# Enzymic Synthesis of Ureidosuccinic Acid from Citrulline *via* Compound X and Carbamyl Phosphate

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The first step in the enzymic synthesis of citrulline involves the formation of an intermediate (compound X) which contains ammonia and $CO_2$ in an activated form [1]. Citrulline is formed by condensation of the intermediate with ornithine. USA ** arises when the same intermediate condenses with aspartic acid [2].

Grisolia and Cohen [1] showed that the presence of ATP, $Mg^{++}$ and an N-substituted derivative of glutamic acid, *e. g.* carbamyl glutamate or acetyl glutamate, was required for the formation of compound X. They proposed originally that the intermediate contained ammonia, $CO_2$ and phosphate bound to an N-substituted glutamic acid in an unknown way. Recently Jones *et al.*[3], however, made the very important demonstration that carbamyl phosphate was formed in an extract of *Streptococcus faecalis* and that CAP ** in liver preparations readily formed citrulline in the presence of ornithine. Indirect evidence indicated that USA also could be formed from CAP and aspartic acid. The possibility was therefore considered that CAP and compound X were identical. Further strong evidence for this concept was presented by Marshall *et al.*[4] On the other hand experiments by Grisolia *et al.*[5] indicated that CAP was not identical with the compound X prepared by these authors.

We have recently demonstrated the formation of USA from citrulline-ureido-$^{14}C$ and aspartic acid in the presence of acetylglutamate, ATP and $Mg^{++}$ in an extract from rat liver mitochondria [6]. The requirement of the reaction for acetyl glutamate implicated the intermediate formation of compound X. We have now studied this question by investigating the formation of radioactive compounds during incubation of citrulline-ureido-$^{14}C$ with acetyl glutamate, ATP and $Mg^{++}$ in mitochondrial extracts.

After deproteinization and addition of carrier CAP the solution was made slightly alkaline (phenolphthalein) and precipitated with barium acetate. After centrifugation two volumes of alcohol were added to the supernatant and the precipitated barium salts were collected and analyzed by paper electrophoresis. The paper strips were scanned for radioactivity (Fig. 1). The presence of acetyl glutamate during incubation resulted in the appearance of two radioactive bands, one of which coincided with CAP. Both bands also depended on the presence of ATP. When the paper strips were sprayed with 0.05 *N* HCl and dried in a stream of warm air the radioactivity in the two fast moving bands disappeared. This type of behaviour would be expected from acid labile compounds such as CAP or CAP bound to acetyl glutamate.

These two radioactive bands were also found in experiments where compound X was formed from $NH_3$, $NaH^{14}CO_3$, ATP, $Mg^{++}$ and acetyl glutamate (*cf.*[1]). Again the bands depended on addition of acetyl glutamate, and spraying with acid removed the radioactivity. In these experiments no carrier CAP was added before barium precipitation.

When compound X was formed from radioactive carbamyl glutamate, $NaHCO_3$, $NH_3$, ATP and $Mg^{++}$, it was found that only the fastest moving band was radioactive and that the radioactivity did not disappear on spraying with acid.

Fractionation of the radioactive compounds was also carried out by ion exchange chromatography on Dowex-2-formate columns. In this case the deproteinized solution was put directly on the column after neutralization to pH 8 without addition of carrier CAP. Gradient elution with ammonium formate, pH 8.0, was performed. Again two radioactive peaks were observed, whose presence depended on the inclusion of acetyl glutamate during incubation. The first of these peaks corresponded in position to CAP, while the second was retained more strongly by the column. Both peaks lost radioactivity after acidification and warming. When ornithine and a mitochondrial extract were added to the material from each peak formation of citrulline could be demonstrated. The second peak was radioactive in experiments with carbamyl glutamate-$^{14}C$.

The evidence presented here indicates that from both $NaH^{14}CO_3$ or citrulline-ureido-$^{14}C$ two radioactive compounds

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** The following abbreviations are used in this paper: ATP, adenosine triphosphate; USA, ureidosuccinic acid; CAP, carbamyl phosphate.

