

Accumulation of Arginine Precursors in *Escherichia coli*: Effects on Growth, Enzyme Repression, and Application to the Forward Selection of Arginine Auxotrophs

M. CRABEEL, D. CHARLIER, R. CUNIN, A. BOYEN, N. GLANSDORFF,* AND A. PIÉRARD
Erfelijkheidsleer en Mikrobiologie, Vrije Universiteit Brussel, Laboratoire de Microbiologie, Université Libre
de Bruxelles and Institut de Recherches du CERIA, B-1070 Brussels, Belgium*

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The accumulation of ornithine, citrulline, and possibly acetylornithine by *Escherichia coli* K-12 arginineless mutants provided with acetylarginine as source of arginine causes severe growth inhibition. This occurs under conditions where comparable derivatives of *E. coli* W (Bollon and Vogel, 1973) show little or no growth inhibition. The same conditions, which have been reported to cause noncorrelative synthesis of acetylornithinase and argininosuccinase in *E. coli* W (Bollon and Vogel, 1973), do not alter the correlative pattern of enzyme synthesis observed in *E. coli* K-12. Moreover, previously reported effects of ornithine and citrulline on repression of the arginine regulon in *E. coli* W are not observed in the K-12 strains examined. The bearing of these observations on possible differences between the mechanism of enzyme repression operating in the two types of strains cannot yet be fully evaluated; it is, however, clear that considerable care should be exercised before extrapolating the results obtained with one type of strain to the other one. The particularly strong inhibition of acetylarginine utilization exerted by ornithine in *E. coli* K-12 allows the forward selection of several classes of arginine auxotrophs from strains deficient in carbamoylphosphate biosynthesis and thus capable of ornithine accumulation. Possible applications of this technique to the genetic analysis of the bipolar *argECBH* operon are discussed.

The synthesis of the eight enzymes involved in the arginine pathway is repressed by arginine through the mediation of one regulatory gene (*argR*) despite the scattering of the *arg* loci on the bacterial chromosome (22). Maximal rates of enzyme synthesis are obtained in conditions of restriction of arginine supply or in *argR* mutants (13, 18, 23). Derepression as well as repression affects the synthesis of all eight enzymes simultaneously, although not in a strictly coordinated fashion; indeed, the ratio between the specific activities of any two enzymes of the pathway is not necessarily the same under all circumstances as it is for the enzymes of a classical operon (11).

This pattern of pleiotropic regulation may be defined as "correlative" or "qualitatively coordinated." In a forthcoming paper (manuscript in preparation) and in a preliminary note (5) we show that this is due to differential transcription effectiveness of genetic units of expression, at least in the case of *argE* and *argCBH*.

Because of the pleiotropy of arginine control

one would not expect to find growth conditions allowing an *argR*⁺ strain to derepress one or more of the arginine enzymes and not the others. Recently, however, Bollon and Vogel (4) reported a case of noncorrelative expression of acetylornithinase (enzyme E, Fig. 1) and argininosuccinase (enzyme H) in *Escherichia coli* W. This paper is concerned with the more commonly used K-12 strain, where the situation is shown to be different.

E. coli argE⁺ strains can use *N*- α -acetylarginine as a source of arginine because enzyme E (acetylornithinase) is able to deacetylate acetylarginine (see reference 2). From Bollon and Vogel's work (4) it appears that an *argA* mutant of *E. coli* W growing at a suboptimal rate on 25 μ g of acetylarginine per ml exhibits full enzyme E derepression but only half the maximal rate of synthesis for enzyme H. More dramatic is the behavior of an *argB argG* double mutant; growth on 25 μ g of acetylarginine per ml brings about 50% derepression of enzyme E, but maintains complete repression of enzyme H. The

addition of massive amounts of *N*- α -acetylornithine (500 μ g/ml), which can be converted into ornithine and citrulline but not further in the double mutant, slows down the growth rate by 50% and causes maximal derepression of *argE*, *argH* remaining repressed. The inhibitory effect of acetylornithine is interpreted as resulting from a feedback exerted by the internally accumulated citrulline on the entry of acetylarginine through the acetylornithine permease.

