THE METABOLISM OF ARGININE IN *SERRATIA MARCESCENS*

I. PATHWAYS OF SYNTHESIS AND DEGRADATION

By K. T. Glasziou*

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Summary

Serratia marcescens has no ornithine cycle, the enzyme arginase being absent. Arginine is not dissimilated in the manner of the streptococci and staphylococci as arginine desmidase is lacking.

Citrulline is synthesized from ornithine and carbamyl phosphate in the presence of acetone-dried cells. Evidence is given that argininosuccinic acid is an intermediate in the formation of arginine in *S. marcescens*.

The arsenolysis and phosphorolysis of citrulline, and the enzymic breakdown of carbamyl phosphate have been studied. The latter reaction proceeds either by hydrolysis or by the transfer of phosphate to adenosine diphosphate (ADP). The reaction sequence in which citrulline is converted to ornithine is discussed.

I. Introduction

The metabolism of arginine and related compounds in animals and micro-organisms has been reviewed by Ratner (1954). A review by Slade (1954) deals with bacterial systems.

Grisolia and Cohen (1953) investigated the catalytic activity of various glutamic acid derivatives having an *N*-carbonyl group in the conversion of ornithine, carbon dioxide, and ammonia to citrulline. Studies by Grisolia and Cohen (1952) indicated that the role of these catalysts was in the production of a phosphorylated intermediate, formed in the presence of adenosine triphosphate (ATP), carbon dioxide, and ammonia.

Ratner and Petrach (1951) found that argininosuccinic acid is an intermediate in the conversion of citrulline to arginine in liver. The reaction required ATP and aspartic acid. Davison and Elliott (1952) presented chromatographic evidence for the formation of argininosuccinic acid from arginine and fumaric acid in extracts from animal and plant tissues and microorganisms. Oginsky and Gehrig (1952*a*) reported the properties of arginine desmidase from *Streptococcus faecalis*. No evidence could be obtained for the participation of inorganic phosphate, ATP, oxygen, or fumarate. The same authors (1952*b*, 1953), and also Knivett (1952, 1954*a*, 1954*b*, 1954*c*), Korzenovsky and Werkman (1953, 1954), and Slade, Doughty, and Slamp (1954) have shown that the reactions occurring in *Strep. faecalis*, *Strep. lactis*, and *Pseudomonas* are:

$$\text{arginine} + H_2O \rightarrow \text{citrulline} + NH_3 \quad \text{.....(1)}$$

$$\text{citrulline} + \text{ADP} + H_3PO_4 \rightarrow \text{ornithine} + CO_2 + NH_3 + \text{ATP} \quad \text{..(2)}$$

$$\text{citrulline} + H_2O \xrightarrow{\text{arsenate}} \text{ornithine} + CO_2 + NH_3 \quad \text{.....(3)}$$

$$\text{citrulline} + H_2O \xrightarrow{\text{phosphate}} \text{ornithine} + CO_2 + NH_3 \quad \text{.....(4)}$$

* Department of Biochemistry, University of Sydney.

Reaction (**1**) is inhibited by magnesium and by arsenate. Magnesium is required for reactions (**2**) and (**4**) but not for reaction (**3**). Arsenolysis (reaction (**3**)) is competitively inhibited by phosphate and ornithine, while phosphorolysis (reaction (**4**)) is competitively inhibited by ornithine. One of the oxygen atoms released as CO_2 in phosphorolysis comes from phosphate (Stulberg and Boyer 1954).

Krebs, Eggleston, and Knivett (1955) have demonstrated reactions (**3**) and (**4**) but not reaction (**2**) in mammalian liver homogenates, and have suggested that those microorganisms which possess the arginine dihydrolase component (reactions (**1**) and (**2**)) have evolved this system to serve as an energy-yielding process if the substrate is present in the medium. Gale (1945) has suggested that the same system in homolactic fermenters may serve as a source of metabolic carbon dioxide for growth purposes.

Jones, Spector, and Lipmann (1955) found that incubation of extracts of *Strep. faecalis* or liver mitochondria with ammonium carbamate, CO_2, and ATP resulted in the formation of carbamyl phosphate (CAP). Transcarbamylation occurred on incubation of the extract, CAP, and ornithine to yield citrulline. The formation of CAP was reversible and required magnesium.

S. marcescens is an aerobe able to utilize ammonia and a simple carbon source for growth purposes. It is of interest therefore to compare arginine metabolism in this organism with that of mammalian tissue, the fungi, and those bacteria which possess the dihydrolase system.

