Arginine Synthesis in *Proteus mirabilis*

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SUMMARY

Twenty-nine arginine auxotrophs of *Proteus mirabilis* strain 13 have been divided into eight groups which represent different biochemical blocks in the arginine biosynthetic pathway. The mutants were classified according to their growth requirements, syntrophism, accumulation of intermediates and enzyme deficiencies. The steps in the pathway are the same as those of *Escherichia coli* although mutants will not utilize *N*- α -acetyl-L-ornithine or *N*-acetyl-L-glutamate. However, these two substances were shown to be intermediates. Ornithine is synthesized via the linear route common to enterobacteria and not by transacetylation between *N*- α -acetyl-L-ornithine and L-glutamate as in some other bacteria and yeasts.

INTRODUCTION

Arginine synthesis has taxonomic significance as differences exist in the biosynthetic steps and enzymic control in various bacteria, yeasts and fungi (Vogel, 1953; Udaka, 1966; Smith, 1966). The arginine pathway as described for bacteria (Vogel, Bacon & Baich, 1963; Glansdorff, 1965; Udaka, 1966) is presented in Fig. 1. Some arginine

Fig. 1. Pathway of arginine synthesis in bacteria. A to H represent enzymic steps and the sites blocked in the corresponding *arg* mutants.

mutants of yeasts and fungi respond to proline (Bonner, 1946; Srb, Fincham & Bonner, 1950) whilst the early intermediates in the bacterial pathway are acetylated and distinct from the precursors in the proline pathway (Vogel, 1953; 1955). In *Micrococcus glutamicus* and some other bacteria transacetylation between L-glutamate and *N*- α -acetyl-L-ornithine (Fig. 1, enzyme E') occurs (Udaka & Kinoshita, 1958; Vogel & Vogel, 1963; Udaka, 1966; Hoare & Hoare, 1966). In these organisms the second enzyme in the pathway, *N*-acetyl- γ -glutamokinase (Fig. 1, enzyme B), is the site of feedback control, whereas in bacteria like *Escherichia coli* which lack this step the first enzyme, acetylglutamate synthetase (Fig. 1, enzyme A), is susceptible (Vyas & Maas, 1963; Udaka, 1966). The pathways of arginine biosynthesis in *E. coli* strains K 12, W and B are identical (Gorini, Gundersen & Burger, 1961; Maas, 1961; Vogel, 1961) although mutants lacking *N*-acetylornithine- δ -transaminase (Fig. 1, enzyme D) have only been isolated in strain W (Vogel *et al.* 1963). Enzyme repression in *E. coli* B

also differs from the mechanism in strains K12 and W (Gorini *et al.* 1961). The pathway of arginine synthesis in *Salmonella typhimurium* resembles that of *E. coli* (Demerec *et al.* 1960).

Baumberg, Bacon & Vogel (1965) concluded that in *Escherichia coli* K12 the four clustered arginine genes, *arg E*, *C*, *B* and *H* have individual repressor recognition sites as repression of these genes was not affected in their mutant which has undergone a separation of these normally closely-linked genes. Vogel & Bacon (1966) have found that mutations tend to occur concomitantly in functionally-related although not closely linked arginine genes, and because of this they suggested that gene aggregates of such genes may be formed by chromosome folding. The finding of a genetic topography in *Proteus mirabilis* similar to that of *E. coli* would confirm the importance and generality of these observations regarding gene sequence and clustering. As a preliminary to genetic studies arginine synthesis in *P. mirabilis* was investigated.

METHODS

Media. The minimal medium was that of Grabow & Smit (1967), with agar (1.75 % w/v) in solid media. When enriched with a mixture of growth factors (Novick & Maas, 1961) this medium was used as a complete defined arginine-free medium (AF), to which various concentrations of arginine could be added when required. The complete medium was Difco SS agar for *P. mirabilis* and Difco MacConkey agar for *E. coli* mutants. The broth was that of Coetzee & Sacks (1960a).

