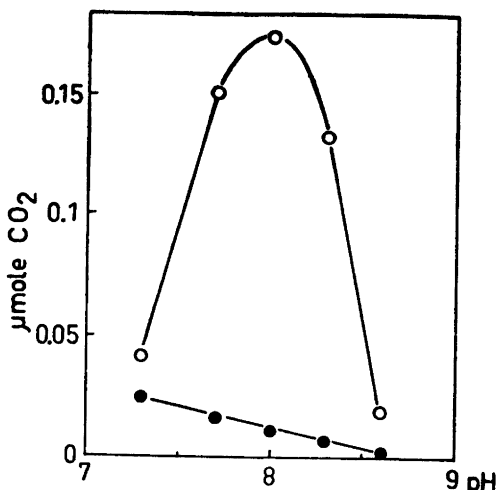


Fig. 1. pH curve of $^{14}\text{CO}_2$ formation from citrulline- ^{14}C .

5 μmoles of citrulline- ^{14}C , 5 μmoles of ATP, 30 μmoles of PGA, 5 mg MP, 50 μmoles of MgSO_4 , 250 μmoles of glycyl-glycine buffer and 20 μmoles of acetyl glutamate were incubated with enzyme (1.1 mg nitrogen) for 20 minutes at 37° at pH values indicated (open circles). The full circles represent experiments where acetyl glutamate was excluded.

Requirements for over-all reaction. After the enzyme was brought into solution by freezing and thawing the rat liver mitochondria in 0.1 M NaHCO_3 , the requirements for the over-all reaction were established (Table 2). CA-aspartate formation from citrulline was dependent on the presence of L-aspartate, and was strongly stimulated by acetylglutamate*, Mg^{++} , and ATP (and an ATP regenerating system). The addition of phosphate was not necessary. The strong stimulation by acetylglutamate suggested the intermediate formation of CAP, which in liver preparations requires the presence of acetylglutamate.

Formation of CAP from citrulline

Since it was demonstrated that free CO_2 was not involved in the reaction, CAP could not be formed from citrulline by way of reaction (1), described above. This suggested a direct transfer of the carbamyl group from citrulline to a phosphate group to form CAP. This reaction was studied directly in soluble extracts from lyophilized mitochondria by the omission of aspartate from the system. The enzyme assay was based on the measurement of the acetylglutamate dependent release of $^{14}\text{CO}_2$ from citrulline-carbamyl- ^{14}C , following the addition of strong acid to break down CAP. In confirmation of the work of Krebs *et al.*¹⁷ it was found that our enzyme preparation contained a "citrullinase" which released CO_2 from the carbamyl group of citrulline inde-

* Stimulation by acetyl glutamate was much more pronounced in mitochondrial extracts than in intact mitochondria. The amount of stimulation varied considerably with different preparations.

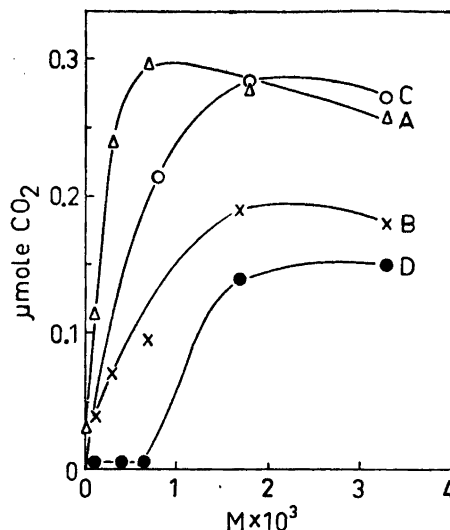


Fig. 2. Dependence of CAP formation on citrulline (curve A), acetylglutamate (curve B) and ATP (curves C and D).

Incubation for 20 minutes at 37°. 50 μ moles of $MgSO_4$ and 250 μ moles of glycylglycine buffer, pH 8.0, in a volume of 3.0 ml for each curve.

Curve A: 10 μ moles of ATP, 20 μ moles of acetyl glutamate, enzyme (1.1 mg nitrogen) and citrulline-¹⁴C as indicated on abscissa.