From genetic and biochemical studies of *E. coli* K-12 it is known that *argE* and *argCBH* are two wings of a bipolar operon transcribed from an internal control region situated between *argE* and *argCBH* (2, 7, 9, 16, 20, 21). Certain operator type mutations occurring in that region cause constitutive expression of the whole gene cluster. In line with the genetic evidence, the expression of *argE* and *argCBH* is correlative, if not strictly coordinated at the transcription level (5; manuscript in preparation). We therefore felt it necessary to undertake with K-12 strains the type of study performed by Bollon and Vogel with *E. coli* W. We found that our strains behaved differently; no physiological conditions could be obtained that brought any significant departure from the correlative pattern of synthesis observed previously. Moreover, the addition of even low concentrations of acetylornithine to a culture of a K-12 *argB argC* mutant inhibited growth dramatically. The latter observation allowed us to find conditions permitting the forward selection of certain *arg* auxotrophs.

MATERIALS AND METHODS

Chemicals. *N*- α -acetylarginine, *N*- α -acetylornithine, arginosuccinate, and carbamoylphosphate were from Sigma Chemical Co., St. Louis, Mo. Arginase was from Boehringer, Mannheim, Germany.

Only L-amino acids were used.

Strains. Symbols for genetic markers are those used by Taylor and Trotter (19) except for the new abbreviation *car*, which designates the locus for the structural genes of carbamoylphosphate synthetase (16). *ppc* is the gene for phosphoenolpyruvate carboxylase (E.C. 4.1.1.3.1). For other abbreviations see below.

The parental K-12 strain of the *argG* mutant P4XSB68 (ultraviolet induced; S. Bourgeois, unpublished data) and of its *argB argG* derivative is Hfr P4X6 (see reference 1); *metB*, *relA*, *Str-s*. The *argB argG* derivative was constructed as follows. Phage 363 was grown on the *Met*⁺ *argBI* K-12 strain 3OSOMA4 (8) and used to transduce P4XSB68; *Met*⁺ recombinants were selected, purified, and checked for the presence of the *argB* mutation by cross-feeding and recombination tests.

P4XB2 is an *argR* (genetically derepressed) deriva-

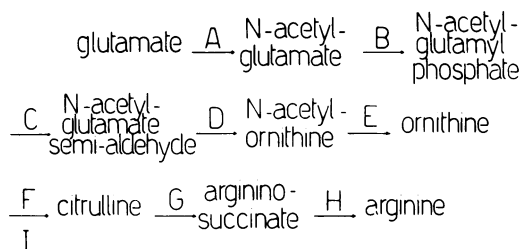


FIG. 1. Gene-enzyme relationship in the arginine regulon of *E. coli* K-12.

tive of HfrP4X. MN42 is a *ppc argECBH* deletion mutant of Hfr P4X (9).

The K-12 strain PA2011 (F⁻, *thi*, *argE*, *argG*, *Str-r*) has been obtained from R. Lavallé. The *carB8* deletion mutant, deficient in carbamoylphosphate biosynthesis, is a derivative of Hfr P4X6 (see above); it has been described previously (19).

Cultivation of the organisms. Cells were grown under agitation at 37 C. Minimal medium 132 was used (10) with glucose 0.4% as the carbon source. Where required, L-methionine was used at 100 μ g/ml and thiamine at 1 μ g/ml. Other additions were as indicated in the text and in Table 1.

Enzyme assays. The preparation of crude extracts, the determination of the protein concentration and the assays for acetylornithinase (*argE* product, E.C. 3.5.1.16), arginosuccinate lyase (*argH* product, E.C. 4.3.2.1), and ornithine carbamoyltransferase (*argF* and *argI* product, abbreviated OTCase [E.C. 2.1.3.2]), have been described previously (9, 21). Enzyme specific activities are expressed as micromoles of product formed per minute per milligram of protein.

ArgECBH messenger ribonucleic acid (RNA) determinations. Cultures were pulse labeled with tritiated uracil, and the RNA was extracted and hybridized with λ *darg* deoxyribonucleic acid as described previously (7, 21).

Mutagenesis. About 2×10^8 cells of the *carB8* strain were poured on selective plates and a crystal of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or a disk of filter paper soaked in diethylsulfate was deposited near the circumference of the plate.