Chemicals. Amino acids, growth factors, arginine precursors, coenzymes and other chemicals were obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.; Sigma Chemical Company, St Louis 18, Missouri, U.S.A.; British Drug Houses Ltd., Poole, England or Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England. *N*- α -Acetyl-L-ornithine was synthesized (Vogel, 1955) from *N*- δ -carbobenzoxy-L-ornithine obtained from Dr Theodor Schuchardt, G.M.B.H. & Co., München, Germany. *o*-Amino-benzaldehyde was synthesized from *o*-nitro-benzaldehyde by the method of Albrecht, Scher & Vogel (1962). Carbamyl phosphate was synthesized according to Jones, Spector & Lipmann (1955) and extemporate hydroxylamine was prepared from hydroxylammonium chloride (E. Merck A.G., Darmstadt, Germany) by the method of Beinert *et al.* (1953).

Bacteria. Arginineless mutants of *Proteus mirabilis* strain 13 and a streptomycin-resistant mutant of this organism, 13 *str-r* (Coetzee & Sacks, 1960b) were obtained (Prozesky & Coetzee, 1966) by treatment with manganese chloride and hydrogen peroxide (Demerec, Bertani & Flint, 1951). A penicillin enrichment technique (Lederberg & Zinder, 1948) was followed by replica plating (Lederberg & Lederberg, 1952). Some of the mutants were selected by the method of Gorini & Kaufman (1960). The nutritional requirements of mutants were determined auxanographically (Lederberg, 1946). Mutant *arg A1*, *B1* with two blocks was obtained by transduction with phage 34/13 (Prozesky, in preparation). *E. coli* K12 arginine mutants W3422, W3679, PA201 were obtained from Dr W. K. Maas (Vyas & Maas, 1963). The *E. coli* K12 arginine mutant GSB1 was obtained from Dr N. Glansdorff (Glansdorff & Sand, 1965). A secondary *E. coli* mutant W4422P able to grow with acetylglutamate was obtained from mutant W3422. It has the same phenotype as mutant W3422B of Vyas & Maas (1963) and was selected in the same way. The mutants and their phenotypes

are listed in Table 1. Cultures were maintained on nutrient agar slopes at 4° and the incubation temperature was 37°. Fluid cultures were aerated with sterile air.

Syntrophism. All the *Proteus mirabilis* and *Escherichia coli* mutants were tested with one another. Overnight broth cultures of mutants were streaked in parallel about

Table 1. *Phenotypes of arginineless mutants*

Mutants	Enzyme block	Reaction to streptomycin (1 mg./ml.)	Growth response with				
			ac-glu	ac-orn	orn	cit	arg
<i>Proteus mirabilis</i>							
<i>arg A</i> 1	A	r	—	—	+	+	+
<i>arg A</i> 2	A	s	—	—	+	+	+
<i>arg A</i> 3	A	s	—	—	+	+	+
<i>arg A</i> 4	A	r	—	—	+	+	+
<i>arg A</i> 5	A	s	—	—	+	+	+
<i>arg A</i> 6	A	s	—	—	+	+	+
<i>arg A</i> 1, <i>B</i> 1	A+B	r	—	—	+	+	+
<i>arg B</i> 1	B	s	—	—	+	+	+
<i>arg B</i> 2	B	s	—	—	+	+	+
<i>arg C</i> 1	C	s	—	—	+	+	+
<i>arg D</i> 1	D	s	—	—	+	+	+
<i>arg D</i> 2	D	s	—	—	+	+	+
<i>arg D</i> 3	D	s	—	—	+	+	+
<i>arg D</i> 4	D	s	—	—	+	+	+
<i>arg D</i> 5	D	s	—	—	+	+	+
<i>arg D</i> 6	D	s	—	—	+	+	+
<i>arg E</i> 1	E	r	—	—	+	+	+
<i>arg E</i> 2	E	s	—	—	+	+	+
<i>arg E</i> 3	E	r	—	—	+	+	+
<i>arg F</i> 1	F	r	—	—	—	+	+
<i>arg F</i> 2	F	r	—	—	—	+	+
<i>arg F</i> 3	F	r	—	—	—	+	+
<i>arg F</i> 4	F	s	—	—	—	+	+
<i>arg F</i> 5	F	s	—	—	—	+	+
<i>arg G</i> 1	G	s	—	—	—	—	+
<i>arg G</i> 2	G	s	—	—	—	—	+
<i>arg H</i> 1	H	s	—	—	—	—	+
<i>arg H</i> 2	H	s	—	—	—	—	+
<i>arg H</i> 3	H	s	—	—	—	—	+
<i>Escherichia coli</i>							
<i>W</i> ₃₄₂₂	A	s	—	+	+	+	+
<i>W</i> ₃₄₂₂ P	A	s	+	+	+	+	+
<i>W</i> ₃₆₇₉	B	s	—	+	+	+	+
GSB I	C+E	r	—	—	+	+	+
PA 201*	E	r	—	—	+	+	+

