

Suppression of Proline Requirement of *proA* and *proAB* Deletion Mutants in *Salmonella typhimurium* by Mutation to Arginine Requirement

TSENG-TONG KUO AND B. A. D. STOCKER

Department of Medical Microbiology, Stanford University, Stanford, California 94305

Received for publication 22 January 1969

Eleven variants able to grow without proline (provided arginine was absent) were obtained by spontaneous mutation from *Salmonella typhimurium* LT7 *proA* and *proAB* deletion mutants. Suppression resulted from mutation at *argG*, which specifies N^α -acetylornithine δ -transaminase. In the absence of exogenous arginine, deficiency of this enzyme would cause derepression of the arginine pathway and accumulation of *N*-acetylglutamic γ -semialdehyde. *N*-acetylglutamic γ -semialdehyde, if deacetylated, would produce glutamic γ -semialdehyde, the proline precursor whose synthesis from glutamate is blocked in *proA* and *proAB* mutants. All of the mutants grew only slowly (some very slowly) if not supplied with arginine. Sonic-treated preparations of eight mutants had no measurable acetylornithine δ -transaminase activity, but those of the three mutants least dependent on arginine had 0.11, 0.28, and 1.48 of wild-type activity; presumably, their enzymes have low specific activity, at least in vivo. Phage P22 cotransduced *argG* and *strA*. Genetic analysis showed that the minor degree of arginine dependence of the mutant with greater than wild-type in vitro enzyme activity was a characteristic of its *argG* allele, not the result of modification of the *argG* phenotype by mutation elsewhere.

Extended or multisite mutations in bacteria are believed to arise by deletion, and genetic information thus lost cannot be regained by further mutation at the affected region, nor by suppressor mutations elsewhere. We were therefore surprised to encounter nonrequiring variants of several proline-requiring *Salmonella typhimurium* mutants inferred from genetic analysis (6, 8) to be deleted for large parts of *proA* or of both *proA* and *proB*. These genes are involved in the conversion of glutamate to glutamic γ -semialdehyde, an intermediate which after spontaneous cyclization is converted to proline by the product of the *proC* gene (Fig. 1). The mutants' recovery of ability to grow without exogenous proline proved to result from the opening up of an alternative biosynthetic route to glutamic γ -semialdehyde by a mutation affecting one of the enzymes of the arginine biosynthetic route.

MATERIALS AND METHODS

Strains. The multisite deletion mutants used were *proAB47* and its derivative *proAB47 purE66*, *proAB126*, and *proA107*. Two *proC* strains, the multi-

site mutant *proC110* and the stable point mutant *proC90*, were also examined. All of these *pro* mutants stem from *S. typhimurium* strain LT7, four of them (*proAB126*, *proA107*, *proC90* and *proC110*) via an LT7 line given the *proA*⁺ *proB*⁺ region from strain LT2. For the origin and genetic analysis of these mutants, see references 6 and 8. Two LT7 *arg* mutants, *argG10* (2) and *argA162* (K. Sanderson, University of Calgary, unpublished data), were used in transduction experiments. Wild-type phage P22 was used for lysogenization experiments, and its nonlysogenizing derivative P22.L4 (11) was used for transduction.

Phage methods. Phages were propagated by the soft-agar layer method. In transduction experiments a multiplicity of 10 to 15 was used, and after 10 min the bacteria were washed and plated on selective media. In lysogenization experiments, clones isolated after exposure to wild-type P22 were tested for phage release and for sensitivity to phages P22.c2, ES18, and 9NA.P22. c2 and ES18 do not attack P22-lysogenic strains; 9NA, a smooth-specific phage, attacks even P22-lysogenic lines.

Enzyme assay. The N^α -acetylornithine δ -transaminase activity of broken-cell preparations was assayed by the method of Albrecht and Vogel (1). Cells were harvested after overnight incubation at 37 C on