II. Materials and Methods

(*a*) *Materials*

(i) *Buffers.*—Phosphate buffers were mixtures of 0·5M Na_2HPO_4 and KH_2PO_4. Arsenate and tris(hydroxymethyl)aminomethane buffers were prepared by mixing molar solutions with N HCl or N KOH and diluting.

(ii) N-*Acetyl Glutamic Acid.*—This substance was prepared according to the method of Bergmann and Zerfas (1928).

(iii) *Carbamyl Phosphate.*—Synthesis was by the method of Jones *et al.* (1955).

(iv) *Amino Acids.*—Commercial preparations of the L-isomers were used.

(*b*) *Methods*

(i) *Cell Preparations.*—A laboratory strain of *S. marcescens* was grown in Bunting's (1946) glycerol-ammonium citrate-salts medium. An arginine-requiring mutant was obtained by irradiation of the prototroph with ultraviolet light, and was isolated by the method described by Lederberg and Tatum (1946). Acetone-dried cells were prepared by pouring a thick suspension into 20 volumes of acetone at −15°C and collecting by filtration after 6 min. Drying was completed *in vacuo* over conc. H_2SO_4, and the preparations stored at −15°C. Dialysis was against distilled water for 3–4 hr at 2°C.

(ii) *Urease and Arginase.*—Experiments for the detection of these enzymes were carried out under the conditions described by Srb and Horowitz (1944).

(iii) *Estimations.*—Urea was determined by the method of Krebs and Henseleit (1932), arginine by the method of Dubnoff (1941), citrulline by the modification of Archibald's method described by Knivett (1954*b*), and CAP by the method of Jones *et al.* (1955). In experiments in which citrulline was estimated, deproteinization was carried out with a final concentration of 10 per cent. (w/v) trichloroacetic acid.

(iv) *Paper Chromatography.*—To identify argininosuccinic acid, the incubated material was deproteinized with 2 vol. of ethanol and evaporated to dryness *in vacuo* over conc. H_2SO_4. The residues were then taken up in 20 per cent. (v/v) ethanol and spotted on Whatman No. 1 filter paper. The chromatograms were developed with phenol-water solvent (4:1) or butanol-acetic acid-water (4:1:1) for a period of 15 hr with ascending flow. To detect citrulline synthesis, and ornithine as a breakdown product of citrulline, the same procedure for deproteinization was adopted and the chromatograms run in butanol-acetic acid-water. Suitable markers were selected for each experiment, those used being ornithine, arginine, citrulline, glycocyamine, and glutamic and aspartic acids. After drying, the papers were sprayed with 0·2 per cent. (w/v) ninhydrin in ethanol and developed in the dark, either for 24 hr at room temperature, or for 15 min at 60-70°C.

To test for monosubstituted guanidyl derivatives, 0·04 per cent. (w/v) α-naphthol in ethanol, followed by 0·1 per cent. (v/v) bromine in 5 per cent. (w/v) NaOH was used (Eden, Harrison, and Linnane 1954). Carbamyl derivatives of amino acids were detected with 1 per cent. (w/v) *p*-dimethylaminobenzaldehyde in N HCl (Fowden 1951).

III. Results

(*a*) *Growth Characteristics of the Arginine-requiring Mutant*

The mutant was found to grow on arginine or citrulline but not on ornithine when these substances were added to the minimal medium (Bunting's). CAP and *N*-acetyl glutamic acid were inactive, as were other amino acids, uracil, glycocyamine, creatine, and urea. Growth in the presence of arginine was proportional to the concentration up to 1 μmole/ml of Bunting's medium. It was found that acetone-dried cells of the mutant were unable to carry out the arsenolysis of citrulline (Table 2). When a large inoculum was placed in minimal medium, growth was usually observed after a delayed period. Acetone-dried cells of the organism grown in this manner were found to have regained the ability to decompose citrulline in the presence of arsenate. When the mutant was streaked out on minimal-agar medium, a few colonies appeared after 4 days incubation. In none of many colonies tested was this growth due to back-mutation.

(*b*) *Urease and Arginase*

Bergey (1948) states that *S. marcescens* will not grow on urea, KCl, and glucose. As the organism is capable of synthesizing all of its nitrogenous requirements from ammonia and a simple carbon source, urease cannot be present except the organism be impermeable to urea. Urease could not be detected in whole cells of the parent strain. The lack of arginase activity was shown in experiments extending over a 20-hr period at pH 9·5 and 27°C, no accumulation of urea being detected when

washed cells were incubated with arginine in an atmosphere of nitrogen. Arginine was recovered quantitatively. The permeability of whole cells to arginine was indicated by the formation of argininosuccinic acid (see Section III (*c*)).