RESULTS

For all determinations of growth rates and enzyme activities (Table 1, Fig. 2 and 3, and data not shown) the following procedure is followed: a culture pregrown overnight in a particular medium is centrifuged, washed with medium 132, inoculated in medium of the same composition (for Table 1, see "Preculture") at a density of about 2×10^7 cells per ml, and incubated till mid-exponential phase (4×10^8 cells/ml). The bacteria are then centrifuged for 3 min at room temperature in the Sorvall SPX at 5,000 rpm and inoculated into prewarmed

TABLE 1. Specific activities of arginine enzymes in *arg* mutants provided with acetylarginine as a restrictive arginine source and arginine precursors (acetylornithine, ornithine or citrulline) at varied concentrations

Strains ^a	Additions to minimal medium ($\mu\text{g/ml}$)		Enzyme sp act ($\mu\text{mol per min/mg}$ of protein)			Doubling time (min)
	Preculture ^b	Culture	Acetylornithinase	Argininosuccinase	Carbamoyltransferase	
<i>argR</i> ⁻ (P4XB2) ^b	Arg (100)	Arg (100)	1.40	0.162	18.33	50
<i>argR</i> ⁺ (P4X)	Arg (100)	Arg (100)	0.07	0.003	0.025	50
<i>argR</i> ⁺ <i>argB argG</i> (P4XSB68)	Ac.arg (100)	Ac.arg (1,000)	0.55	0.028		50
	Arg (100)	Ac.arg (250)	1.03	0.042	12.12	- ^c
	Ac.arg (250)	Ac.arg (25)	1.41	0.128	21.65	55
	Ac.arg (100)	Ac.arg (25)	1.33	0.138		
	Ac.arg (25)	Ac.arg, (25) + ac.orn, (50)	1.22	0.090	13.54	
		Ac.arg (25) + ac.orn (100)	0.86	0.072		Not exponential (see Fig. 3)
		Ac.arg (25) + ac.orn (150)	1.03	0.098		
	Arg, (100)	Ac.arg (250) + ac.orn (25)	0.80	0.047	2.78	
		Ac.arg (250) + orn (25)	0.28	0.027	2.75	>700
		Ac.arg (250) + citr (25)	1.14	0.083	6.37	- ^c
		Ac.arg (25) + ac.orn (10) ^{d,e}	1.63	0.105	10.87	120
		Ac.arg (25) + ac.orn (10) + ac.orn (15) ^{d,e}	1.92	0.124	13.05	120
		Ac.arg (25) + ac.orn (10) + ac.orn (15) + ac.orn (100) ^{d,e}	1.60	0.125	11.50	Not exponential (see Fig. 3)
	Arg (100)	Ac.arg (250) + ac.orn (75) ^d	1.07	0.065	3.70	60
		Ac.arg (25) + citr (25) ^d	1.53	0.130	9.17	195
Arg (100)	Chemostat arg (10)		0.98	0.103	8.22	210
			0.90	0.135	9.41	210
		Chemostat arg (10) + citr (300), after 5 h	1.27	0.138	6.73	210
		21 h	1.58	0.105		210
<i>argE argG</i> (PA 20II)	Arg (100)	Chemostat arg (10)			4.27	85
		Chemostat arg (10) + orn (100), after 20 h			4.66	85
		Chemostat arg (10)			6.13	95
		Chemostat arg (10) + orn (100), after 10 h			5.55	95

^a Genetically derepressed.

^b Arg, Arginine; Ac.arg, acetylarginine; Ac.orn, acetylornithine; orn, ornithine; and citr, citrulline.

^c Growing but not yet fully adapted.

^d Arginine (2 $\mu\text{g/ml}$) present at inoculation.

^e Sequential addition of increasing amounts of acetylornithine.

test medium (for Table 1, see "Culture"). Except when indicated, cells are harvested for enzyme assays from cultures having reached a steady state of growth in the new medium. As one of our main purposes is to examine to what extent the rate of synthesis of enzymes E and H remain correlated over a wide range of conditions, no attempt is made to determine exactly when enzyme-specific activities reach their equilibrium values in the new medium.

Utilization of acetylarginine by *E. coli* K-12. The *argB argG* derivative of *E. coli* K-12 strain P4X grows at a suboptimal rate on 25

μg of acetylarginine per ml (Fig. 2). At 250 $\mu\text{g/ml}$ or higher concentrations, the same rate as in excess arginine is achieved. In a similar mutant of *E. coli* W, the addition of as much as 250 μg of acetylornithine per ml to 25 μg of acetylarginine per ml does not affect the growth rate (4). In contrast, as little as 50 μg of acetylornithine per ml stops exponential growth of a culture of the K-12 derivative fully adapted to acetylarginine (25 $\mu\text{g/ml}$; Fig. 3). Half this amount suffices to prevent organisms precultivated in the presence of arginine—thus in conditions of repression—to start growing at the ex-