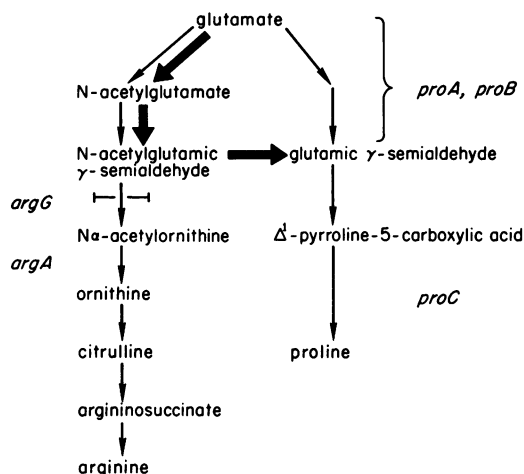


FIG. 1. Arginine and proline biosynthetic pathways. Gene symbols as used in *S. typhimurium* (9). The range lines (by *argG*) indicate the step blocked in suppressed *proAB* mutants by loss of acetylornithine δ -transaminase. The large arrows indicate the postulated bypass route to glutamic semialdehyde in suppressed *proAB* mutants.

solid defined medium supplemented with proline (50 $\mu\text{g/ml}$) and adenine (20 $\mu\text{g/ml}$), with glycerol (5 mg/ml) as carbon source. The protein concentration of sonic-treated preparations was determined by a spectrophotometric measurement of absorption at 280 nm, corrected for scattering by extrapolation from the 340- and 360-nm readings.

RESULTS

Colonies able to grow on defined medium lacking proline (though most of them only slowly) were repeatedly obtained from the three *proA* and *proAB* deletion mutants; the average number of colonies after 2 days at 36 C was about 1 per 10^8 cells inoculated. None was obtained from either of the stable *proC* strains. We investigated 11 proline-nonrequiring variants obtained as spontaneous mutants (Table 1). All of six independent derivatives of *proAB47 purE66* retained the parental purine auxotrophy. The deleted region in the three multisite *proA* or *proAB* mutants includes the normal integration site for prophage P22 (10). We tested *proAB47 purE66* and a proline-nonrequiring variant of it for susceptibility to lysogenization by P22; as additional controls, two nonlysogenic transductants, one *pro*⁺ and the other *pur*⁺, obtained from it by transduction with P22.L4, were also tested. All of the tested clones from the P22-treated cultures of the parent strain, its *pur*⁺ transductant, and its proline-nonrequiring variant were nonlysogenic, whereas most of those from the phage-exposed

culture of the *pro*⁺ transductant were P22-lysogenic. Thus, the P22 integration site is still deficient even when the proline requirement caused by the deletion mutation is no longer apparent. The nonrequiring variants are therefore neither contaminants nor wild type for the *pro* region, and their ability to grow without proline must result from suppressor mutation.

All 11 variants, when growing on defined medium without proline, caused syntrophic growth of neighboring *proAB* bacteria (blocked between glutamic acid and glutamic γ -semialdehyde), but not of *proC* bacteria (blocked between Δ^1 -pyrroline-5-carboxylic acid and proline). This suggested that they excreted either glutamic semialdehyde or some substance which could be converted to it. Crystals of arginine placed on surface-inoculated plates of defined medium without proline caused wide zones (diameter, 12 to 20 mm) of inhibition of the growth of all 11 variants; none other of the 20 common amino acids produced this effect. The presence of proline (20 $\mu\text{g/ml}$) in the defined medium completely prevented this inhibition. The "arginine sensitivity" of the mutants suggested that their ability to grow without exogenous proline might result from an aberration of arginine biosynthesis. The addition of arginine, or of one of its three immediate precursors, citrulline, ornithine, and *N* ^{α} -acetylornithine, to proline-supplemented defined medium caused increased growth of all the variants; the earlier intermediates, *N*-acetylglutamate and glutamate, did not have this effect. Citrulline, ornithine and acetylornithine, but not acetylglutamate or glutamate, inhibited the growth of the mutants in the absence of proline but not in its presence—that is, arginine and its late precursors improved growth in the presence of proline but prevented growth in its absence. The growth responses suggested that the mutants had defects either in the conversion of *N*-acetylglutamate to *N*-acetylglutamic γ -semialdehyde or of the latter to acetylornithine (Fig. 1). If the defect was in the latter conversion, which is catalyzed by the enzyme acetylornithine δ -transaminase (α -*N*-acetyl-L-ornithine:2-oxoglutarate amino-transferase, EC 2.6.1.11), growth in the absence of exogenous arginine would cause derepression of the arginine biosynthetic pathway and accumulation of *N*-acetylglutamic semialdehyde. Deacetylation of *N*-acetylglutamic semialdehyde would produce glutamic semialdehyde and thus bypass the blocked step in the proline pathway. The provision of an exogenous supply of arginine, or of a precursor which could be converted to arginine, would be expected to prevent the accumulation of acetylglutamic semialdehyde by causing repression of the arginine biosynthetic pathway (4, 7,

