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Studies on the Mechanism of Repression of Arginine Biosynthesis in *Escherichia coli*

III.† Repression of Enzymes of Arginine Biosynthesis in Arginyl-tRNA Synthetase Mutants

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Canavanine-resistant mutants of *Escherichia coli* K12 have been isolated which produce a defective arginyl-tRNA synthetase, the activity of the enzyme in various mutants ranging from about 3 to 50% of that in the wild type. On the basis of their growth rates in the presence and absence of arginine, the mutants can be put into two groups. Class I mutants (slow growth in the absence of arginine, normal growth in the presence of arginine) have an altered arginyl-tRNA synthetase with a decreased affinity for arginine. No marked changes were noticed in the enzyme obtained from class II mutants (slow growth in the absence or presence of arginine).

Repression by arginine of the enzymes of arginine synthesis, as measured by the rate of formation of ornithine transcarbamylase and argininosuccinase, was not impaired in either class. Levels of charged $tRNA^{Arg}$ in growing cells showed no correlation between the state of repression and the degree of charging. Thus for each strain tested there was no appreciable difference in the level of charging between repressed cells, growing with arginine, and de-repressed cells, growing without arginine. For class II mutants this level was about 20% of the total arginine acceptor capacity of tRNA, for class I mutants about 75%, and for the parent strains with a normal arginyl-tRNA synthetase, 80 to 90%. We conclude that the bulk of $tRNA^{Arg}$ is not involved in repression, but we have not ruled out participation of a small fraction of this tRNA.

1. Introduction

It has been established for most biosynthetic pathways of *Escherichia coli* that the formation of enzymes in a given pathway is controlled by end-product repression. For the biosynthesis of amino acids, evidence has recently become available which indicates that in some cases the substance responsible for repression of the biosynthetic enzymes is not the amino acid itself, but rather its aminoacyl-tRNA ester or a substance derived from it. These studies have involved the synthesis of histidine and valine and are based on the observation that interference with the conversion of the amino acid to its tRNA ester leads to de-repression. In the case of histidine,

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this phenomenon was first demonstrated by the use of an inhibitor of histidyl-tRNA synthetase (Schlesinger & Magasanik, 1964) and later in mutants with a defect in this enzyme (Roth, Anton & Hartman, 1966; Silbert, Fink & Ames, 1966). The evidence for a possible role of valyl-tRNA in repression of valine biosynthesis comes from studies on temperature-sensitive valyl-tRNA synthetase mutants (Eidlic & Neidhardt, 1965; Yaniv & Gros, 1967). However, there are other amino acids, such as tyrosine and phenylalanine, for which conversion to the aminoacyl-tRNA derivative does not appear to be necessary for repression of the enzymes of biosynthesis (Ravel, White & Shive, 1965; Neidhardt, 1966).

These studies have prompted us to look for mutants affected in arginyl-tRNA synthetase. Repression in the pathway of arginine biosynthesis has been investigated extensively in our laboratory (for review, see Maas, 1961,1965). The two previous papers in the present series (Maas, Maas, Wiame & Glansdorff, 1964; Maas & Clark, 1964) dealt with mutants of a regulatory gene *argR*. It was shown that the product of the wild-type allele *argR*$^+$, in conjunction with arginine or a derivative of arginine, is able to elicit repression of the eight enzymes involved in arginine formation. In *argR* mutants, in which formation of these enzymes is not repressible by arginine, no change in arginyl-tRNA synthetase could be detected (Boman, Boman & Maas, 1961).

The present paper describes the isolation and characterization of mutants that are defective in arginyl-tRNA synthetase and the effect of arginine on the formation of enzymes of arginine biosynthesis in these strains. In conjunction with these studies, the intracellular concentrations of "arginine-charged" tRNA (arginyl-tRNA^{Arg}) were measured in cells growing under repressed and de-repressed conditions.