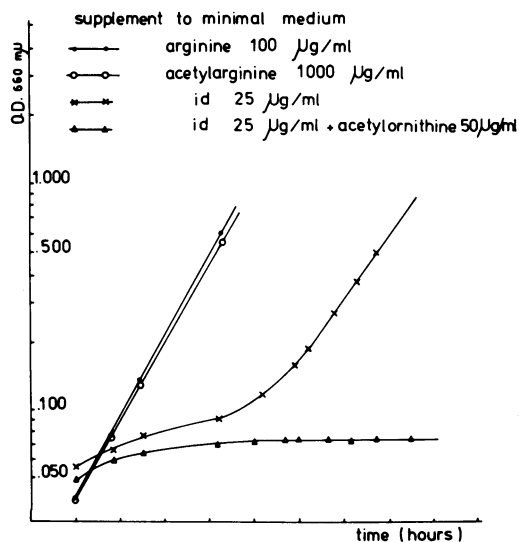


FIG. 2. Growth of the *argB argG E. coli* K-12 mutant in minimal medium supplemented as indicated. The cells were pregrown in the presence of arginine (100 $\mu\text{g/ml}$) until a cell density of $5 \times 10^8/\text{ml}$ was reached, and were centrifuged, washed, and inoculated in prewarmed medium. Only L-amino acids were used. OD, Optical density. Supplement to minimal medium: \bullet , arginine (100 $\mu\text{g/ml}$); \circ , acetylarginine (1,000 $\mu\text{g/ml}$); \times , acetylarginine (25 $\mu\text{g/ml}$); and Δ , acetylarginine (25 $\mu\text{g/ml}$) + acetylornithine (50 $\mu\text{g/ml}$).

pense of acetylarginine (25 $\mu\text{g/ml}$; Fig. 2). The growth response becomes positive only if the relative proportions of acetylarginine and acetylornithine are reversed (Table 1). The addition of arginine to a culture fully inhibited by acetylornithine reverses this inhibition, indicating that the cells are not irreversibly intoxicated.

The addition of citrulline also reduces the growth rate but never quite as much as ornithine, not tried on W, which is the most effective inhibitor tested up to now (Table 1, line 11).

The effect of arginine precursors accumulation readily explains why the *argG* parental strain grows more slowly on acetylarginine than the double mutant (92 min doubling time instead of 55 min).

The same pattern of growth inhibition is observed whether the cultures are stationary (Bollon and Vogel's conditions) or grown under agitation as in all experiments reported here.

Correlative enzyme synthesis in the presence of acetylarginine and acetylornithine. The syntheses of acetylornithinase and argininosuccinase remain broadly correlative under all circumstances investigated (Table 1).

In the range of conditions allowing balanced growth to proceed, the lower the growth rate (and thus the more stringent the restriction in the arginine supply) the higher the level of derepression achieved. When growth inhibition is too severe to measure significant variations in the rate of enzyme synthesis, the determination of the percentage of pulse-labeled RNA hybridizing with *argECBH* deoxyribonucleic acid (as described in references 8 and 21) nevertheless indicates that the cluster is at least partly derepressed; 0.42% hybridizable RNA can be recovered from extracts of cultures incubated in 25 μg of acetylarginine and 150 μg of acetylornithine per ml; derepressed (*argR*) mutants of *E. coli* give between 0.4 and 1%; the repression level is around 0.1% (8, 21).

We also tested the effect of ornithine and citrulline in continuous cultures (chemostats) of arginine auxotrophs. In one experiment the same *argB argG* mutant as above was grown at a dilution rate allowing near maximal derepression with a limiting arginine concentration of 8 $\mu\text{g/ml}$. The addition of citrulline (300 $\mu\text{g/ml}$) to the chemostat did not appreciably change the

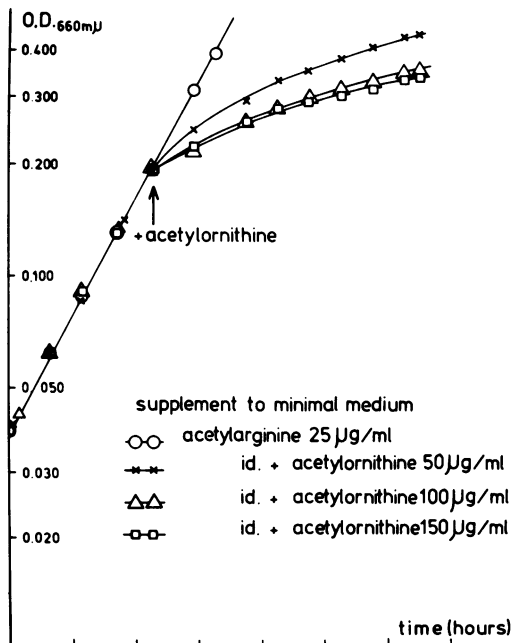


FIG. 3. Effect of acetylornithine addition to cultures of the *argB argG E. coli* K-12 mutant adapted to acetylarginine. Only L-amino acids were used. Supplement to minimal medium: \circ , acetylarginine (25 $\mu\text{g/ml}$); \times , acetylarginine + acetylornithine (50 $\mu\text{g/ml}$); Δ , acetylarginine + acetylornithine (100 $\mu\text{g/ml}$); and \square , acetylarginine + acetylornithine (150 $\mu\text{g/ml}$).

