71-12,620

STROMAN, David Womack, 1944-

EXPRESSION OF THE CLUSTERED ARGININE GENES IN ESCHERICHIA COLI.

The University of Oklahoma, Ph.D., 1970
Biochemistry

THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

EXPRESSION OF THE CLUSTERED ARGININE GENES IN *ESCHERICHIA COLI*

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

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Oklahoma City, Oklahoma

1970

EXPRESSION OF THE CLUSTERED ARGININE

GENES IN ESCHERICHIA COLI

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ACKNOWLEDGMENTS

I wish to express my appreciation to Dr. Leon Unger for his professional guidance, criticism, encouragement and friendship during the course of this study.

My thanks also go to my fellow graduate students, Edward Schneider, David Cheung, and Ron Martin and to medical students, John Tipton and Gaylen Toews for many hours of valuable discussions concerning this project. A special word of thanks goes to Lynn DeOgny for her excellent technical assistance.

Numerous faculty members of the Department of Biochemistry and Molecular Biology have generously given of their time and ideas during the past four years. My gratitude is extended to the members of my reading committee for their help in preparing this dissertation.

A particular note of gratitude is due the Oklahoma Medical Research Foundation, without whose support and facilities this project would not have been accomplished.

I also thank my wife, Janice, for making these years of graduate study possible and enjoyable.

My graduate study has been financed by a grant to Dr. Leon Unger (AI08175 from the National Institutes of Health) and by a Glenn Rapp Foundation Scholarship.

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LIST OF ABBREVIATIONS

Gene Symbol	Phenotypic Trait Affected
argA	N-acetylglutamate synthetase
argB	N-acetyl-γ-glutamokinase
argC	N-acetylglutamic-γ-semialdehyde dehydrogenase
argD	Acetylornithine δ-transaminase
argE	Acetylornithinase
argF and arg I	Ornithine transcarbamylase
argG	Argininosuccinate synthetase
argH	Argininosuccinase
argM	"Inducible" acetylornithine δ-transaminase
argP	Arginine permease
argR	Arginine regulatory gene
argS	Arginyl-tRNA synthetase
aceA and aceB	Acetate utilization
adeK	Adenine requirement
aroE	Dehydroshikimate reductase
cya	Adenyl cyclase
cyc	Resistance or sensitivity to D-cycloserine
glpK	Glycerol kinase
his	Requirement for histidine
ilv	Isoleucine-valine biosynthesis
lac	Lactose fermentation
leu	Requirement for leucine
lysA	Diaminopimelic acid decarboxylase
metA	Homoserine O-transsuccinylase
metB	Cystathionine synthetase
metC	Cystathionase
metF	N^5, N^{10}-methyltetrahydrofolate reductase
pgi	Phosphoglucoisomerase
ppc	Phosphoenolpyruvate carboxylase
proA and proB	Block prior to L-glutamate semialdehyde
purD	Phosphoribosylglycineamide synthetase
purH	Phosphoribosyl-aminoimidazole-carboxamide formyltransferase
rad	Resistance or sensitivity to x-rays

LIST OF ABBREVIATIONS continued

Gene Symbol	Phenotypic Trait Affected
rhaA	L-Rhamnose isomerase
rhaB	L-Rhamnulokinase
rhaC	Rhamnose regulatory gene
rhaD	L-Rhamnulose-1-phosphate aldolase
rna	RNA polymerase
rts	Electrophoretic mobility of 50S ribosomal subunit
serA	3-Phosphoglyceric acid dehydrogenase
strA	Resistance, dependence, or sensitivity to streptomycin
supM	Suppression of ochre mutations
thi	Thiamine requirement
trp	Tryptophan biosynthesis

EC	Enzyme Commission
g	gram
l	liter
mg	milligram
μg	microgram
ml	milliliter
mμ	millimicrons
μmoles	micromoles
OTC	ornithine transcarbamylase
Tris	tris (hydroxymethyl) aminomethane

EXPRESSION OF THE CLUSTERED ARGININE GENES IN *ESCHERICHIA COLI*

CHAPTER I

INTRODUCTION

Arginine Pathway

In *Escherichia coli*, the synthesis of arginine from glutamate is mediated by eight biosynthetic enzymes (Fig. 1). The first half of the pathway proceeds via a series of acetylated intermediates and is unique to bacteria; however, the conversion of ornithine to arginine is universal in nature. The acetylation of glutamate functionally separates the arginine pathway from the proline pathway (Fig. 2).