2. Materials and Methods

(a) *Strains, media and conditions of growth*

The strains of *E. coli* K12 used in the experiments described in this paper are listed in Table 1.

TABLE 1

List of strains used

Strain	Genotype of arginine loci					Nutritional requirements				Response to		Mating type
	argA	argB	argG	argR	argS	arg	his	ilv	thi	can	str	
MA 5000	+	−	+	+	+	br	+	+	+	S	S	F$^+$
MA 5001	−	+	+	−	+	br	+	−	+	S	S	F$^+$
MA 5002	−	+	+	−	−	br	+	−	+	R	S	F$^+$
MA 5003 to MA 5009	+	−	+	+	−	br	+	+	+	R	S	F$^+$
MA 15	+	+	−	+	+	br	−	+	−	S	R	F$^-$
MA 18	+	+	−	+	−	br	+	+	−	R	R	F$^-$
MA 1010	+	+	−	+	+	−	+	+	−		S	Hfr

Abbreviations used: arg, arginine; his, histidine; ilv, isoleucine and valine; thi, thiamin; can, canavanine; str, streptomycin; br, bradytrophic; S, sensitive; R, resistant. The conventions suggested by Demerec, Adelberg, Clark & Hartman (1966) have been followed as closely as possible. Since no numbers have been assigned to the *arg* mutant alleles used, wild-type alleles are denoted by $^+$, mutant alleles by $^-$.

Cells were grown in an enriched arginine-free medium (AF) identical to that described by Novick & Maas (1961), except that histidine and lysine were omitted, since growth of the arginine bradytroph MA 5000 and MA 5001, from which the arginyl-tRNA synthetase mutants were derived, is inhibited by these two amino acids. This medium is referred to as HALF medium. For the growth of the histidine auxotroph MA 15 and its derivative, MA 18, histidine was added to HALF medium at 60 μg/ml. L-Arginine and L-canavanine (Mann Research Laboratories) were added to a final concentration of 100 μg/ml. unless specified. For solid media, Difco agar was added to HALF medium to a final concentration of 2%.

Liquid cultures were grown with aeration at 37°C, and unless stated otherwise the bacteria were harvested in the late exponential phase. Growth was measured by determining the optical density (O.D.) with a Lumetron colorimeter at 580 mμ.

(b) *Measurements of enzyme activities*

Ornithine transcarbamylase activity was determined by a variation of the method of Jones, Spector & Lipmann (1955), in suspensions of toluenized cells (Maas *et al.*, 1964). One unit of OTC† activity represents an amount of enzyme which catalyzes the production of 1 μmole of citrulline in 1 hr at 37°C under the standard assay conditions.

For the determination of argininosuccinase, the bacteria were harvested by centrifugation, washed once with 0·1 M-potassium phosphate buffer (pH 7·5) and resuspended in 7 ml. of the same buffer. The suspension was treated in a Branson sonifier, model 125, at setting 5 for 1 min. The resulting sonic extract was centrifuged at 15,000 *g* for 15 min and the pellet was discarded. For the assay, 0·65 ml. of the extract was incubated with 0·35 ml. of the reaction mixture containing the following ingredients: 5 μmoles of potassium argininosuccinate (Calbiochem); 50 μmoles of potassium phosphate (pH 7·5) and 0·3 mg arginase (Sigma Chemical Co.). After incubation at 37°C for 20 min, 2 ml. of 7·5% trichloroacetic acid was added in the cold, the mixture was centrifuged and the precipitate was discarded. 1 ml. of supernatant was used to determine urea by the method of Archibald (1945). A unit of enzyme activity is defined in the same way as for OTC.