TABLE 1. Properties of *proA* and *proAB* suppressor mutants

Strain no.	Mutation	Parent	Arginine dependence ^a		Transaminase assay ^a	Cotransduction rate ^d
			Colony size ^b	Doubling time ^c (min)		
SL3715	<i>argG454</i>	<i>proAB47</i>	+		None	0.36/0.15
SL3716	<i>argG459</i>	<i>proAB47</i>	+		None	0.50/0.22
SL3709	<i>argG455</i>	<i>proAB47 purE66</i>	+	390	None	0.12/0.10
SL3710	<i>argG456</i>	<i>proAB47 purE66</i>	++++	110	0.59 (= 148%)	0.14/0.15
SL3711	<i>argG457</i>	<i>proAB47 purE66</i>	+		None	0.21/0.08
SL3714	<i>argG458</i>	<i>proAB47 purE66</i>	+		None	
SL3791	<i>argG461</i>	<i>proAB47 purE66</i>	+		None	0.21/0.13
SL3792	<i>argG462</i>	<i>proAB47 purE66</i>	++		None	0.30/0.10
SL3717	<i>argG460</i>	<i>proAB126</i>	+++		0.06 (= 11%)	0.19/0.10
SL3841	<i>argG463</i>	<i>proA107</i>	++++	130	0.15 (= 28%)	0.33/0.18
SL3842	<i>argG464</i>	<i>proA107</i>	++	348	None	0.37/0.15

^a N^α-acetylornithine δ-transaminase activity of sonic extracts, in units/mg of protein (and as percentage of activity of extract of *arg*⁺ parent). Extracts of parent strains, *proAB47*, *proAB47 purE66*, *proAB126*, and *proA107*, had activities of, respectively, 0.43, 0.41, 0.54, and 0.53 units/mg of protein.

^b Approximate diameter of colonies on defined medium, with or without proline, after 2 days at 37 C: + = 0.2 mm or less; ++++ = 1 to 2 mm.

^c Doubling time in defined medium supplemented with proline and other nutrients except arginine; doubling time for *arg*⁺ parent strain was 42 min.

^d Proportion of *arg*⁺ transductants with donor *str* allele/proportion of *str-r* transductants with donor *arg*⁺ allele (among ca. 60 transductant clones tested, in most cases).

14). End-product inhibition of the first enzyme of the pathway might also result (16). Failure to accumulate acetylglutamic semialdehyde would account for the inhibition of growth on medium lacking proline caused by arginine, citrulline, ornithine, and acetylornithine. We inferred that the suppression of the proline requirement of the *proA* or *proB* mutants resulted from mutational loss of acetylornithine δ-transaminase, and we assigned *arg* allele numbers to the suppressed *pro* mutants (Table 1).

Extracts of all 11 proline-independent mutants and their proline-requiring parent strains were tested for acetylornithine δ-transaminase activity (Table 1). The activities for the four parent strains ranged from 0.41 to 0.53 units per mg of protein. Eight mutants gave extracts which were inactive or had activities too low to be detected. Three mutants, *argG460*, *argG463*, and *argG456*, had activities of, respectively, 0.11, 0.28, and 1.48 of the activities of their *arg*⁺ parent strains. The mutants differed in their degree of arginine dependence as judged by their growth on solid defined medium (Table 1); in the absence of arginine, the presence or absence of proline had no obvious effect on the amount or rate of growth. The three mutants with measurable in vitro enzyme activity were least dependent on arginine, as judged from size of colony, etc., on solid defined medium. To obtain a more precise measurement of the degree of arginine dependence, doubling times were

measured for two suppressor mutants which grew poorly and for two which grew well without arginine, and for an *arg*⁺ parent strain. The growth of shaken flask cultures was followed by absorption measurements; the defined medium used was supplemented with proline (20 μg/ml) and adenine (10 μg/ml), and with Difco arginine assay medium (10 mg/ml) as a convenient source of growth-accelerating nutrients other than arginine. The two mutants without measurable enzyme activity had doubling times of about 6 hr, compared with about 42 min for the *arg*⁺ parent. Mutants *argG463* and *argG456*, with, respectively, 0.28 and 1.48 of normal enzymatic activity, both had doubling times of about 2 hr.