The first enzyme in the pathway is N-acetylglutamate synthetase (EC 2.3.1.1), specified by gene *argA*, which converts L-glutamate to N-acetyl-L-glutamate. The initial evidence for the existence of this enzyme came in 1953 (86, 132). Vogel showed that N-acetylglutamic-γ -semialdehyde, a subsequent intermediate, could not arise from glutamic-γ -semialdehyde (the first intermediate in the proline biosynthetic pathway) because N-acetylornithine was excreted into the growth medium by a mutant blocked in both enzyme 5 of the arginine pathway and enzyme 1 of the proline pathway (132, see Fig. 2). Maas, *et al*. (86)

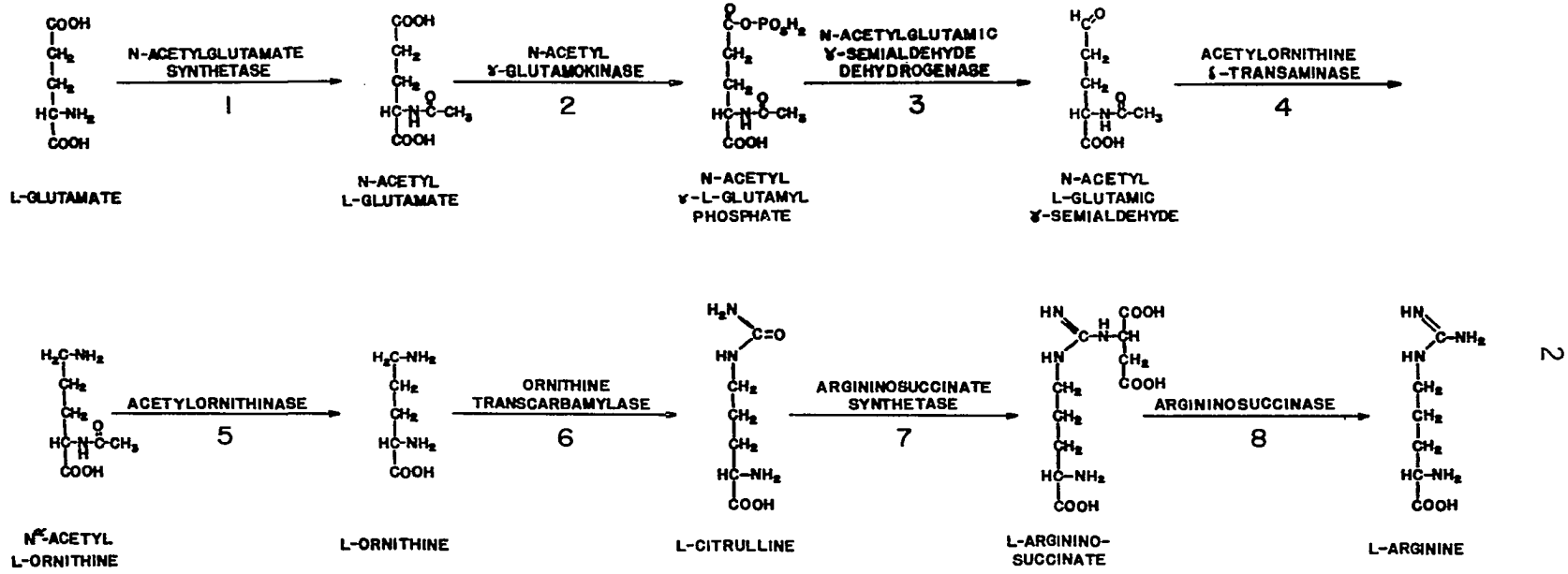


Figure 1. Arginine pathway in Escherichia coli.