Arginyl-tRNA synthetase activity was determined by measuring the incorporation of L-[14C]arginine (obtained from either New England Nuclear Corp. or from the International Chemical & Nuclear Corp.) into tRNA (General Biochemicals). To obtain extracts, the cells after harvesting by centrifugation were washed in a solution containing 0·01 M-Tris–HCl (pH 7·3), 0·01 M-$MgCl_2$ and 0·006 M-mercaptoethanol (TMM buffer). The washed cells were suspended in TMM buffer and disrupted by sonication as described above for argininosuccinase. After centrifugation, the supernatant fluid was dialyzed overnight in the cold against TMM buffer. For storage at −20°C, 0·25 vol. of glycerol were added to 0·75 vol. of extract (Kelmers, Novelli & Stulberg, 1965). The assay system contained, in a total volume of 0·1 ml.: 10 μmoles of Tris–maleate buffer (pH 7·0), 2·0 μmoles of phosphoenolpyruvate (tricyclohexylammonium salt), 0·1 μmole of ATP, 1·0 μmole of $MgCl_2$, 1·0 μmole of KCl, 0·4 μmole of glutathione, 40 μg of pyruvate kinase (Sigma Chemical Co.), 0·02 μmole of L-[14C]arginine (20 μc/μmole), 0·02 μmole of each of 19 [12C]amino acids, 1·0 mg of *E. coli* tRNA, and cell-free extract, containing 5 μg of protein. After incubation at 37°C for 10 min, 50 μl. were pipetted onto 3 MM Whatman filter paper discs, diameter 24 mm. The discs were plunged immediately into ice-cold 5% trichloroacetic acid containing 1% non-radioactive arginine. After a waiting period of 15 min, they were treated twice with fresh solution of the same composition, each time for 15 min, 3 times with 5% trichloroacetic acid for 10 min, and 3 times with 1 : 1 ethanol–ether for 10 min. Finally, they were washed twice with ether at room temperature, dried and placed into glass vials containing 15 ml. of 24 times diluted Liquifluor with final concentrations of 4 g PPO/l. and 50 mg POPOP/l. for counting in a Nuclear Chicago 720 scintillation counter. The efficiency of counting was 42%. One unit of enzyme activity represents the amount of enzyme which catalyzes the incorporation of 1 mμmole of arginine into tRNA in 10 min at 37°C.

† Abbreviation used: OTC, ornithine transcarbamylase.

(c) *Concentration of charged tRNA in growing cells*

To determine the fraction of $tRNA^{Arg}$ that is charged, 500-ml. cultures were grown in 2-l. Erlenmeyer flasks to a density of about 3×10^8 cells/ml. (O.D. of 0·15 to 0·20). RNA was extracted and treated according to the procedure of Yegian, Stent & Martin (1966) with the following modifications: (1) the periodate concentration was 2×10^{-3} M, and the RNA was exposed to periodate for 1 hr; (2) the RNA was "stripped" by incubation in 0·4 M-Tris–HCl (pH 7·4) for 2 hr at 37°C. (3) After the final ethanol precipitation, the RNA was dissolved in 0·1 M-Tris–HCl (pH 7·4) at a concentration of 5 to 15 mg/ml., determined spectrophotometrically assuming $O.D._{260}$ of 24 equivalent to 1 mg RNA/ml. To determine the acceptor capacity of $tRNA^{Arg}$, the method described above for determination of arginyl-tRNA synthetase activity was used, except that the concentration of tRNA was lowered to 0·2 mg/ml. and the incubation period was extended to 30 min to ensure complete charging of $tRNA^{Arg}$. The source of enzyme was a crude extract of a strain with a wild-type synthetase.

(d) *Protein determination*

This was carried out according to the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin as standard.