(*c*) *The Formation of Argininosuccinic Acid*

When the arginine-requiring mutant was plated out on Bunting's agar medium and a very small amount of citrulline placed on the surface of the agar, a halo of growth appeared around the site of the addition after 14 hr incubation. When whole, washed cells or acetone-dried cells of the parent strain were incubated with arginine and fumarate, but not with arginine alone, paper chromatograms showed the presence of a second ninhydrin-reacting substance. With phenol-water as the mobile phase the R_F value of this substance, which appeared below arginine, was 0·24. Ratner, Petrach, and Rochovansky (1953) gave a value of 0·27 for pure argininosuccinic acid. The evidence is compatible with a pathway of conversion of citrulline to arginine proceeding via argininosuccinic acid.

TABLE 1

THE SYNTHESIS OF CITRULLINE

The complete system contained 25 mg acetone-dried cells of the parent strain, 500 μmoles tris(hydroxymethyl)aminomethane buffer pH 7·4, 20 μmoles ornithine, 10 μmoles CAP. Final vol. 3·0 ml; system incubated for 30 min at 30°C

System Components	Citrulline Synthesized
No enzyme	None
Complete (zero time)	None
Complete	10·1 μmoles
No ornithine	None
No CAP	None

(*d*) *Arginine Desmidase*

This enzyme hydrolyses arginine without the intermediate formation of argininosuccinic acid. Under conditions in which a rapid breakdown of arginine occurs in several other microorganisms and also under conditions which give rise to rapid phosphorolysis of citrulline in *S. marcescens*, no breakdown of arginine was detected with either whole or acetone-dried cells of the parent strain.

(*e*) *The Synthesis of Citrulline from Ornithine*

Attempts to demonstrate the aerobic synthesis of citrulline by whole washed cells of the parent strain in the presence of ornithine, ammonia, and bicarbonate with fumarate as the oxidizable substrate were unsuccessful. Additions of glutamic acid, *N*-acetyl glutamic acid, separately or together, and the growth of the cells in the presence of ornithine made no difference. However, when acetone-dried cells of the parent strain were incubated with ornithine and CAP, citrulline synthesis occurred (Table 1). The identity of citrulline was verified by paper chromatography.

(*f*) *The Arsenolysis and Phosphorolysis of Citrulline*

The arsenolysis and phosphorolysis of citrulline are carried out by many bacterial and animal tissue preparations. Phosphorolysis by bacteria which possess the arginine dihydrolase component is stimulated by the addition of adenosine phosphates. No such stimulation occurs in the liver homogenate reported by Krebs *et al.* (1955). Phosphorolysis and the effect of added adenosine phosphates using acetone-dried cells of the parent strain is shown in experiment A of Table 2. The rate of evolution of CO_2 was not enhanced by the presence of phosphate acceptors.

The arsenolysis of citrulline occurred with acetone-dried cells of the parent but not the mutant strain (experiment B, Table 2). The Michaelis constant for this reaction at 40°C was $2{\cdot}1 \times 10^{-2}$M. Krebs *et al.* (1955) gave a value of $1{\cdot}4 \times 10^{-2}$M for the liver enzyme under the same conditions of pH and temperature.

TABLE 2

THE ARSENOLYSIS AND PHOSPHOROLYSIS OF CITRULLINE

Expt. A—100 Mg dialysed acetone-dried cells, 500 μmoles phosphate buffer pH 6·1, 20 μmoles $MgSO_4$. Expt. B—50 Mg dialysed acetone-dried cells of the parent strain or arginine-requiring mutant, 500 μmoles arsenate buffer pH 6·7. Final vol. 3·3 ml; systems incubated in an atmosphere of N_2 at 40°C

Expt.	Additions to System	Free CO_2 Produced in 60 Min (μl)
A	None	3
	Citrulline, 160 μmoles	78
	Citrulline, 160 μmoles; AMP, 20 μmoles	77
	Citrulline, 160 μmoles; ADP, 20 μmoles	71
B	None	None
	Citrulline, 160 μmoles	154
	Citrulline (enzyme fraction from mutant), 160 μmoles	None

(*g*) *The Breakdown of Carbamyl Phosphate*

Jones *et al.* (1955) demonstrated the synthesis of citrulline from ornithine and CAP, and also the formation of ATP from CAP and ADP, the reactions being catalysed by extracts of *Strep. faecalis* and liver mitochondria. The breakdown of CAP by *S. marcescens* has been studied using acetone-dried cells of the mutant strain dialysed against distilled water (Table 3). The experiments were carried out under conditions comparable with those used for the breakdown of citrulline.