specific activities of enzyme E, H or of OTCase (Table 1). In another experiment, the *argE argG* mutant strain PA2011 was grown at a dilution rate allowing approximately 25% derepression; the addition of ornithine (which can be converted into citrulline but not further in this particular strain) did not appreciably modify the rate of OTCase synthesis (Table 1).

The results of the latter experiments also are at variance with the data reported for *E. coli* W by Bollon and Vogel (according to them, citrulline represses acetylornithinase synthesis in arginine-limited chemostat) and by Gorini (12) who very early reported that, in *E. coli* W, ornithine antagonized repression of OTCase synthesis in a chemostat run at such a dilution rate that the culture was maintained in a state of partial repression.

There is thus no suggestion in our data that intracellular accumulation of arginine precursors provokes any differential repression of genes investigated, nor that ornithine or citrulline by themselves modify the rate of synthesis of arginine enzymes in *E. coli* K-12. The latter point will however have to be more critically tested in vitro.

Application of acetylarginine-ornithine antagonism to the forward selection of arginine auxotrophs. The inhibition of acetylarginine utilization by ornithine provides an explanation for one of our previous unpublished observations, namely that the requirements of a carbamoylphosphate synthetase mutant—which requires arginine and uracil—are not satisfied by a mixture of acetylarginine and uracil. Indeed, a carbamoylphosphate synthetase mutant incubated with a restrictive arginine source—in this case acetylarginine—is bound to accumulate ornithine, substrate of ornithine carbamoyltransferase (see Fig. 1). If the explanation is correct: (i) a strain harboring a carbamoylphosphate synthetase defect and a mutation in one of the genes involved in the synthesis of acetylornithine should grow on acetylarginine and uracil. A double *argB1 carB8* mutant was constructed and found to exhibit this phenotype indeed. (ii) One should be able to select *arg* auxotrophs blocked in the conversion of glutamate into acetylornithine as derivatives of a carbamoylphosphate synthetase mutant able to grow on acetylarginine and uracil.

The deletion mutant *carB8* (19) was plated on minimal medium supplemented with 50 μ g of uracil per ml and 100 μ g of acetylarginine per ml. Spontaneous and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or diethylsulfate-induced mutants were then selected (see Materials and Methods). The frequency of spontaneous mutants appear-

ing after 2 days was 3×10^{-9} .

Twenty-eight such mutants (2 induced by diethylsulfate, 3 by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and 23 spontaneous ones) were purified and transduced to *Car*⁺ by replica plating on a minimal medium plate devoid of uracil but supplemented with arginine and seeded with 5×10^8 particles of a λ *Car*⁺-transducing phage constructed in this laboratory (14; manuscript in preparation).

Thirteen of the 28 *Car*⁺ recombinants proved to be *arg* auxotrophs; 12 gave a positive growth response with acetylornithine; and one required arginine. The latter organism probably carries a deletion extending from *argC* into *B* and *H*. Two of the other twelve mutants were analyzed further and found to be *argB* or *C*; indeed, all *Ppc*⁺ recombinants recovered from a transduction between those mutants and the *ppc argECBH* deletion mutant MN42 used as recipient were found to be *Arg*⁻. Two diethylsulfate-induced mutants were *argA*, as proved by cotransduction of their *arg* marker with *cysC* (about 2%). Out of the three *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced mutants, one was *argA*, two were *Arg*⁺.

DISCUSSION

(i) **Correlative enzyme synthesis in the *arg* pathway.** The present observations establish that in *E. coli* K-12 strain P4X6 the syntheses of acetylornithine and argininosuccinase remain correlative under conditions (accumulation of arginine precursors) where a marked decoordination is reported to occur in *E. coli* W.