r = resistant; s = sensitive; ac-glu = acetylglutamate; ac-orn = acetylornithine; orn = ornithine; cit = citrulline; arg = arginine; + = growth; — = no growth.

* Also requires threonine, leucine, thiamine, histidine.

2 mm. apart on minimal agar enriched with 0.01 % Difco nutrient broth powder and incubated for 48 hr (Grabow & Smit, 1967). Results were confirmed by auxanography with isolated accumulated precursors obtained from a representative of each of the *P. mirabilis* mutant types (see below). These precursors were tested for growth response with all the *E. coli* mutants and a representative of each of the *P. mirabilis* mutant types.

Isolation of accumulated precursors. Cultures were grown in 1600 ml. minimal

medium containing L-arginine hydrochloride (8 $\mu\text{g./ml.}$). L-Citrulline or L-ornithine was substituted for arginine when possible to minimize possible effects of repression or feedback control (Gorini & Maas, 1958; Vogel, 1961). After incubation for 36 hr at 37° the cultures were Seitz-filtered and any argininosuccinate, citrulline, ornithine and acetylornithine which had accumulated in the medium was extracted using a column (25 $\times$ 3.5 cm.) of cation exchange resin (Amberlite IR 120, H⁺ form). The column was washed with 500 ml. water and eluted with 150 ml. N-NH₄OH (Grabow & Smit, 1967). The eluates were vacuum-evaporated to dryness and the residues dissolved in 0.5 ml. water for preliminary chromatography. *N*-Acetylglutamic- γ -semialdehyde and acetylglutamate were concentrated and purified as described by Vogel (1953) and Vyas & Maas (1963) respectively.

Chromatography. A modification of the technique of Grabow & Smit (1967) had to be used because of the residual salts content of the sample. Duplicate one-dimensional chromatograms of samples and standards were developed for 16 hr on Whatman No. 3MM paper with *n*-butanol + pyridine + water (1 + 1 + 1, by vol.) as solvent by the descending technique. Standards were located by spraying with ninhydrin (0.2 %, w/v, in acetone) and heating at 100° for 5 min. Acetylglutamate was located with 0.05 % (w/v) bromocresol-green in 95 % ethanol (Vyas & Maas, 1963). Corresponding areas on duplicate chromatograms containing the samples were cut out, eluted with water and the eluates vacuum-evaporated to dryness. The residues were taken up in 0.1 ml. water and re-chromatographed on Whatman No. 1 paper in two dimensions. The solvents were: first dimension, *n*-butanol + formic acid + water (77 + 10 + 13 by vol.); second dimension, *n*-butanol + pyridine + water as above. Acetylglutamate gave elongated spots with these solvents when applied to chromatograms in sufficient quantities for clear definition with the bromocresol-green method. This precursor was therefore only chromatographed in one dimension with each solvent which is sufficient for identifying this substance (Vyas & Maas, 1963).

Precursor identification. Substances on chromatograms were identified by position and by mutant response. The mutant response technique was a modification of the method of Winsten & Eigen (1950). Minimal agar was seeded with about 10 ml. washed bacteria from an overnight broth culture of a mutant strain of *Escherichia coli* or *Proteus mirabilis* with a known growth response. The seeded medium was poured as plates of 30 $\times$ 30 $\times$ 1 cm. (Maré, Coetzee & de Klerk, 1964). Chromatograms were cut into squares, sterilized with ultraviolet light and placed on the agar for 5 min. After removal of the paper the plates were incubated for 48 hr and areas of growth were compared with stained chromatograms of samples and standards which had been developed concurrently. *N*-acetylglutamic- γ -semialdehyde was identified by its colour reaction with *o*-amino-benzaldehyde and by preparing its hydrazone derivative with 2,4-dinitro-phenylhydrazine (Vogel, 1953).