The reaction involving CAP in the presence of acetone-dried cells and $MgSO_4$ was found to be a first order reaction, whilst that involving the addition of ADP followed second order kinetics in the initial stages. The velocity constant for the spontaneous decomposition of CAP at 40°C and in the presence of 0·15M phosphate buffer pH 6·1, was calculated from the measurement of CO_2 output as $3{\cdot}7 \times 10^{-5}$ sec^{-1}. The half-life is therefore 5·2 hr.

IV. Discussion

The evidence obtained indicates that the metabolism of arginine and its precursors in *S. marcescens* is directed towards synthesis. Arginine is not dissimilated under conditions in which *Strep. faecalis*, *Staphylococcus aureus*, etc. break down arginine to form ornithine, CO_2 and ammonia with the concomitant synthesis of ATP. The lack of arginase activity shows that there is no ornithine cycle comparable to that found in mammals and certain fungi. Argininosuccinic acid is formed from arginine and fumarate, and the growth of an arginine-requiring mutant on citrulline suggests that the compound is an intermediate in the citrulline to arginine conversion. No arginine desmidase, the enzyme hydrolysing arginine, could be detected.

Table 3

THE BREAKDOWN OF CARBAMYL PHOSPHATE

85 Mg dialysed acetone-dried cells of the arginine-requiring mutant, 500 μmoles phosphate buffer pH 6·1; 10 μmoles CAP. Final vol. 3·3 ml; systems incubated in an atmosphere of N_2 at 40°C

Enzyme Fraction	Additions	Free CO_2 (μl)	
		14 Min	30 Min
None	None	18	21
None	$MgSO_4$, 20 μmoles	18	21
Boiled enzyme	None	18	21
Boiled enzyme	$MgSO_4$, 20 μmoles	19	25
Enzyme	None	45	94
Enzyme	$MgSO_4$, 20 μmoles	52	123
Enzyme	ADP, 20 μmoles	45	102
Enzyme	$MgSO_4$, 20 μmoles; ADP, 20 μmoles	134	194
Enzyme	ATP, 20 μmoles	35	100
Enzyme	$MgSO_4$, 20 μmoles; ATP, 20 μmoles	59	138

Arsenolysis and phosphorolysis of citrulline by acetone-dried cells of *S. marcescens* has been demonstrated, the latter reaction being similar to that in liver extracts in that adenosine monophosphate (AMP) and ADP do not enhance the rate. Jones *et al.* (1955) demonstrated the synthesis of citrulline from ornithine and CAP, and phosphorylation of ADP by CAP in extracts of *Strep. faecalis* and liver mitochondria. The reaction between ADP and CAP yielded ATP, CO_2, and ammonia, and required magnesium. The synthesis of citrulline from CAP and ornithine has been demonstrated in *S. marcescens*. The breakdown of CAP under conditions in which phosphorolysis of citrulline occurs has been studied. CAP is broken down in the absence of an added phosphate acceptor, the reaction rate being increased by the addition of magnesium. In the presence of ADP and magnesium a very marked stimulation of CO_2 output occurred.

If the reactions involved in citrulline breakdown are formulated as

$$\text{citrulline}+\text{phosphate} \rightleftharpoons \text{ornithine}+\text{CAP} \quad \ldots\ldots(5)$$

$$\text{CAP}+H_2O \xrightarrow{Mg^{++}} CO_2+NH_3+\text{phosphate} \quad (6)$$

$$\text{CAP}+\text{ADP} \overset{Mg^{++}}{\rightleftharpoons} CO_2+NH_3+\text{ATP} \quad \ldots(7)$$

then the lack of stimulation of phosphorolysis by adenosine phosphates would be explained if reaction (**5**) is the rate-limiting reaction. The data of Jones *et al.* (1955) and the data for *S. marcescens* show that the equilibrium position for reaction (**5**) lies well to the left. Reaction (**6**) has been shown to be a first order reaction, and reaction (**7**) a second order reaction.

It is suggested that the differences between the mammalian system which splits citrulline, and the system in bacteria possessing the arginine dihydrolase component, may be explained in terms of the two reactions in which CAP is dissimilated. Preliminary experiments using cell-free extracts indicate that reactions (**6**) and (**7**) are catalysed by different enzymes.*

The reaction mechanism proposed by Krebs *et al.* (1955) for the citrulline-ornithine conversion, made prior to the identification of CAP as a phosphorylated intermediate, should now be written

$$E_1+\text{citrulline} \rightleftharpoons E_1\text{-citrulline} \overset{\text{phosphate}}{\rightleftharpoons} E_1\text{-ornithine}+\text{CAP} \quad \ldots(8)$$

$$E_1\text{-citrulline} \xrightarrow{\text{arsenate}} E_1\text{-ornithine}+CO_2+NH_3 \quad \ldots\ldots(9)$$

$$E_2+\text{CAP} \rightleftharpoons E_2 \begin{cases} \text{carbamyl} \rightleftharpoons NH_2COOH \\ \text{phosphoryl} \rightleftharpoons \text{ATP} \end{cases} \quad \ldots\ldots\ldots(10)$$

$$E_3+\text{CAP}+H_2O \rightarrow E_3+CO_2+NH_3+\text{phosphate} \quad \ldots\ldots(11)$$

where E_1, E_2, and E_3 are the enzymes catalysing the respective reactions.