The locus for acetylornithine δ-transaminase is termed *argD* in *Escherichia coli* (12) and *argG* in *S. typhimurium* (9). Demerec and his colleagues encountered only a single mutant of this class, *argG10*, in *S. typhimurium* (2). Phage P22.L4 grown on the *argG455* suppressed derivative of *proAB47* failed to evoke any arginine-independent transductants from strain LT7 *argG10*, whereas phage grown on the unsuppressed *proAB* parent evoked many. By contrast, both lysates evoked *arg*⁺ transductants from LT7 *argA162*, blocked in the deacetylation of acetylornithine by mutation at a locus remote from *argG* (2, 9). This result would be expected if suppression of *proAB47* resulted from mutation at *argG*.

Mutant *argG10* became at one step both argi-

nine-exacting and (somewhat) resistant to streptomycin, and all *arg*⁺ transductants obtained from it by treatment with phage grown on *S. typhimurium* wild type were streptomycin-sensitive (2). We therefore tested 10 of our suppressed mutants for linkage of their suppressor genes, inferred to be *argG*⁻, with *strA210*, which determines resistance to even high concentrations of streptomycin. Phage P22.L4 grown on an LT2 *arg*⁺ *strA210* donor was mixed with each suppressed mutant. Selection was made for *arg*⁺ transductants and for *str-r* transductants, by plating on Nutrient Agar with streptomycin (1 mg/ml) after a delay to permit phenotypic expression. The *arg*⁺ transductants are recognizable as rapid-growing colonies on a background of slow-growing recipient on defined medium supplemented with proline. Transductants selected as *arg*⁺ were tested for streptomycin sensitivity. Those selected as *str-r* were tested for *argG* character, both by comparing their growth on proline-supplemented medium with or without arginine, and by testing for ability to grow on defined medium lacking both proline and arginine, i.e., for suppression of *pro*. The frequency of cotransduction of *argG* with *str-r* ranged from 0.08 to 0.22, and that of *str-r* with *arg*⁺, from 0.12 to 0.50 (Table 1). Thus, the *arg* sites which suppress *proA* or *proAB* are closely linked to *strA*. However, they are separable from it, in distinction from the situation reported (2) for *argG10*, which may have arisen by a multi-site mutation affecting both *argG* and *strA*. Cotransduction was also demonstrated with *argG* and *strA* in linkage. Two suppressed *pro* strains were given *strA210*, but not *arg*⁺, by transduction. When these *pro argG strA* derivatives were treated with P22.L4 grown on a wild-type strain (*arg*⁺ *strA*⁺), many (27 to 58%) transductants selected as *arg*⁺ were found to be streptomycin-sensitive, in consequence of cotransduction of *strA*⁺ with *argG*⁺. Similarly, some of the streptomycin-resistant transductants obtained from an *arg*⁺ *strA*⁺ strain by treatment with phage grown on a *pro argG strA* were *argG*⁻.

We considered the possibility that the suppressor mutants which could grow rapidly on solid defined medium without arginine owed this ability to modification of the *argG*⁻ phenotype by a second mutation, at some other locus, for instance, a mutation altering the specificity of another transaminase. Two experiments tested this possibility in the case of the least arginine-dependent of the suppressor mutants, SL3710 (= *proAB47 purE66 argG456*). This strain was first made *arg*⁺, and therefore proline-dependent, by the cotransduction of *argG*⁺ with *strA210*; new proline-nonrequiring (suppressor) mutants were then selected.

All of four such mutants were much more dependent on arginine than was the ancestral suppressor mutant carrying *argG456*, even though all would still have possessed the hypothetical modifier if it mapped elsewhere than the *argG-strA* region. In a second experiment, strain SL3710 was given *strA210*, but not *argG*⁺, by transduction, and then was used as transductional donor of *strA210* to *proAB47 argG*⁺. Those streptomycin-resistant transductants which became proline-nonrequiring by acquisition of the donor *argG456* allele resembled the donor strain by their ability to grow rapidly on defined agar without arginine. Both tests, therefore, indicate that the relatively minor degree of the arginine dependence of the suppressor mutant carrying *argG456* is determined at *argG*, not by a modifier at some other locus.