Curve B: 10 μ moles of ATP, 5 μ moles of citrulline-¹⁴C, enzyme (0.9 mg nitrogen), and acetyl glutamate as indicated.

Curve C: 5 μ moles of citrulline-¹⁴C, 20 μ moles of acetyl glutamate, enzyme (1.1 mg nitrogen), 30 μ moles of PGA, 5 mg MP, and ATP as indicated.

Curve D: 5 μ moles of citrulline-¹⁴C, 20 μ moles of acetyl glutamate, enzyme (1.1 mg nitrogen), and ATP as indicated.

All values are given for one experimental point in each curve.

pendent of acetylglutamate concentration. This background release of CO_2 was greatly diminished by the exclusion of phosphate from the system, which did not affect CAP formation. The citrullinase activity was furthermore sharply decreased by carrying out the reaction at pH 8.0, the pH optimum for CAP formation from citrulline (Fig. 1). The pH optimum for citrullinase is 6.8¹⁷. The closed circles in Fig. 1 represent the release of carbamyl- CO_2 from citrulline in the absence of added acetyl glutamate, *i.e.* the maximum value for citrullinase in the present system under our experimental conditions.

Requirements of the reaction. Fig. 2 demonstrates the dependence of the reaction on acetylglutamate, citrulline, and ATP. As the different curves were not obtained with the same enzyme preparation, no significance should be attached to the absolute height of the curves, with the exception of curves C and D. These two curves show that the presence of an ATP regenerating system gives better synthesis than ATP alone, presumably because of the presence of ATP-ases. The S-shaped curve D was observed in several experi-

Table 3. Comparison of ATP, ADP, AMP and phosphate in the synthesis of CAP from citrulline-¹⁴C.

Additions	μmoles of ¹⁴ CO ₂ formed	
	+ acetylglutamate	no acetylglutamate
None	0.008	—
20 μmoles of phosphate	0.006	0.011
20 μmoles of ATP	0.105	0.002
20 μmoles of ADP	0.033	0.004
20 μmoles of AMP	0.001	0.001
20 μmoles of ATP + 30 μmoles of PGA + 5 mg MP	0.163	—

5 μmoles of citrulline-¹⁴C, 50 μmoles of MgSO₄, 250 μmoles of glycyl-glycine buffer, pH 8.0 and additions as indicated were incubated with enzyme (1.3 mg nitrogen) in 3 ml volume for 20 minutes at 37°.

ments and maximum synthesis was sometimes not achieved below 3×10^{-3} M concentration of ATP.

The reaction was also found to be dependent on the presence of Mg⁺⁺. Enzyme saturation was achieved between 6×10^{-3} — 3×10^{-2} M concentration of MgSO₄ without apparent inhibition at higher concentration. Some "versene" was always left in the enzyme (from the preparation of the mitochondria) and undoubtedly influenced the amount of Mg⁺⁺ necessary for optimum synthesis.

The specific requirement for ATP is also demonstrated by Table 3. A small amount of synthesis was observed also with ADP (myokinase action), while AMP or phosphate were without effect. Best synthesis was obtained when ATP was regenerated by addition of PGA and a muscle enzyme fraction.

Fig. 3 represents a time curve of the reaction. The dependance on the amount of enzyme is shown in Fig. 4.

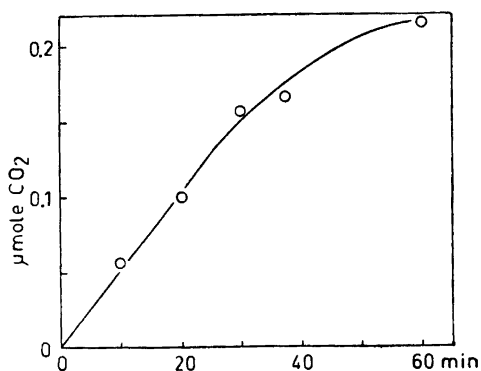


Fig. 3. Time curve of CAP formation.

Substrate and conditions as in Fig. 1 (pH 8.0). 1.1 mg of enzyme nitrogen.