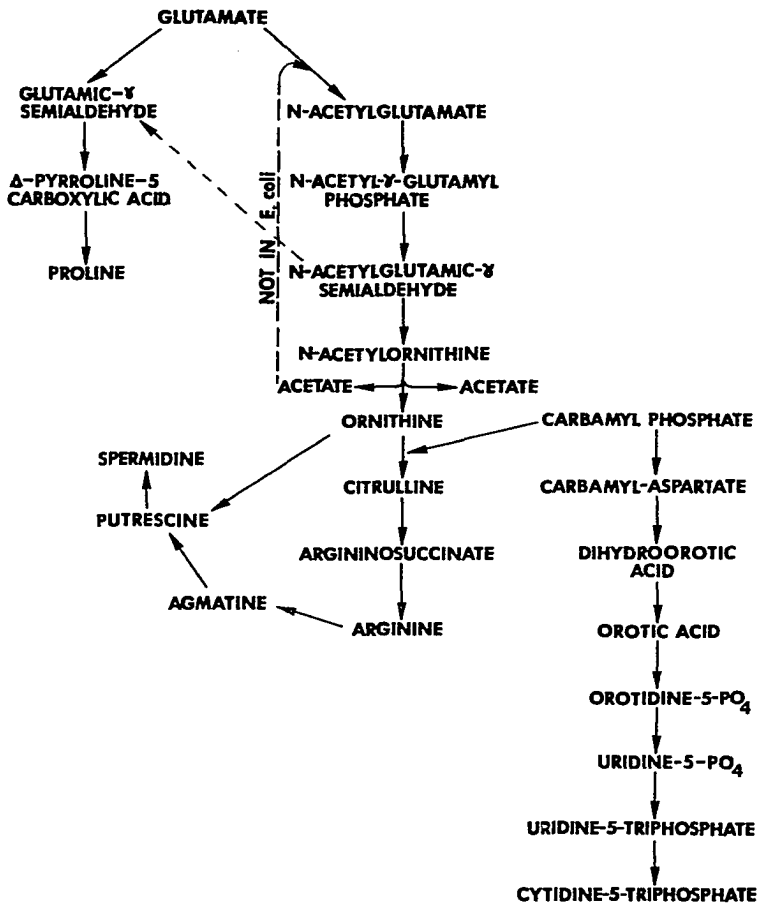


Figure 2. Interrelationships for arginine pathway with other metabolic pathways.

reported the acetylation of glutamic acid by a cell-free extract of E. coli, W. However, to date it has not been possible to repeat this work (143). The assay consisted of Tris buffer, pH 8.0; $MgCl_2$; CoA; H_2S; acetylphosphate; and cell-free extract. Acetylglutamate was detected as the acethydroxamate derivative by the hydroxylamine method (72). Under these conditions, more aspartic acid was acetylated than glutamic acid. In 1963, Vyas and Maas (145) demonstrated by an in vivo method the acetylation of glutamic acid. This assay consisted of glutamate; phosphate buffer, pH 7.5; glucose; and resting cells of E. coli, K12. To obtain resting cells, cells (an argB or argC and an argA which was permeable to acetylglutamate) were suspended in minimal medium minus a nitrogen source. After a ninety minute incubation at $37^{\circ}C$, the reaction was stopped with HCl, centrifuged, the pH of the supernatant fluid adjusted to 7.0, and the amount of acetylglutamate determined by bioassay. The activity could not be demonstrated on a cell-free extract. Since the enzyme is assumed to require acetyl-CoA as the acetyl donor, none of the other known acetyl donors have been investigated. Other information (121) would indicate that, at least in E. coli, W, N-acetylglutamate synthetase is temperature-sensitive since at certain temperatures the wild type becomes auxotrophic for arginine (or any of its intermediates including N-acetylglutamate).

N-acetyl-γ-glutamokinase, the second enzyme in the pathway, is specified by argB and converts N-acetylglutamate to N-acetyl-γ-glutamyl phosphate. The reaction is dependent upon ATP and Mg^{+2} and has a pH optimum from 6.7 to 7.4 (9).

N-acetylglutamic-γ-semialdehyde dehydrogenase, encoded in argC,

catalyzes a NADPH-dependent formation of N-acetylglutamic-γ-semialdehyde from N-acetyl-γ-glutamyl phosphate with a pH optimum of 9.3 (9). Enzymes 2 (109) and 3 (87) have both been purified although the results have yet to be published. Prior to 1962, the formation of N-acetylglutamic-δ-semialdehyde from N-acetylglutamate was presumed to be catalyzed by a single enzyme. With the demonstration of two separate enzymes, the conversion of acetylglutamate to its semialdehyde becomes analogous to the conversion of aspartate to its corresponding semialdehyde.

Enzyme 4, acetylornithine δ-transaminase (EC 2.6.1.11), specified by argD, converts N-acetylglutamic-γ-semialdehyde to N-acetylornithine with glutamate serving as the amino donor. The enzyme was partially purified (3), and shown to be dependent on glutamate and pyridoxal-5-phosphate for maximal activity, has a pH optimum of 8.1 and yields as a by-product, α-ketoglutarate.

Acetylornithinase, the fifth enzyme of the arginine pathway, encoded in argE, catalyzes the hydrolytic cleavage of acetylornithine to ornithine, and was partially purified in 1956 (142). Its activity is dependent upon the cobaltous ion and glutathione, has a pH optimum of 7.0 and produces one mole of acetate for each mole of acetylornithine cleaved.

It has been shown by Itikawa, et al. (64) that if N-acetylglutamic-γ-semialdehyde accumulates due to a block in argD, acetylornithinase will deacylate it to form glutamic-γ-semialdehyde. If a strain contains a block in the proline pathway before glutamic-γ-semialdehyde, then an argD mutation will phenotypically "revert" the proline mutation. If this double mutant is grown in the presence of arginine, however, it again requires proline since acetylornithinase

activity is repressed to a level so low that not enough acetylglutamic- γ-semi-aldehyde is deacylated to supply the proline requirements of the strain. Acetyl-ornithinase is responsible for the ability of a histidine auxotroph to utilize acetyl-histidine as a source of histidine in E. coli (13). This enzyme also appears to be the primary source of acetylmethioninase activity in E. coli (142). It has also been shown to hydrolyze formylmethionine (90, 91) in vitro and acetylarginine (20) in vivo.