3. Results

(a) *Isolation and characterization of arginyl-tRNA synthetase mutants*

Mutants with a defective arginyl-tRNA synthetase can be isolated on the basis of resistance to the growth-inhibitory action of canavanine. This analog of arginine has been shown to prevent growth by inhibiting competitively the utilization of arginine in protein synthesis (Schwartz & Maas, 1960). In fact, canavanine is a substrate for arginyl-tRNA synthetase, forms an ester with tRNA (Mitra & Mehler, 1967) and is incorporated into protein (Schachtele & Rogers, 1965; Hirshfield, unpublished experiments). Canavanine competes poorly with arginine and this is reflected in the conditions that are necessary for inhibition of growth. With cells of *E. coli* K12 growing in minimal medium, only a slight inhibition of growth can be produced, and it is only under conditions in which the rate of arginine synthesis in the absence of canavanine becomes growth-limiting that sufficient inhibition can be achieved to permit the isolation of resistant mutants. With wild-type cells, such conditions are provided by using AF medium (Novick & Maas, 1961) for growth. Another way of achieving the same end result is to grow a bradytrophic mutant, with a partial block in arginine synthesis in the absence of arginine (Novick & Maas, 1961).

Arginyl-tRNA synthetase mutants are the usual kind that is obtained by canavanine selection from bradytrophic parent strains, whereas selection from wild-type cells on AF-canavanine medium results in the isolation of mostly non-repressible (*argR*) mutants (Maas, 1961). The reason for this difference seems to be that wild-type cells growing in AF medium are only partially de-repressed, so that further de-repression with a concomitant increase in the rate of arginine formation is possible; on the other hand, with most bradytrophs that we have studied, growth in the absence of arginine leads to complete de-repression, and the level of the arginine-forming enzymes becomes as high as that in *argR* mutants. Thus, in the latter case mutation to non-repressibility does not result in an increased rate of arginine production. That this interpretation is likely to be correct is shown by the finding that canavanine-resistant (*argR*) mutants isolated from a wild-type strain become again sensitive to canavanine when a second, bradytrophic mutation is introduced. From such a double mutant one can again isolate canavanine-resistant mutants, and these are found to be defective in arginyl-tRNA synthetase.

For the experiments described in the present paper, the parent strains used for the isolation of arginyl-tRNA synthetase mutants were two bradytrophs, MA 5000, a repressible (*argR*+) strain with a partial block in enzyme B, (conversion of *N*-acetylglutamate to *N*-acetylglutamyl phosphate) and MA 5001, a non-repressible (*argR*) strain with a partial block in enzyme A (conversion of glutamate to *N*-acetylglutamate). Spontaneously occurring canavanine-resistant mutants were selected on HALF-agar plates containing 100 μg of canavanine/ml. Of the colonies that grew we picked the smaller ones on the assumption that a strain with a reduced growth rate would have a good chance of being an arginyl-tRNA synthetase mutant. The strains that were picked were purified by re-streaking on the same medium and then grown in liquid cultures for the preparation of cell-free extracts. Assays for arginyl-tRNA synthetase activity were carried out on sonic extracts by measuring the incorporation of L-[^{14}C]arginine into tRNA. Using this procedure, about 60 arginyl-tRNA synthetase mutants were identified. They had activities ranging from 2·5 to 50% of that of the parental strains. Table 2 lists the mutants that have been studied extensively.

TABLE 2

Characteristics of arginyl-tRNA synthetase mutants

Strains		Doubling time = G (min) HALF	HALF + arg	% of parental arginyl-tRNA synthetase activity
Parent (P)	MA 5000	75	43	100
Parent (P)	MA 5001	125	52	100
Growth class I	MA 5003	145	45	14
Growth class I	MA 5004	143	47	14
Growth class I	MA 5009	128	49	10
Growth class II	MA 5002	190	145	2·5
Growth class II	MA 5005	150	76	5
Growth class II	MA 5006	165	75	8
Growth class II	MA 5007	150	88	11
Growth class II	MA 5008	158	75	11

All mutants (except mutant 5002 derived from MA 5001) were derived from MA 5000.

To demonstrate that the lower activities observed with the extracts from these mutant strains were not due to the presence of an inhibitor, mixtures of mutant and parental extracts were assayed. In all cases tested, the addition of the extract of a mutant strain had no effect on the activity of the extract of the parental strain.