DISCUSSION

Our observations indicate that suppression of the proline requirement of *S. typhimurium proA* or *proAB* deletion mutants results from mutation at *argG*. This mutation causes loss or alteration of the arginine biosynthetic enzyme acetylornithine δ -transaminase, and therefore accumulation of *N*-acetylglutamic γ -semialdehyde, deacetylation of which produces the proline precursor whose normal synthesis is blocked. This mechanism of suppression, first proposed by D. F. Bacon and H. J. Vogel (Federation Proc. 22, 476, 1963), has recently been demonstrated in both *E. coli* strain W and *S. typhimurium* strain LT2 by Itikawa, Baumberg, and Vogel (5). They reported that suppressed mutants usually have no detectable acetylornithine δ -transaminase activity and suggested that (slow) growth of these mutants in the absence of arginine probably results from nonspecific activity of some other transaminase. Eight of the suppressor mutants which we tested had no measurable *in vitro* activity, but the other three had activities of 0.11, 0.28, and 1.48 the activity of their *arg*⁺ parents. These three mutants with measurable enzyme activity were the mutants least dependent on arginine, as judged by growth on solid defined medium; but, even in the two mutants with highest enzyme activities, the presence of the *argG* mutation caused a threefold increase in the doubling time in arginine-deficient supplemented defined medium. The two mutants with measurable though reduced *in vitro* activities presumably carry leaky *argG* alleles, coding for enzymes of low specific activity. Even a partial enzyme defect would presumably cause accumulation of the last intermediate before the incomplete block, which would account for the suppression of *proAB*. The arginine pathway of a

leaky mutant would be completely derepressed by growth without arginine, whereas that of an *arg*⁺ strain is partly repressed by endogenous arginine; the specific activity of these mutant enzymes, relative to wild-type enzyme, is therefore presumably less than the in vitro activities found for these two mutants, i.e., 0.11 and 0.28 of those of the *arg*⁺ control.

We were surprised to find that the sonic extract of one suppressed mutant, carrying *argG456*, had greater than wild-type activity in the in vitro enzyme assay. Genetic tests showed that the relatively minor degree of arginine dependence of this mutant was determined at *argG*, not by a modifying gene elsewhere. The suppression of *proAB* by *argG456* shows that the enzyme which it specifies is relatively inefficient as a catalyst of the conversion of acetylglutamic semialdehyde to acetylornithine in vivo. A mutation which reduced the affinity of the enzyme for acetylglutamic semialdehyde would have low in vivo activity (at least until the internal concentration of acetylglutamic semialdehyde reached a high level), but it might not affect activity in the in vitro assay, which measures the effect of the enzyme on the rate of the reverse reaction.

Itikawa and his colleagues (5) demonstrated by enzyme assay the repression of the arginine biosynthetic enzymes by exogenous arginine, which we assume accounts for the growth-inhibiting effect of arginine in the absence of proline. They also showed that mutation to "arginine resistance" (a phenomenon we observed but did not investigate) results from mutation at the arginine regulatory locus, making the arginine pathway constitutive. They also cited evidence that the deacetylation of acetylglutamic semialdehyde results from a slight nonspecific activity of another enzyme of the arginine pathway, acetylornithinase.

The suppression of proline auxotrophy of *proA* or *proAB* by loss of acetylornithine δ -transaminase is an example of what has been termed indirect suppression (3), since the ability to grow without proline results not from partial or complete repair of the damaged *pro* region but from mutation elsewhere which circumvents the effect of the genetic defect. The opening up of the alternative route from glutamic acid to glutamic semialdehyde is possible in *Salmonella* and *Escherichia* because, in bacteria, though not in higher organisms (13), there are separate routes from glutamate to proline and to arginine, the first steps being similar except that in the arginine pathway the intermediates are acetylated. The paradoxical ability of arginine to inhibit the growth of the suppressed *pro* mutants, even though these are

arginine auxotrophs, is a consequence of the substitute route to the proline precursor remaining under the control of the repressor mechanism of the arginine pathway.

The suppression of *proAB* provides a convenient method for the isolation of acetylornithine δ -transaminase mutants in *S. typhimurium*. Demerec and his colleagues (2) found only one such mutant among 39 arginine auxotrophs tested. Perhaps all such mutants resemble those isolated as suppressors of *pro*, and grow fairly well without exogenous arginine, possibly because the function of the missing enzyme is sufficiently effected by other transaminases. Mutants with such a "leaky" phenotype might well be overlooked or discarded, which would account for their apparent rarity. (We found that the single *argG* mutant recognized by Demerec was aberrant in that it grows only slowly, even on arginine-supplemented medium or nutrient agar. Perhaps it is a multisite mutant.)