Ornithine transcarbamylase (EC 2.1.3.3), enzyme 6, specified by argF and argI, was purified from E. coli in 1962 (107). This enzyme (OTC) which requires carbamyl phosphate, has a pH optimum of 8.5-8.9, and has a sedimentation value of 7.4S corresponding to a molecular weight of approximately 60,000. It apparently has neither a specific metal ion requirement nor the necessity of free sulfhydryl groups for activity.

Enzyme 7, argininosuccinate synthetase (EC 6.3.4.5), encoded in argG, has not been studied in E. coli except for some preliminary repression-derepression studies. This enzyme converts citrulline to argininosuccinate and is measured by using a radioactive assay. Citrulline-5-^{14}C is converted to argininosuccinate-5-^{14}C by enzyme 7. An excess of argininosuccinase, arginase, and urease were added to release $^{14}CO_2$ from the argininosuccinate formed.

Argininosuccinase (EC 4.3.2.1), enzyme 8, specified by argH, converts argininosuccinate to arginine. It has been purified (102) although the results have yet to be published. It has no cofactor requirements and is assayed at pH 7.5 (analogous to animal systems).

Genetics of Arginine System

The location on the E. coli chromosome of the eight genes which code for the biosynthetic enzymes, other genes associated with the arginine system, and reference markers are shown in Fig. 3. The figure was drawn using data from references 24, 41, 42, 45, 50, 68, 82, 83, 85, 124, and 140. The genes coding for the eight biosynthetic enzymes are located in six regions on the E. coli chromosomes, with argE, argC, and argB, and argH being clustered at minute 77.3. Thus, the arginine biosynthetic pathway provides an opportunity to study the regulation of expression of both scattered and clustered genes which are functionally related. There are a few special points which should be made concerning the location of some of these loci on the chromosome map.

The argD gene has been mapped in E. coli, W, by conjugation (140). Despite efforts, there have been isolated no mutants of argD in E. coli, K12. ArgM is the gene in E. coli, W, responsible for production of an inducible transaminase (63, 139, 140); this gene does not appear to be another structural gene for a second acetylornithine δ-transaminase (125). Ornithine transcarbamylase is coded for by two structural genes argF and argI (45). Both genes must be nonfunctional in order for a strain to be OTC-less. Vogel (135) has reported an acetylornithine permease whose synthesis is repressible by arginine, but its gene has not been mapped. The arginine permease specified by argP is responsible for the uptake of arginine, lysine, and ornithine as well as canavanine (83, 118); hence, argP mutants are also phenotypically canavanine resistant. The other two genes which, when mutated, give rise to the phenotype of canavanine resistance are the