The mutants could be grouped into two classes according to their growth rates. Class I mutants grow at a reduced rate in the absence of arginine, but nearly at the parental growth rate in the presence of arginine. Class II mutants grow at reduced rates both in the absence and in the presence of arginine. The two types of mutants occur at about equal frequencies.

In order to characterize the mutant strains further, various parameters of arginyl-tRNA synthetase were measured in crude extracts, such as the values of K_m for arginine, temperature and pH optima, and inhibition of enzyme activity by canavanine. The K_m for arginine of the wild-type synthetase was found to be $1{\cdot}5$ to $2{\cdot}0 \times 10^{-5}$ M. The enzymes extracted from two class I mutants, MA 5003 and MA 5004, had

K_m values of $2{\cdot}5 \times 10^{-4}$ M. Three class II mutants (MA 5005, MA 5007 and MA 5008) were found to have enzymes with K_m values very similar to that of the wild-type enzyme. The enzyme from a fourth class II mutant (MA 5002) was found to have a slightly higher K_m ($4{\cdot}0 \times 10^{-5}$ M). As might be expected from the similarity between the wild-type and class II mutant enzymes, canavanine inhibited their activities to the same degree. With a concentration of arginine of 2×10^{-4} M in the reaction mixture, 50% inhibition required a canavanine concentration of 2·55-mM. The enzyme extracts of class I mutants, on the other hand, required 7·75 mM canavanine for 50% inhibition under these conditions.

The pH optima for the incorporation of arginine into tRNA was 8·0 to 8·5 with the wild-type enzyme and all mutant enzymes except one (MA5007). With the enzyme from this strain, there was a broad plateau between pH 6·5 and 9·0, with a slight elevation at the lower end of this range.

The temperature optima for activity of the synthetases from the two classes of mutants differ from each other (Table 3). Enzyme activities of class II mutant

TABLE 3

Effect of temperature on arginyl-tRNA synthetase activity of wild-type and mutant strains

Strain	Class	Temperature of incubation 15°C	25°C	37°C	45°C
		Units/mg protein			
MA 5000	P	12·1	33·6	55·6	56·2
MA 5003	I	2·5	6·9	6·3	1·6
MA 5004	I	2·7	8·8	8·2	1·9
MA 5005	II	—	1·1	2·5	2·2
MA 5007	II	—	2·8	5·4	7·0

The arginyl-tRNA synthetase extracts of the various strains were incubated in the standard reaction mixture for 10 min at the indicated temperatures. The specific activity of L-[^{14}C]arginine was 20 μc/μmole and the arginine concentration 2×10^{-4}M.

extracts varied in the same way with temperatures as those of wild-type extracts, whereas enzymes extracted from class I mutants had lower optima for activity. A more detailed analysis of the characteristics of wild-type enzymes in purified preparations is now in progress and will be reported subsequently.

Class I and class II mutations have been mapped. They are located near each other (0·4 recombination unit apart) between *his* and *aroD* on the map of Taylor & Trotter (1967). We have assigned them tentatively to one locus, *argS*. Details of the mapping experiment will be published separately. It should be noted that *argS* is not near any of the other *arg* genes.

(b) *Repressibility of enzymes of arginine biosynthesis*

Data on the repressibility of OTC in representatives of the two classes of mutant and their corresponding parent strains are shown in Table 4. After growth in the presence of arginine, the formation of OTC is repressed as completely in the mutants as it is in the parent strains. Similar results were obtained with the formation of argininosuccinase (enzyme H) which was tested in wild-type and class II mutants.

TABLE 4

Repressibility of OTC formation in arginyl-tRNA synthetase mutants

Expt	Strain	G(min)	Additions to growth medium	OTC (units/mg protein)
A	MA 5000	75	none	286
	(parent)	52	orn	37
		43	arg	1·5
	MA 5007	150	none	280
	(class II)	133	orn	8·3
		88	arg	1·3
B	MA 5000	75	none	270
	(parent)	44	orn	61
		40	arg	2·6
	MA 5003	145	none	275
	(class I)	70	orn	37
		45	arg	1·3
C	MA 15	60	none	442
	(parent)	40	arg	2·6
	MA 18	165	none	26
	(class II)	140	arg	2·8

All procedures are described in Materials and Methods.
MA 18 is an *argR*⁺ recombinant carrying the *argS* mutation of MA 5002.