Enzymes. The enzymes and their assay methods are listed in Table 2. In general the specific activities of enzymes in cell-free extracts of all the auxotrophs were compared with those of the prototroph. However, the wild-type level for glutamate acetylation (enzyme A) was measured with resting cell suspensions of mutant *arg B* 1 (Vyas & Maas, 1963) and the mutant enzyme level was determined with the double mutant, *arg A* 1, *B* 1; acetylglutamate was assayed with *Escherichia coli* w 3422P. To eliminate interference from acetylornithinase, transacetylation between acetylornithine and glutamate (enzyme E') was measured in mutant *arg E* 1.

Table 2. *Enzymes of the arginine pathway of Proteus mirabilis*

Enzyme		Trivial name	E.C. no.	Position in pathway	Reaction product measured	Reference for method	Enzyme specific activity in prototroph (units)
Systematic name							
AcetylCoA:L-glutamate N-acetyltransferase		Acetylglutamate synthetase	2.3.1.1	A	N-Acetylglutamate	Vyas & Maas (1963): measured with mutant <i>arg B1</i>	59.3*
ATP:N-acetylglutamate 5-phosphotransferase		N-Acetyl- γ -glutamokinase	—	B	N-Acetyl- γ -glutamyl hydroxamate	Baich & Vogel (1962) as used by Glansdorff (1965)	3.5†
α -N-Acetyl-L-glutamate- γ -semialdehyde:NADP oxidoreductase		N-Acetylglutamic- γ -semi-aldehyde dehydrogenase	1.2.1.4	C	NADPH	Baich & Vogel (1962); Glansdorff & Sand (1965)	0
L-Ornithine: 2-oxoacid aminotransferase		N-Acetylornithine- α -transaminase	2.6.1.11	D	N-Acetyl-glutamic- γ -semialdehyde	Albrecht & Vogel (1964)	3.6‡
α -N-Acetyl-L-ornithine amidohydrolase		Acetylornithinase	3.5.1.4	E	Ornithine	Vogel & Bonner (1956)	22.7†
N-Acetylornithine; L-glutamate N-acetyl-transferase		Acetylornithine-glutamate transacylase	—	É	Ornithine	Udaka (1966): measured with mutant <i>arg E1</i>	0.05†
Carbamoylphosphate: L-ornithine carbamoyl-transferase		Ornithine transcarbamylase	2.1.3.3	F	Citrulline	Jones (1962)	3.1†
L-Citrulline: L-aspartate-lyase		Condensing enzyme	6.3.4.5	G	Disappearance of citrulline	Ratner (1955)	0.5†
L-Argininosuccinate-arginine lyase		Argininosuccinase	4.3.2.1	H	Ornithine	Baumberg <i>et al.</i> (1965)	1.0†

* 1 unit = 1 μ mole/mg. protein/hr.† 1 unit = 1 μ g./mg. dry bacteria/hr.‡ 1 unit = 0.1 increase in absorbancy at 440 m μ /mg. protein/hr.

NADPH = Reduced nicotinamide-adenine dinucleotide phosphate.

E.C. no. = Enzyme Commission number.

Preparation of cell-free extracts. Organisms (approximately 500 mg. dry wt) were harvested from 1600 ml. of medium AF supplemented with L-arginine hydrochloride (8 μ g./ml.) after 36 hr. They were washed twice in the buffer recommended for the enzymic assay (Table 2) and resuspended in 25 ml. of the same buffer. Suspensions were disrupted in a Raytheon model S102A oscillator (9 kcyc./sec. for 45 min.) and centrifuged at 30,000 *g* for 30 min. in a Sorvall SS-4 centrifuge. The supernatant fluid was dialyzed against 2 l. of the corresponding buffer with 2 changes. The above manipulations were all performed at 4°. Protein content of the extracts was determined by the method of Gornall, Bardawill, & David (1949).