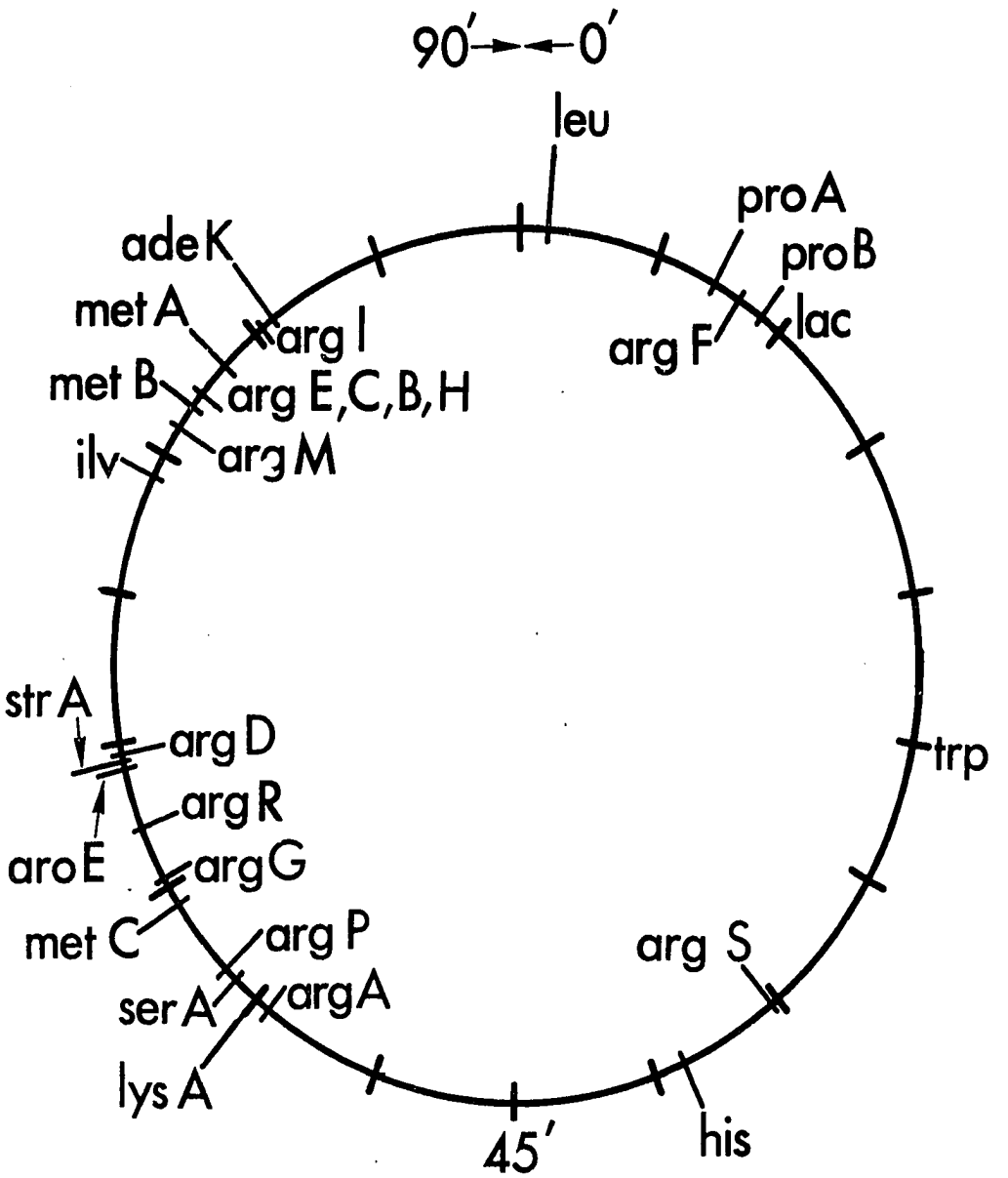


Figure 3. Linkage map showing genes of the arginine system in E. coli and reference markers.

argR gene and the argS gene, both of which are discussed below.

Figure 4 gives a more detailed diagram of minutes 75-79 within which the arginine cluster lies (41, 42, 44, 124, 144, 150). The indicated gene order for the arginine cluster comes from the work of Glansdorff (41). The gene order was determined by three-point crosses between ppc and one argE, one argB, two argC, and two argH point mutations. However, the author points out that, "Further studies with a larger number of arg mutants will help clarify this point (whether the four genes are arranged on the chromosome without any discontinuities); in particular, the occurrence of deletion mutants extending over more than one locus (which have not yet been discovered in this system) should be investigated."

Regulation of Arginine Biosynthesis

In biosynthetic pathways, regulation of protein synthesis is effected by the "active" repressor which consists of the corepressor - a small molecule, usually the end-product of the pathway being regulated - and the aporepressor which is coded for by a regulatory gene and in every case studied thus far, is a protein. There is evidence accumulating which would indicate that tRNA is also part of the "active" repressor. In the arginine system, the corepressor is arginine and the aporepressor is coded for by the argR gene. There is evidence that arginyl-tRNA or its synthetase is involved in the "active" repressor. Since argR and argS (whose products comprise part of the "active" repressor) mutants are resistant to the arginine analogue, canavanine, the phenomenon known as "canavanine death" will be discussed before the information concerning the "active" repressor of the arginine system.