As this pattern was observed in all mutants listed in Table 2, we conclude from these experiments that the mutations in arginyl-tRNA synthetase do not impair repressibility by arginine of the arginine-biosynthetic enzymes.

This conclusion is supported further by the findings of enhanced repression in arginyl-tRNA synthetase mutants under certain conditions even in the absence of added arginine (Table 4). The requirement for this enhancement is that the block in arginyl-tRNA synthetase be drastically more limiting to growth than any other limitation of arginine synthesis, thus permitting accumulation of endogenously formed arginine. This condition is particularly evident in bradytrophs with early blocks (MA 5003, MA 5007) in the presence of ornithine and in a bradytroph with a very slight block in arginine synthesis (MA 18) even without any arginine precursors added. Under both of these conditions, there is much more repression in the mutant strain than in its parent strain (Table 4). It should be noted here that in order to explain canavanine resistance of class II mutants, in which the extracted arginyl-tRNA synthetase is as sensitive to canavanine inhibition as is the wild-type enzyme, it is necessary to assume greater accumulation of endogenously formed arginine than in their canavanine-sensitive parent strain.

(c) *Concentrations of arginyl tRNA^{Arg} in growing cells*

Because of the postulated role of aminoacyl-tRNA in repression, it seemed of importance to see whether or not, in spite of the low arginyl-tRNA synthetase activities in the mutants, the concentrations of arginyl-tRNA^{Arg} in growing cells are of similar magnitude in the mutants as in the wild-type strains. Charged tRNA^{Arg} could still be the co-repressor provided that the enzyme activity in the mutants was sufficient to effect the degree of charging required for repression. We therefore

measured the concentrations of arginyl-tRNA^{Arg} in the wild-type and mutant strains under conditions of repression and de-repression.

The method used was that described by Yegian *et al.* (1966) and consists of measuring the ratio of arginine acceptor activity of tRNA surviving periodate oxidation to the total arginine acceptor activity. These measurements determine the fraction of the total tRNA^{Arg} the terminal adenosyl 3^1 (or 2^1 —) hydroxyl of which is esterified. It should be noted that the esterification may not always be due to arginine. In fact, Yegian *et al.* (1966) found that a certain amount of tRNA was esterified by other constituents than the expected amino acid, including short peptides.

The results of determinations carried out on a strain of each mutant class and its wild-type parent strain are shown in Table 5. The tRNA was extracted from the

TABLE 5

Levels of arginine-charged tRNA in wild-type and mutant strains

Strain	Arginine during growth	G(min)†	Amino acid esterified	Acceptor capacity Total‡	Protected‡	P/T × 100
MA 15	−	30	arg	582	460	79
(patent)	+	27	arg	397	338	85
	−	30	leu	386	291	75
	+	27	leu	295	234	79
MA 18	−	90	arg	462	110	24
(class II)	+	78	arg	593	111	19
	−	90	leu	390	350	90
	+	78	leu	455	440	97
MA 5000	−	40	arg	539	488	90
(parent)	+	25	arg	595	476	80
MA 5003	−	90	arg	445	338	75
(class I)	+	25	arg	390	295	76

† In these experiments, because of improved aeration, growth was more rapid than in the other experiments described in this paper.