RESULTS

Arginineless auxotrophs. A total of 103 arginine-requiring mutants were obtained from 30 isolation experiments and a group of 29 mutants was chosen for investigation on the basis of one phenotype per isolation.

Growth requirements. The growth requirements of these mutants are given in Table 1, and three classes of *Proteus mirabilis* mutant can be distinguished. They responded to ornithine, citrulline or arginine and appear to be blocked in the synthesis of ornithine, the conversion of ornithine to citrulline or the conversion of citrulline to arginine, respectively (Fig. 1). None of the mutants responded to glutamate, acetylglutamate, acetylornithine, acetylglutamic- γ -semialdehyde, argininosuccinate or proline. No secondary mutants (Vyas & Maas, 1963) able to grow with acetylglutamate, acetylornithine, acetylglutamic- γ -semialdehyde or argininosuccinate were isolated after exposure to these intermediates, although the selection method proved satisfactory for the isolation of *Escherichia coli* W3422P from mutant W3422.

Syntrophism. The above results were confirmed by studies of syntrophism with all the mutants; the same three mutant classes were identified. Mutants subsequently defined as types *G* and *H* fed all other mutants but were fed by none. Type *F* mutants were fed by type *G* and *H* mutants and fed the class of mutants which included types *A*, *B*, *C*, *D*, *E*. This latter class of mutants did not feed the other classes of mutant nor was syntrophism exhibited between pairs of mutants within this class despite the fact that they accumulate precursors (see below). These mutants were further classified by syntrophism tests with well-characterized *A*, *B* and *E* mutants of *Escherichia coli*. *Proteus mirabilis* mutants types *F*, *G* and *H* fed all the *E. coli* mutants, while none of the *E. coli* mutants fed any of the *P. mirabilis* mutants. *Proteus mirabilis E* mutants fed *E. coli A* and *B* mutants but not *E* mutants. *Proteus mirabilis B* and *C* mutants only fed the secondary *E. coli* mutant of type *A* (W3422P which responds to acetylglutamate). *Proteus mirabilis A* and *D* mutants did not feed any *E. coli* mutants nor did the *P. mirabilis C* mutant feed the *E. coli* type *B* mutant. The results of syntrophism tests with the auxanographic technique gave identical results. Positive identification of the following groups of mutants was possible on the basis of growth response and syntrophism: *A + D*; *B + C*; *E*; *F*; *G + H*.

Accumulation of precursors. All the mutants were tested for accumulation of intermediates. Apart from group *arg A* mutants the other groups accumulated preceding precursors in the medium. Only in the case of *arg H* mutants was more than one accumulated precursor identified. The precursors, their *R_p* values, the mutants which excrete them and the mutants used for their identification are listed in Table 3. The

Table 3. *Precursors accumulated by arginineless mutants*

<i>Proteus mirabilis</i> and <i>Escherichia coli</i> mutant groups	Accumulated precursor	R_F value in solvent:		Mutants used for identification*	
		Butanol- formic acid water	Butanol- pyridine- water	Positive growth response	Negative growth response
<i>Arg A</i>	None	—	—	—	—
<i>Arg B, arg C</i>	<i>N</i> -acetylglutamate	0.65	0.38	<i>E. coli</i> w 3422 P	<i>E. coli</i> w 3422
<i>Arg D</i>	<i>N</i> -acetylglutamic- γ -semialdehyde	†	†	—	—
<i>Arg E</i>	<i>N</i> - α -acetylornithine	0.04	0.06	<i>E. coli</i> w 3679	<i>E. coli</i> PA201, <i>arg D</i> 1
<i>Arg F</i>	Ornithine	0.01	0.15	<i>E. coli</i> PA201	<i>arg G</i> 1, <i>arg H</i> 1
<i>Arg G, arg H</i>	Citrulline	0.03	0.21	<i>arg F</i> 1	<i>arg G</i> 1, <i>arg H</i> 1
<i>Arg H</i>	Argininosuccinate	0.01	0.04	None	All classes

* *Proteus mirabilis* mutants except where noted.† Chromatography not done. Identified by a colour reaction with *o*-aminobenzaldehyde and by preparing the hydrazone derivative (see 'methods').

results of the growth requirement experiments, syntrophism tests and accumulation studies made it possible to identify the following groups of mutants: *A*; *B*+*C*; *D*; *E*; *F*; *G*; *H*. Distinction between the *B* and *C* mutants could only be made by enzyme assays.