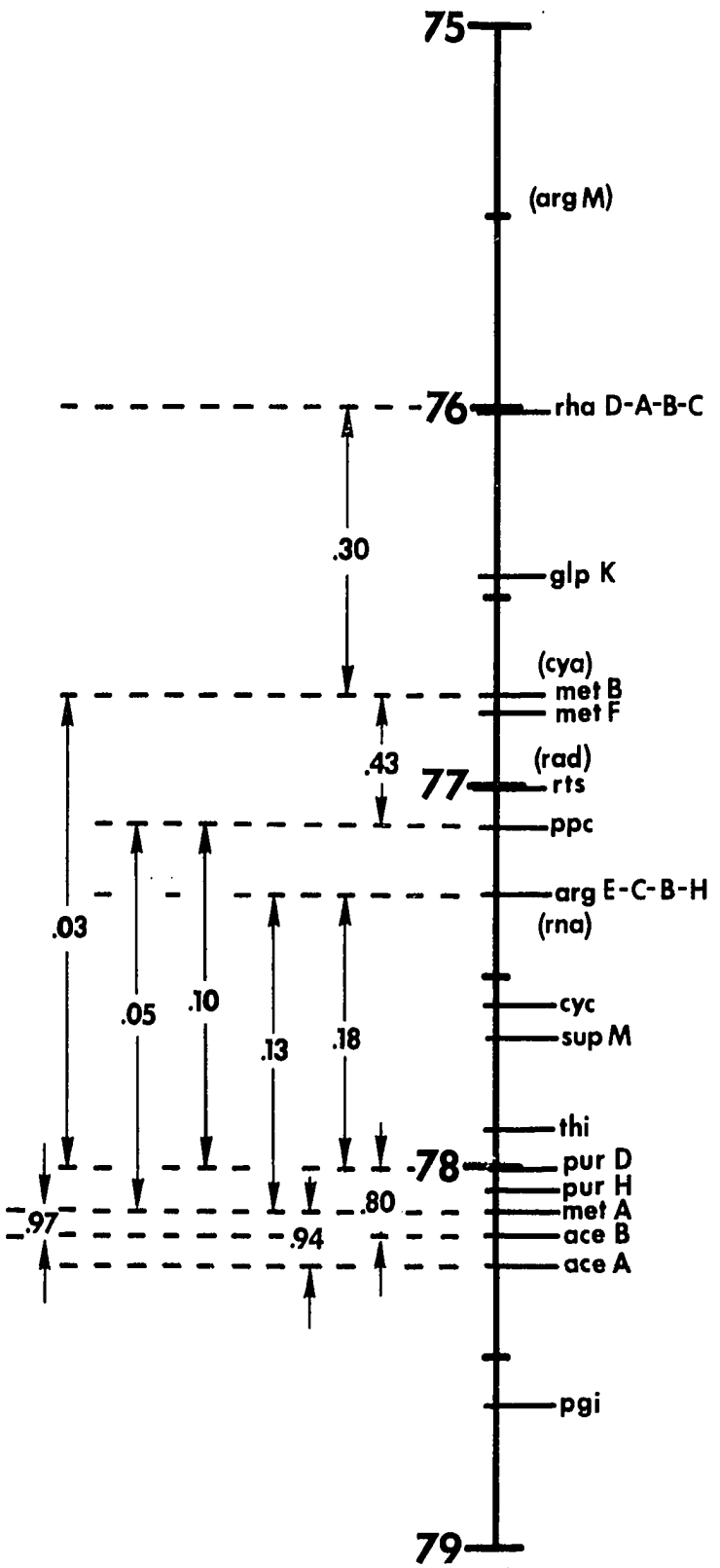


Figure 4. Minutes 75 to 79 of the E. coli chromosome.

Canavanine Death

Canavanine, a naturally occurring analog of arginine (Fig. 5), was first demonstrated as an inhibitor of protein synthesis in E. coli in 1960 (117). As mentioned above, resistance to canavanine can arise by mutation of any of three genes: (1) argP, which produces the permease responsible for concentrating canavanine inside the cell; (2) argR, which leads to pathway-wide derepressed and non-repressibility of the synthesis of the arginine enzymes and thus to a high level of intracellular arginine which overcomes the affects of canavanine; and (3) argS, which codes for arginyl-tRNA synthetase. Canavanine death, that is, the loss of viability of cells grown in canavanine which have a low intracellular concentration of arginine, was first thought to occur because of an interference by canavanine with the utilization of arginine in protein synthesis (117). Schwartz and Maas showed that the extent of inhibition by canavanine was dependent upon the intracellular concentration of arginine at the time of exposure to canavanine. In 1965, Schactele and Rogers (112) put forth evidence to support their hypothesis concerning the mechanism of canavanine death:

> 1) "Under conditions of arginine starvation, canavanine is incorporated in place of arginine into a protein or proteins vital for a new round of DNA replication.
>
> 2) Once the canavanine-containing protein is formed, the organism cannot commence a new round of DNA synthesis, since the protein made is faulty in some as yet unknown way.
>
> 3) The faulty protein itself cannot be replaced by a functional arginine-containing protein until a new