‡ Radioactivity (cts/min) per 0·2 mg RNA sample. Specific activities of [^{14}C]arginine and [^{14}C]leucine were 16,000 cts/min per mμmole.

cultures in the exponential phase of growth. The experiment was repeated at least once for each strain, and the results of duplicate experiments were generally in good agreement. Control experiments were carried out which showed that the periodate treatment after stripping resulted in almost complete removal of acceptor activity of charged tRNA. Furthermore, periodate treatment of completely charged tRNA^{Arg}, followed by stripping and recharging caused a reduction of about 20% in the subsequent acceptor activity of tRNA^{Arg}. This reduction is small compared to the differences between wild-type and mutant strains described in Table 5. These data show first of all that the total arginyl-tRNA acceptor activities per unit of total RNA are about the same for all strains. In both parent strains, the tRNA^{Arg} is charged almost completely, after growth with or without arginine. The class II mutant shows a low level of charging under both conditions of growth, whereas the class I mutant shows a high level of charging under both conditions. Other class I and class II

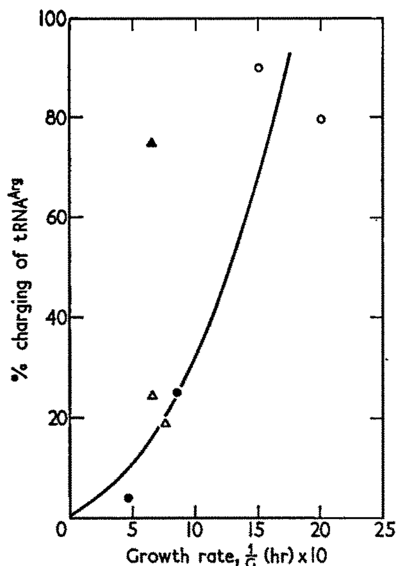

FIG. 1. Relationship between level of charged $tRNA^{Arg}$ and growth rate during arginine-limited growth.

(1) ●, *argS*⁺ auxotroph grown in chemostat (Novick & Szilard, 1950). (2) ○, *argS*⁺ bradytroph grown without exogenous arginine. (3) △, class II *argS* mutant, grown with or without exogenous arginine. (4) ▲, class I *argS* mutant grown without exogenous arginine.

Data for 2, 3 and 4 obtained from experiments described in Table 5.

mutants gave similar results to the one shown in Table 5. To be sure that the low level of charging in the class II mutant was not due to a general effect of the mutation on the charging of all tRNA species, the *in vivo* level of leucyl-$tRNA^{Leu}$ was measured and found to be as high as in the wild type (Table 5). These results show that the formation of the arginine-forming enzymes can be repressed fully under conditions in which only about 20% of the $tRNA^{Arg}$ is esterified.

An unexpected finding was the high level of charging in class I mutants grown in the absence of arginine. Under these conditions, these mutants grow at a reduced rate, being limited by the rate of arginine synthesis. One would expect that with different degrees of arginine limitation the concentration of arginyl-$tRNA^{Arg}$ would be a function of the growth rate. To test this expectation, we grew an arginine auxotroph (MA 1010) in a chemostat with arginine limiting the growth rate, and determined the levels of charged $tRNA^{Arg}$ at several growth rates. The results for various conditions of arginine-limited growth for the arginine auxotroph, arginine bradytrophs and *argS* mutants are shown in Figure 1. There is a clear interdependence between the growth rate and levels of charging for all strains except the class I mutant. Especially the class II mutant has a level of charged tRNA expected for its growth rate. So far the unusual behavior of class I mutants has remained a mystery.

4. Discussion

(a) *Involvement of $tRNA^{Arg}$ in repression of arginine synthesis*

In the arginyl-tRNA synthetase mutants described in this paper, formation of the enzymes of the arginine pathway is fully repressed during growth with arginine. In this respect the arginyl-tRNA synthetase mutants resemble phenylalanyl-tRNA