The cotransducibility of the *argG* locus with that determining streptomycin resistance provides a tool for the marking of the chromosome of any *arg*⁺ *str*⁺ (wild-type) strain with *argG* and *strA*. This can be done by selection of streptomycin-resistant transductants after treatment with phage P22 grown on a streptomycin-resistant suppressed *pro* (i.e., *pro argG strA*) donor. Of the two marker alleles thus introduced, *strA* is convenient as a selective character in conjugational crosses, and *argG*, despite its leakiness, can be successfully used as a contraselective marker.

ACKNOWLEDGMENTS

Tseng-tong Kuo was supported by a predoctoral traineeship from the National Institutes of Health. The work was supported by Public Health Service research grant AI-07168 and Public Health Service Training Grant AI-82 from the National Institute of Allergy and Infectious Diseases.

We thank K. E. Sanderson, H. O. Smith, and P. E. Hartman for provision of bacterial strains and phages, and H. J. Vogel, H. Itikawa, and S. Baumberg for communication of their unpublished results.

LITERATURE CITED

1. Albrecht, A. M., and H. J. Vogel. 1964. Acetylornithine δ -transaminase. *J. Biol. Chem.* **239**:1872-1876.
2. Demerec, M., E. L. Lahr, E. Balbinder, T. Miyake, J. Ishidzu, K. Mizobuchi, and B. Mahler. 1960. Bacterial genetics. Carnegie Inst. Wash. Yearbook **59**:438-440.
3. Gorini, L., and J. R. Beckwith. 1966. Suppression. *Ann. Rev. Microbiol.* **20**:401-422.
4. Gorini, L., W. Gundersen, and M. Burger. 1961. Genetics of regulation of enzyme synthesis in the arginine biosynthetic pathway of *Escherichia coli*. Cold Spring Harbor Symp. Quant. Biol. **26**:173-182.
5. Itikawa, H., S. Baumberg, and H. J. Vogel. 1968. Enzymic basis for a genetic suppression: accumulation and deacetylation of N-acetylglutamic γ -semialdehyde in enterobacterial mutants. *Biochim. Biophys. Acta* **159**:547-550.

6. Itikawa, H., and M. Demerec. 1968. *Salmonella typhimurium* proline mutants. *J. Bacteriol.* **95**:1189-1190.
7. Maas, W. K. 1961. Studies on repression of arginine biosynthesis in *Escherichia coli*. Cold Spring Harbor Symp. Quant. Biol. **26**:183-191.
8. Miyake, T., and M. Demerec. 1960. Proline mutants of *Salmonella typhimurium*. *Genetics* **45**:755-762.
9. Sanderson, K. E. 1967. Revised linkage map of *Salmonella typhimurium*. *Bacteriol. Rev.* **31**:354-372.
10. Smith, H. O., and M. Levine. 1965. Gene order in prophage P22. *Virology* **27**:229-231.
11. Smith, H. O., and M. Levine. 1967. A phage P22 gene controlling integration of prophage. *Virology* **31**:207-216.
12. Taylor, A. L., and C. D. Trotter. 1967. Revised linkage map of *Escherichia coli*. *Bacteriol. Rev.* **31**:332-353.
13. Umbarger, E., and B. D. Davis. 1962. Pathways of amino acid synthesis, p. 186. In I. C. Gunsalus and R. Y. Stanier (ed.), *The bacteria*, vol. 3. Academic Press Inc., New York.
14. Vogel, H. J. 1961. Aspects of repression in the regulation of enzyme synthesis: pathway-wide control and enzyme-specific response. Cold Spring Harbor Symp. Quant. Biol. **26**:163-172.
15. Vogel, H. J., D. F. Bacon, and A. Baich. 1963. Induction of acetylornithine δ -transaminase during pathway-wide repression, p. 293-300. In H. J. Vogel, V. Bryson, and J. O. Lampen (ed.), *Informational macromolecules*. Academic Press Inc., New York.
16. Vyas, S., and W. K. Maas. 1963. Feedback inhibition of acetylglutamate synthetase by arginine in *Escherichia coli*. *Arch. Biochem. Biophys.* **100**:542-546.