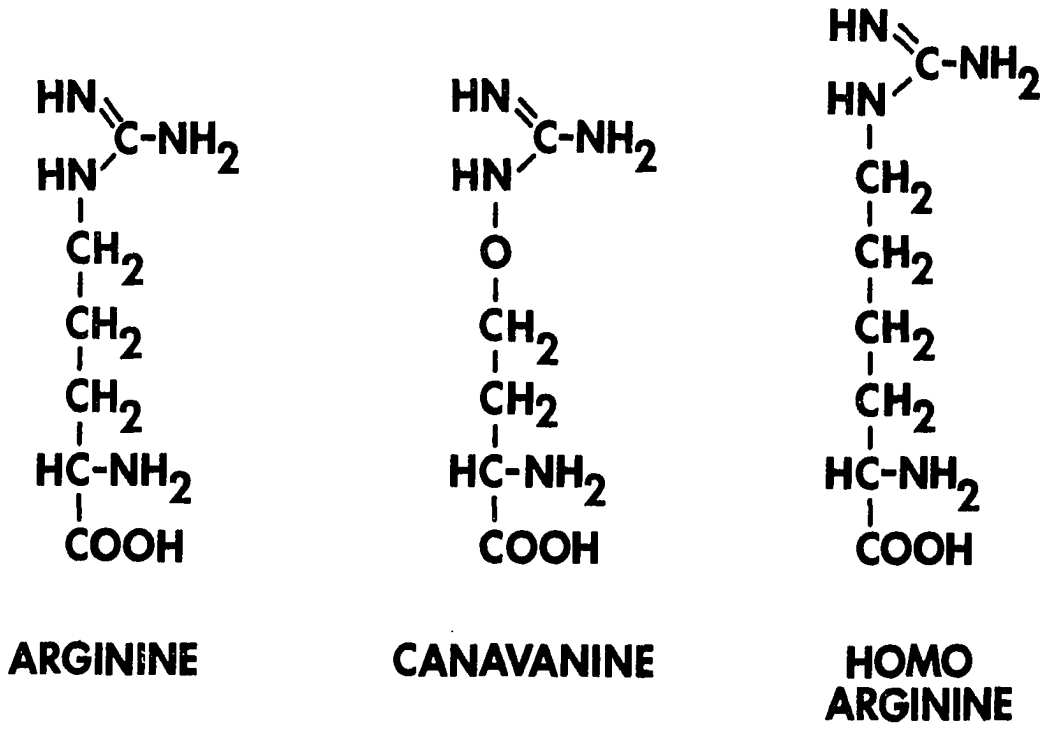


Figure 5. Chemical structures of arginine, canavanine, and homo-arginine.

cycle of DNA synthesis has been completed. . .

4) The bacterium becomes inviable when all of its genomes have completed replication of DNA and the canavanine-containing faulty protein is synthesized and positioned at the point of DNA replication."

This mechanism has recently been revised (110, 111, 113) to include the observations that the bulk of new protein in which arginine is replaced by canavanine concentrates as amorphous masses attached to the inside of the cell membrane. These canavanyl-protein-membrane complexes also contain attached high molecular weight DNA which can be freed by treatment with either pronase or sodium lauryl sulfate. There is also an abnormal dispersion of ribosomes and no distinct nuclear region in canavanine-dead cells. Canavanine-dead cells are incapable of propagating T-bacteriophage although adsorption occurs. It should be recalled at this point that T-even phages are capable of growth on E. coli rendered non-viable by agents such as ultraviolet light (6, 81), x-rays (73, 74), mustard gas (55), and colicin E2 (98, 99, 100). Canavanyl-proteins appear to block all sites in bacteria capable of initiating DNA replication.

A mutant of an arginine auxotroph of E. coli, K12, was isolated which was canavanine-resistant but appeared not to have a mutation in argR or argP (110). The possibility of an argP mutation was eliminated because the strain was able to concentrate ^{14}C-arginine. The possibility of an argR mutation was discarded due to the fact that OTC synthesis was normally repressible by arginine; however, it has been shown (82, 121) that some canavanine-resistant argR mutations have normal arginine repressible synthesis of the arginine enzymes. The authors proposed that the mutation altered a specific protein involved in organizing the bac-

terial "nucleus" and that this protein remains functional after canavanine substitution. Thus, it is possible that there is a fourth genetic locus which can produce the phenotype of canavanine-resistance although the authors did not attempt to eliminate the possibility that their mutant had an altered arginyl-tRNA synthetase (argS) (146).

Corepressor

That arginine could act as the corepressor for enzymes in the arginine biosynthetic pathway was first observed in 1953 (131) when it was discovered that arginine added to growing cultures of strain W of E. coli repressed the formation of acetylornithinase. Sercarz and Gorini (119) reported the occurrence of an unusual behavior with regard to the arginine biosynthetic enzymes in a hybrid of E. coli, B and K12 (the argR gene from K12 into a B recipient). This strain, BC28, had fully derepressed enzyme levels when grown on minimal medium and repressed levels when grown in the presence of exogenous arginine. The K12 donor of the argR gene behaved normally (that is, enzymes were partially derepressed in minimal medium). Their studies with BC28 provides evidence for the existence of two functional arginine compartments, one involving endogenous arginine and related to protein formation and the other involving exogenous arginine associated with arginine-permease and related to repressor formation. The intermixing of these two compartments is rapid in the wild-type but is greatly, perhaps completely, impaired in BC28. The possibility exists that some of Maas' canavanine-resistant, arginine non-excreting strains exhibiting derepressed and part-

ially repressible synthesis of the arginine enzymes (82) might be examples of BC28-type behavior. However, his strains were obtained by direct mutation rather than by conjugation between two E. coli strains. Tabor and Tabor recently observed a partial separation of two arginine pools when measuring spermidine synthesis (122, 123).