The evidence presented in this paper is consistent with the hypothesis that the arginine-requiring mutant lacks the enzyme E_1. Reaction (**10**), in which the enzyme anhydride link is to phosphoryl, would account for the evidence of Stulberg and Boyer (1954) who demonstrated the incorporation of ^{18}O from labelled inorganic phosphate into CO_2 arising during the phosphorolysis of citrulline.

V. Acknowledgment

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* The enzyme catalysing reaction (**7**) has been obtained free of detectable phosphatase activity, and the stoichiometry of the reaction established.

VI. References

Bergey, D. H. (1948).—"Bergey's Manual of Determinative Bacteriology." 6th Ed. (Ed. R. S. Breed, E. G. D. Murray, and A. P. Hitchins.) (The Williams and Wilkins Co.: Baltimore.)

Bergman, M., and Zerfas, L. (1928).—*Biochem. Z.* **203**: 280.

Bunting, M. I. (1946).—*Cold Spr. Hbr. Symp. Quant. Biol.* **11**: 25.

Davison, D. C., and Elliott, W. H. (1952).—*Nature* **169**: 313.

Dubnoff, J. W. (1941).—*J. Biol. Chem.* **141**: 711.

Eden, E., Harrison, D. D., and Linnane, A. W. (1954).—*Aust. J. Exp. Biol. Med. Sci.* **32**: 333.

Fowden, L. (1951).—*Biochem. J.* **48**: 327.

Gale, E. F. (1945).—*Brit. J. Exp. Path.* **26**: 225.

Grisolia, S., and Cohen, P. P. (1952).—*J. Biol. Chem.* **198**: 561.

Grisolia, S., and Cohen, P. P. (1953).—*J. Biol. Chem.* **204**: 753.

Jones, M. E., Spector, L., and Lipmann, F. (1955).—*J. Amer. Chem. Soc.* **77**: 819.

Knivett, V. A. (1952).—*Biochem. J.* **50**: xxx.

Knivett, V. A. (1954*a*).—*Biochem. J.* **56**: 602.

Knivett, V. A. (1954*b*).—*Biochem. J.* **56**: 606.

Knivett, V. A. (1954*c*).—*Biochem. J.* **58**: 480.

Korzenovsky, M., and Werkman, C. H. (1953).—*Arch. Biochem. Biophys.* **42**: 204.

Korzenovsky, M., and Werkman, C. H. (1954).—*Biochem. J.* **57**: 343.

Krebs, H. A., Eggleston, L. F., and Knivett, V. A. (1955).—*Biochem. J.* **59**: 185.

Krebs, H. A., and Henseleit, K. (1932).—*Hoppe-Seyl. Z.* **210**: 33.

Lederberg, J., and Tatum, E. L. (1946).—*J. Biol. Chem.* **165**: 381.

Oginsky, E. L., and Gehrig, R. H. (1952*a*).—*J. Biol. Chem.* **198**: 799.

Oginsky, E. L., and Gehrig, R. H. (1952*b*).—*J. Biol. Chem.* **198**: 791.

Oginsky, E. L., and Gehrig, R. H. (1953).—*J. Biol. Chem.* **204**: 721.

Ratner, S. (1954).—*Advances in Enzymology* **15**: 319.

Ratner, S., and Petrach, B. (1951).—*J. Biol. Chem.* **191**: 693.

Ratner, S., Petrach, B., and Rochovansky, O. (1953).—*J. Biol. Chem.* **204**: 94.

Slade, H. D. (1954).—"Streptococcal Infections." (Columbia Univ. Press: New York.)

Slade, H. D., Doughty, C. C., and Slamp, W. C. (1954).—*Arch. Biochem. Biophys.* **48**: 338.

Srb, A., and Horowitz, N. H. (1944).—*J. Biol. Chem.* **154**: 129.

Stulberg, M. P., and Boyer, P. D. (1954).—*J. Amer. Chem. Soc.* **76**: 5569.