Enzyme studies. Seven enzymes in the pathway could be demonstrated in the wild-type organism (Table 2), while all the mutants in each of these seven mutant groups showed complete absence or only traces of the corresponding enzyme activity. The enzyme deficiencies in these mutants positively confirmed their classification based on the results of the preceding investigations. These results also made a distinction between the *arg B* and *arg C* mutants although *N*-acetylglutamic- γ -semialdehyde dehydrogenase (enzyme C) could not be demonstrated. Unsuccessful attempts were made to demonstrate it alone and also in combination with enzymes B or D as for *Escherichia coli* (Baich & Vogel, 1962; Glansdorff & Sand, 1965). Previous work with *Proteus mirabilis* strain 18 also yielded negative results (Smith, 1966). Failure to demonstrate enzyme C may be due to oxidation of reduced nicotinamide-adenine dinucleotide phosphate (Smith, 1966) and attempts to separate it from this activity were unsuccessful. However, mutant *arg C*1 has the wild-type level of *N*-acetyl- γ -glutamokinase (enzyme B), activity while enzyme B activity is completely lacking in *arg B* mutants. The low level of acetylornithine-glutamate transacetylase (enzyme E') and the high level of acetylornithinase (enzyme E) found indicate that the transacetylation reaction probably does not participate in the proteus pathway. The absence of any *arg E'* mutants is additional evidence for this conclusion.

DISCUSSION

The findings indicate that the route of arginine synthesis in *Proteus mirabilis* is identical to that found in *Escherichia coli* and *Salmonella typhimurium* (Demerec *et al.* 1960; Vogel *et al.* 1963; Baumberg *et al.* 1965). The proteus mutants differ from the *E. coli* mutants by their inability to grow with acetylornithine. However, it appears that this substance is an intermediate in the pathway because it is accumulated by *arg E* mutants and it is the substrate for acetylornithinase (enzyme E) which is present in *P. mirabilis*. The failure of mutants to respond to acetylglutamate is in accordance with similar findings in *S. typhimurium* (Demerec *et al.* 1960). Secondary mutants of *E. coli* are able to utilize acetylglutamate which may reflect the acquisition of permeability for this substrate; an acetylornithine permease has been identified in *E. coli* (Vogel, 1960). Consequently, it appears that *P. mirabilis* is cryptic (Cohen & Monod, 1957) for acetylornithine and acetylglutamate. Although some experimental conditions were different the specific activities for the enzymes in the arginine biosynthetic pathway (Table 2) are similar to those reported for *E. coli* (Vyas & Maas, 1963; Glansdorff & Sand, 1965; Vogel & Bonner, 1956; Udaka, 1966 and Gorini, 1963). The low specific activity of enzyme E' (Table 3) indicates that transacetylation between acetylornithine and glutamate does not play an important part in the biosynthesis of arginine in *P. mirabilis*. Udaka (1966) encountered a similar situation with *P. vulgaris*. The inability to demonstrate *N*-acetylglutamic- γ -semialdehyde dehydrogenase activity in *P. mirabilis* does not exclude the existence of two steps between acetylglutamate and *N*-acetylglutamic- γ -semialdehyde (Baich & Vogel, 1962). There is strong evidence that reaction C (Fig. 1) exists: Mutant *arg C*1 accumulates acetylglutamate and has

the wild-type level of *N*-acetyl- γ -glutamokinase (enzyme B) activity (enzyme B activity is completely lacking in *arg B* mutants). The observed syntrophism and accumulation of arginine precursors is in marked contrast with the absence of these features in the methionine pathway of *P. mirabilis* strain 13 (Grabow & Smit, 1967) which indicates that this is a peculiarity of the latter pathway in this organism and not a common property of *P. mirabilis*.

Linked transduction with some of these mutants has been described (Prozesky & Coetzee, 1966) and the genetic studies in progress may allow further comparison between arginine synthesis in *Escherichia coli* and *Proteus mirabilis*.

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