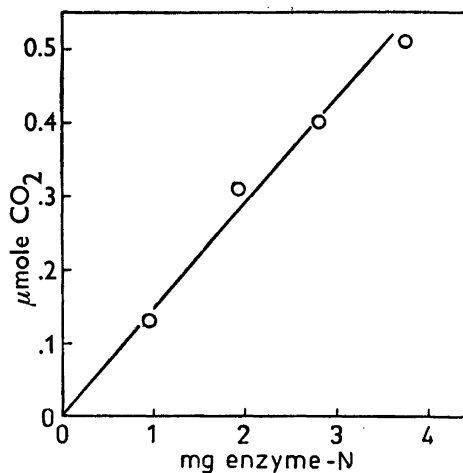


Fig. 4. Dependence of CAP formation on the amount of enzyme. Substrate and conditions as in Fig. 1 (pH 8.0).

Products of the reaction. The presence of CAP at the end of an experiment was demonstrated by the enzymic formation of CA-aspartate after addition of an excess of L-aspartate and aspartate carbamyl transferase from *E. coli*⁸. This enzyme catalyzed the formation of CA-aspartate from CAP and aspartate, but was incapable of forming CA-aspartate from citrulline + aspartate. From several such experiments it was calculated that a minimum of one fourth to one half of the ¹⁴CO₂ released by the addition of acid in the enzyme assay corresponded to CAP present at the end of the incubation. The rest of the ¹⁴CO₂ corresponded presumably to CAP broken down chemically and enzymically¹⁸ during the course of the incubation.

Further evidence for the formation of CAP was obtained by paper chromatography and paper electrophoresis. Citrulline-¹⁴C (750 000 ct/min/μmole in the carbamyl group) was incubated under the conditions described in Fig. 3. At the end of the incubation the reaction mixture was chilled and 20 μmoles of synthetic CAP were added as carrier. All subsequent steps were carried out at 0–5°. Protein was precipitated by the addition of 0.5 N PCA to pH 1–2 and the supernatant after centrifugation was neutralized to pH 8.0 with M NaOH (phenolphthalein). Then 0.5 ml of M barium acetate were added and the resulting precipitate was discarded after centrifugation. Two volumes of alcohol were added and the Ba-CAP was centrifuged and dried.

The barium salt was extracted with 0.25 ml of water + 0.05 ml of M sodium sulfate in the cold. After centrifugation aliquots of 0.01 ml of supernatant were subjected to paper electrophoresis¹² (saturated sodium tetraborate containing 0.01 M versene, 4 hours at 0°, 12 volts/cm) and ascending paper chromatography with three different solvents (methanol: 2 M NH₃: versene¹⁹, 7:3:0,01;

5 % K_2HPO_4 ; isocamyl alcohol²⁰; propanol: water; conc. NH_3 : versene²¹, 6:1:3:0.01). After drying the paper strips were scanned for radioactivity.

In all four solvents the CAP obtained by the carrier technique from the citrulline- ^{14}C incubation showed a radioactive spot which coincided with that obtained from synthetic CAP (*cf.* Ref. ⁸). When acetylglutamate or ATP was not included during the incubation no radioactivity appeared in the chromatograms at the place of CAP. The best separation was obtained by paper electrophoresis and results with this method have been published in a preliminary communication¹². After paper electrophoresis one more radioactive spot was observed whose presence depended on the inclusion of acetylglutamate and ATP during the incubation. This compound moved faster towards the anode than CAP and it could also be obtained from carbamylglutamate- ^{14}C . It was therefore suggested that the compound was CAP bound to a glutamic acid derivative¹².

The incorrectness of this idea has now been demonstrated by experiments in which CAP was formed in the presence of ^{32}P labeled ATP and ^{32}P labeled inorganic phosphate, respectively (conditions as in Fig. 3, but including 100 μ moles of K_2HPO_4 and excluding PGA and MP). When added carrier CAP was reisolated and subjected to electrophoresis as described above no radioactivity was found in the position of the fast moving band.

Ion exchange chromatography of the reisolated CAP showed that this substance had received ^{32}P from ATP while in the experiment with labeled inorganic phosphate only a very small incorporation of isotope into CAP was observed (*ca.* 12 % of that found in the ATP experiment).