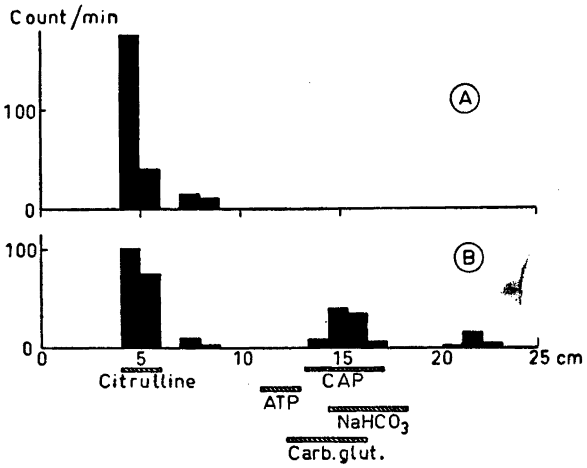


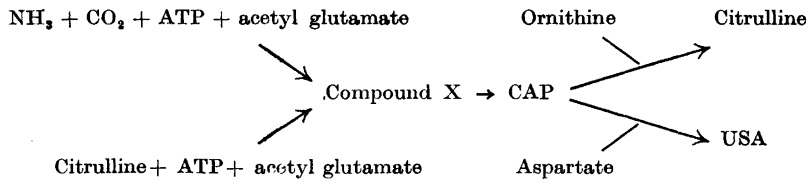

*Fig. 1. Five ml of an extract of rat liver mitochondria (7 mg protein nitrogen) were incubated at 37° for 20 min. with 20 µmoles of citrullineureido-$^{14}C$ (90 000 ct/min/µmole), 40 µmoles of acetyl glutamate, 80 µmoles of ATP, 200 µmoles of $MgSO_4$ and 1 000 µmoles of glycyl-glycin buffer, pH 8.0, in a final volume of 9 ml. An alcohol insoluble barium fraction was prepared as described in the text. After decomposition with $Na_2SO_4$ an aliquot was subjected to paper electrophoresis with saturated sodium tetraborate at 0° (12 volts/cm, 4 hours). After drying at room temperature the paper strips were directly scanned for radioactivity.*

*A = acetyl glutamate excluded from incubation mixture.*
*B = complete substrate.*
*The figure gives also the positions of different compounds on the paper strips as determined in separate parallel experiments.*
*The distance was measured from the starting line towards the anode.*

were formed in the presence of acetyl glutamate. The more acid compound was also formed from carbamyl glutamate-$^{14}C$. Both compounds could form citrulline in the presence of ornithine. One of the compounds behaved like synthetic CAP during paper electrophoresis, ion exchange chromatography and also paper chromatography. The other more acid compound seemed to contain CAP bound to acetyl glutamate (or carbamyl glutamate) and thus would be identical in structure with the originally formulated compound X. This second compound amounted in our experiments to between 5 and 20 % of the CAP at the end of the incubation. It is likely, however, that different proportions are obtained under other experimental conditions.

Since acetyl glutamate was necessary for the formation of CAP it seems reasonable to assume that compound X (= CAP bound to acetyl glutamate) is the first active intermediate formed in citrulline and USA synthesis and that CAP arises from it. The following tentative reaction sequence is put forward: See below.

The intermediate position of CAP in USA synthesis was established by enzyme



experiments where $^{15}NH_4Cl$, ATP, $MgCl_2$, acetyl glutamate and aspartate-$^{14}C$ were substrates. The influence of increasing amounts of $^{13}CAP$ on the isotopic pattern of the formed USA was studied. The results were in full agreement with the scheme, since the $^{15}N$-content of USA sharply decreased with increasing amounts of CAP, while the $^{13}C$-content increased and finally reached the same level as the $^{14}C$-content. Similar results were obtained with labeled citrulline in place of ammonia.

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Received June 22, 1955.

## The Preparation of 2,6-Dichlorophenylacetic Acid

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*Ortho* halogen substituted benzoic, phenylacetic and phenoxyacetic acids have in recent years been frequently tested as to their auxin activity, among others by Thimann [1]. The activity of a benzoic acid is greatly increased by di-substitution of halogen atoms in 2,6-position, while the same substitution in a phenoxyacetic acid completely destroys the activity.

The 2,4,6-trichlorophenylacetic acid was recently prepared by Jönsson [2], but the 2,6-dichlorophenylacetic acid is not earlier described. By a method related by Fierz-David and Blangey [3] 2,6-dichlorotoluene was prepared. This compound was chlorinated to benzyl chloride which was treated with potassium cyanide to benzyl cyanide and hydrolysed to phenylacetic acid as Staedel [4] has described.

For the sake of control a sample of the 2,6-dichlorotoluene was oxidized, yielding pure 2,6-dichlorobenzoic acid with m. p. 140–141°.

*Experimental: 2,6-Dichlorobenzyl chloride.* 16.1 g of 2,6-dichlorotoluene was chlorinated with dry chlorine at 180° in ultra-violet rays until an increase of the weight of 3.5 g had been obtained. The product was fractionated *in vacuo* yielding 12 g (61 %) of colourless oil, boiling at 114—119°/13 mm, melting point 11—12°.

*2,6-Dichlorobenzyl cyanide.* 6.5 g of benzyl chloride was refluxed 5 hours with 2.7 g of potassium cyanide and 30 ml alcohol. The alcohol was distilled off from the solid.

*2,6-Dichlorophenylacetic acid.* The before mentioned cyanide was refluxed with 1 *N* sodium hydroxide overnight. After extraction with ether the mixture was acidified with 2 *N* hydrochloric acid. The dichlorophenylacetic acid was obtained as a colourless solid (4.0 g; 60 %) which was repeatedly crystallised from aqueous ethanol. Melting point 158—159°. (Found: Equiv. wt. 205.4; Cl 34.41 %. Calc. for $C_8H_6O_2Cl_2$: Equiv. wt. 205.0; Cl 34.59 %).

The author whishes to express his thanks to Professor Arne Fredga for valuable discussions.

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Received June 21, 1955.

## The Structure of Tellurium Dibenzenethiosulphonate

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This compound [1], $Te(S_2O_2C_6H_5)_2$, is an analogue of tellurium dimethanethiosulphonate, $Te(S_2O_2CH_3)_2$, and of salts of telluropentathionic acid, $Te(S_2O_2OH)_2$. The crystal structure has been worked out and found to be closely analogous to that of