synthetase mutants and differ from valyl- and histidyl-tRNA synthetase mutants. To explore further a possible involvement of $tRNA^{Arg}$ in repression of arginine biosynthesis, we studied *in vivo* concentrations of charged $tRNA^{Arg}$. With a temperature-sensitive valyl-tRNA synthetase mutant, it was found that at elevated temperatures under conditions of de-repression the concentration of charged $tRNA^{Val}$ dropped (Bock, Faiman & Neidhardt, 1966), whereas with a temperature-sensitive phenylalanyl-tRNA synthetase mutant under similar conditions there was neither a de-repression nor a decrease in the level of charged $tRNA^{Phe}$ (Neidhardt, 1966). In our system we found no correlation between repression and level of charging. Thus with any of the strains tested, the level of charged $tRNA^{Arg}$ is about the same under conditions of repression and de-repression. In class II mutants this level is low (about 20%), in class I mutants it is higher (about 75%) and in strains with a normal arginyl-tRNA synthetase it is slightly higher (about 80 to 90%). The conclusion we draw from these findings is that the bulk of the $tRNA^{Arg}$ is not involved in repression; but we have not ruled out the possible participation of a small fraction of the total $tRNA^{Arg}$.

(b) *Nature of the mutational changes*

In class I mutants, arginyl-tRNA synthetase is altered as a result of the mutation. It differs from the corresponding wild-type enzyme in the K_m value for arginine, sensitivity to canavanine and temperature optimum for activity. The gene in which these mutations have occurred presumably codes for the structure of the enzyme molecule. In class II mutants, on the other hand, no clear-cut qualitative changes in the enzyme have been demonstrated which would permit one to say definitely that the enzyme is different from the wild-type enzyme. These mutations map in close proximity to class I mutations, but they may or may not have occurred in the same cistron. Furthermore, they may be located either in a structural gene or in a regulatory gene concerned with controlling the amount of enzyme produced. Provisionally we have assigned both types of mutants to one locus, *argS*.

(c) *Mechanism of canavanine resistance*

In non-repressible (*argR*) mutants, the resistance to inhibition of growth by canavanine was found to be due to an increase in the internal level of arginine as a result of de-repression of the arginine-forming enzymes (Maas, 1961). Such an increase could also result from a partial block in the further conversion of arginine, and this appears to occur in class II mutants and to be responsible for their canavanine resistance. As a result of the mutational block, class II mutants grow more slowly than their parent strains, either in the absence or in the presence of arginine. In extracts, the class II arginyl-tRNA synthetase is as sensitive to canavanine inhibition as the wild-type enzyme. Since arginine competitively reverses canavanine inhibition of the extracted enzyme, the most plausible mechanism for resistance to inhibition of growth by canavanine is therefore the above mentioned increase in the arginine pool in growing cells. In class I mutants, accumulation of arginine may also be partially responsible for resistance. However, in these strains the extracted enzyme was found to be less sensitive to canavanine than the wild-type enzyme, and this would provide an additional cause for resistance. One cannot say to what extent each of these factors, accumulation of internal arginine and decreased sensitivity of the enzyme, contributes to the over-all resistance to the growth-inhibitory action of canavanine.

(d) *Correlation between growth rate and level of charged tRNA^Arg^*

With strains having a normal arginyl-tRNA synthetase and with class II mutants, it was found that during arginine-limited growth the level of charged $tRNA^{Arg}$ is a function of the growth rate. This is what one would expect on the basis of current concepts of protein synthesis. In class I mutants, however, the level of charged $tRNA^{Arg}$ was higher than one would expect from their growth rate. This is similar to the situation in temperature-sensitive phenylalanyl-tRNA synthetase mutants described by Faiman & Neidhardt (1966). Here, at elevated temperatures growth slows down and finally comes to a halt, but the level of charged $tRNA^{Phe}$ remains high. So far no explanation is available for these unexpected findings. One operationally important consideration in evaluating these results is that the method used to measure the level of charging of tRNA shows that the tRNA is esterified, but does not specify the substance responsible for the esterification. It is conceivable that the mutation in these strains has relaxed the specificity of the aminoacyl-tRNA synthetase, so that some of the $tRNA^{Arg}$ is now charged by substances other than arginine.

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