Ornithine was demonstrated as a product of the reaction by ion exchange chromatography by the method of Hirs *et al.*²² In one experiment when 0.80 μ moles of $^{12}CO_2$ were liberated after acid treatment (corresponding to CAP), 1.04 μ moles of ornithine were formed, thus demonstrating a rough stoichiometry for the two products of the reaction. In the absence of acetylglutamate ornithine formation was negligible.

Ornithine was a very potent inhibitor of CAP formation from citrulline and ATP. At 1.7×10^{-3} M concentration of citrulline a 50 % inhibition of the reaction rate was observed at *ca.* 0.7×10^{-4} M concentration of ornithine. No similar inhibition was observed with CAP.

DISCUSSION

It is evident from the present investigation that the utilization of citrulline for the biosynthesis of pyrimidines in rat liver represents a relatively small fraction of the total pyrimidines formed. In liver slices it was found that only 0.14 μ moles of orotic acid were synthesized from citrulline-CA- ^{14}C under conditions that permitted synthesis of 3–5 μ moles of orotic acid from ammonia. This low rate of pyrimidine synthesis from citrulline was the reason why in earlier experiments with citrulline-CA- ^{15}N it was concluded that the utilization of ammonia for pyrimidine synthesis did not proceed *via* citrulline.⁶

The present experiments demonstrate that in rat liver preparations the carbamyl group of citrulline can be utilized for the synthesis of CA-aspartate. This process could be separated into at least two enzymic steps. In the first

step the CA-group of citrulline gave rise to CAP, which in the second step reacted with aspartate to form CA-aspartate. Since the second step has been described earlier⁶⁻⁸, this paper is mainly concerned with the first step, *i.e.* the formation of CAP.

Using intact mitochondria and comparing the utilization of labeled bicarbonate and citrulline for CA-aspartate synthesis it could be clearly demonstrated that the carbamyl group of citrulline was used relatively directly and not after breakdown to ammonia and CO₂ (Table 1). The dilution experiments with non-labeled ammonium carbonate — which in solution is in equilibrium with ammonium carbamate — also made the participation of free carbamate in the reaction unlikely.

In addition to citrulline the formation of CAP required ATP, acetylglutamate, and Mg⁺⁺. Addition of phosphate either did not influence or slightly decreased CAP formation as measured in our enzyme assay. This fact, together with the specific requirement for ATP and the pH optimum of 8.0, makes it unlikely that the phosphorolytic cleavage of citrulline¹⁷, which might represent a reversion of reaction (3), was involved in the formation of CAP from citrulline*.

The requirement for acetylglutamate also differentiates this enzyme system from citrullinase. Acetylglutamate, or a similar compound, was previously shown to be required by rat liver preparations for CAP synthesis from CO₂, NH₃ and ATP. The function of acetylglutamate in CAP formation is not understood at the present time. Our earlier evidence for the existence of an intermediate compound, containing CAP bound to acetylglutamate¹² has been invalidated by the present finding that the proposed compound does not contain phosphate.

Two of the products of the reaction have been identified as CAP and ornithine. It seems likely that ADP was also formed but the presence of ATPase and myokinase in the mitochondrial extracts made it impossible to demonstrate the specific formation of ADP. Because of the unusual instability of the enzyme activity attempts to purify the enzyme by ammonium sulfate precipitation were not successful.

CAP formation from citrulline in other rat organs than liver could not be demonstrated**. The apparent low activity in mitochondrial extracts together with the high inhibition by ornithine would seem to indicate that the formation of CAP from citrulline in the rat is of minor quantitative importance for pyrimidine biosynthesis.

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* *Added in proof:* We have recently extensively purified ornithine carbamyl transferase (the enzyme catalyzing reaction (3)) from rat liver and found that the citrullinase of Krebs *et al.*¹⁷ in all probability is identical with this enzyme. Reversal of reaction (3) therefore represents an alternative way for CAP formation from citrulline which — unlike the reaction demonstrated in this paper — does not involve the participation of acetyl glutamate or ATP.

** An acetylglutamate stimulated release of ¹⁴CO₂ from citrulline-carbamyl-¹⁴C was, however, observed with washed cells of *E.coli*.

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