One could at first sight conclude that the regulation of arginine biosynthesis operates differently in the two types of strains. We consider, however, that the situation is not yet clear enough to be forced to such conclusions. The *argB argG* W strain used by Bollon and Vogel produced low enzyme H levels under all conditions investigated by the authors. One should exclude, for example, that the strain originally carried a polar *argB* mutation; from such strains it is relatively easy to select derivatives in which the *argH* gene is duplicated and not submitted anymore to the controlling elements of the cluster; it is then expressed at a fixed rate, which may be high or low, depending on the particular strain investigated (3).

With this reservation in mind it is worth pointing out some undisputable differences between *E. coli* W and K-12, at least those K-12 strains we are more familiar with (Hfr P4X and related organisms).

(a) The amplitude of variation of enzyme *E* and *H* synthesis (but not of OTCase and acetyl-

ornithine transaminase) is considerably less in *coli* W than in K-12: 5 instead of 15 to 20 for enzyme E, and 25 instead of 50 to 70 for enzyme H. This is reminiscent of some partially constitutive operator mutants of the bipolar *argECBH* operon in *E. coli* K-12 (7, 16) and may result from a lower affinity of the internal operator region for the *arg* repressor in W than in K-12.

(b) Acetylarginine, even at 1,000 $\mu\text{g/ml}$, does not create conditions of complete repression in *E. coli* K-12, whereas 500 $\mu\text{g/ml}$ suffices in the case of W. This might reflect a less efficient uptake or utilization of acetylarginine by K-12 than W and explain in part the higher sensitivity of K-12 towards antagonism of acetylarginine utilization (see below).

(c) The *argB argG* K-12 strain is indeed much more sensitive to the antagonism exerted by acetylornithine on acetylarginine utilization than its W counterpart. In K-12 ornithine accumulation appears responsible for the largest part of this effect since exogenous ornithine is most effective in promoting growth inhibition. It is possible that the less considerable inhibition exerted by citrulline is in part due to conversion of some of the citrulline into ornithine by a backwards-working OTCase; however, as citrulline is inhibitory even when OTCase specific activity is low, it most probably exerts an effect of its own.

(d) The arginyl-transfer RNA synthetase of some *E. coli* strains is claimed to be about 50% inhibitable by moderate (10^{-6} M) concentrations of ornithine, citrulline, and argininosuccinate (24). In the K-12 strains which we use, however, it is clear that neither ornithine nor citrulline nor argininosuccinate exert a significant effect on the synthetase (Charlier, unpublished data). Direct inhibition of the latter enzyme by ornithine or citrulline is thus not responsible for the observed antagonism.

The bearing of the present observation on possible differences between the mechanisms of repression or arginine-biosynthetic enzymes in the two types of strains cannot yet be fully evaluated, if only because the nature of the corepressor of the *arg* regulon is still a matter of controversy (6, 15, 25). It is, however, clear that considerable care should be exercised before extrapolating the results obtained with one type of strain to the other one.

The target of the inhibition exerted in our strains by ornithine, citrulline, and possibly acetylornithine is at present unknown; it may be that acetylarginine penetration is affected, as in *E. coli* W (4); the presence of high amounts of ornithine could slow down the deacylation

reaction considerably; part of the effect of acetylornithine may be due to a direct competition between acetylornithine and acetylarginine for the active site of acetylornithinase.

(ii) Forward selection of *arg* auxotrophs.

We have seen that the particularly strong inhibition of acetylarginine utilization exerted by ornithine allows the forward selection of arginine auxotrophs (*argA*, *B*, *C*, and possibly *D*) from strains deficient in carbamoylphosphate biosynthesis and thus capable of ornithine accumulation. This technique may prove particularly useful for the genetic analysis of the bipolar *argECBH* operon. A method for the forward selection of *argE* mutants is also available (17).

An absolute requirement of the present technique is a functional *argE* gene, but if one uses a strain carrying a second exemplar of *argE* on an episome, it becomes possible to select mutations which would affect *argB* or *C* expression and at the same time exert an effect on *argE*. If the control region contains one or two overlapping operators flanked by two promoters, each one on the far side of the operator region with respect to the corresponding structural genes (9), one may indeed wonder if some types of polar insertions or deletions occurring between the two promoters could not be of that type. The *argH* gene may be affected, whether this occurs directly (by a deletion) or as the result of a promoter or polar mutation.

The technique is of potential use in other organisms where an antagonism between acetylarginine utilization and arginine precursors would come to be discovered.

Those acetylarginine- and uracil-utilizing strains which remain Arg^+ may provide useful medications on the nature of the inhibition exerted by ornithine. They are under current investigation.

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