It has been shown that canavanine can repress the synthesis of OTC, however, in concentrations forty times as high as the concentration of arginine necessary to get full repression (36). Homoarginine (Fig. 5) has been shown to be capable of repressing the synthesis of acetylornithinase and OTC at concentrations comparable to arginine in E. coli, W (38). However, in E. coli, B, homoarginine inhibits growth and does not lead to repression (103). E. coli, B, has very low levels of the arginine enzymes and thus a low intracellular concentration of arginine. Homoarginine inhibition can be overcome physiologically by addition of exogenous arginine and genetically by mutation of E. coli, B, to homoarginine-resistance. These resistant strains contain mutations in the argR gene. The authors showed, however, that the allele of the argR gene present in E. coli, B, is not necessary for homoarginine sensitivity. Therefore, it appears that homoarginine can act as a corepressor of the arginine system, but in E. coli, B, it has an additional effect which is indirectly related to corepressor action.

The involvement of aminoacyl-tRNA synthetases in the regulation of protein synthesis has been reported (33, 39, 108, 115). Sixty arginyl-tRNA synthetase mutants showed no alteration in the repression-derepression behavior of OTC (59). It was concluded that arginyl-tRNA synthetase was not involved in the

regulation of the synthesis of the arginine biosynthetic enzymes. Williams (146) on the other hand, using a different selection procedure, selected mutations in argS which led to nonrepressibility for the arginine biosynthetic enzymes, thus implicating arginyl-tRNA synthetase in repression of the arginine biosynthetic enzymes.

Aporepressor

In 1960, it was first reported (48) that there was a definite difference in the ability of arginine to repress the synthesis of the arginine biosynthetic enzymes in E. coli, W, as compared to B. E. coli, B, had very low levels of the arginine enzymes and upon the addition of arginine, the synthesis of these enzymes was induced rather than repressed. Very quickly it became apparent that the differences in repressibility of the arginine enzymes among E. coli, B, K12, and W, were due to a locus which became known as $argR^+$ for arginine repressible and $argR^-$ for not repressible by arginine (35, 50, 82, 136, 138). Hereafter, $argR^-$ will mean derepressed and nonrepressible rates of enzyme synthesis. Before discussing the differences between E. coli, B, and K12, with respect to their argR loci, it is necessary to discuss the evidence concerning the K12 locus.

In E. coli, K12, $argR^-$ mutations were first isolated as phenotypically canavanine-resistant mutants (82) which would cross-feed an arginine auxotroph because of increased arginine production and subsequent excretion. Such cross-feeders show derepressed (high) and nonrepressible (by arginine) rates of synthesis of the arginine enzymes. ArgR mutants of E. coli, B, have been reported (50) to be resistant to 9 mg/ml lysine which completely prevents growth of strains

carrying the wild-type argR allele. Maas (82) also reported a significant number of canavanine-resistant, arginine non-excreters which have mutations which map in the argR region but either have (1) normal argR$^+$ levels of enzymes 6, 7 and 8 grown with or without arginine, or (2) partially derepressed and partially repressible (by arginine) rates of synthesis for enzyme 6 (OTC). These last two classes of argR mutations (non-excretors) have not been studied further since this first report.

In 1964, it was reported that in transient argR$^+$/argR$^-$ zygotes, argR$^+$ (or repressibility) is dominant over argR$^-$ (non-repressibility) (85). In permanent merodiploids for the argR region, arginine was able to repress synthesis of OTC, showing again that the argR$^+$ was trans-dominant to argR$^-$ (84). The first report of a temperature-sensitive mutation in argR was by Udaka and Horiuchi (128). They isolated a mutant of E. coli, K12, which has fully derepressed levels of OTC and argininosuccinase when grown at 42^{0}C in minimal medium plus arginine, but which has nearly fully repressed levels of OTC and enzyme 8 when grown at 20^{0}C in the same medium. The existence of a temperature-sensitive mutation in the argR gene is suggestive that the product of the gene might well be protein in nature. In 1969, Jacoby and Gorini (69) reported the isolation of an amber (UAG) mutation in the argR locus in E. coli, B, suppressible by the amber suppressors su I which inserts serine and su III which inserts tyrosine. They also isolated a temperature-sensitive mutation in the argR gene. All the above information indicates that the argR gene codes for a diffusible, cytoplasmic product which is a protein.

The relationships which have been elucidated between E. coli, K12,

and B (68, 69, 71, 128) can best be summarized by the use of a diagram (Fig. 6). The argR locus in E. coli, W, is probably very similar, if not identical, to the K12 locus, although not much work has been done with it other than to isolate argR$^-$ mutations.

Repression-Derepression Behavior of Arginine Enzymes

The ability of E. coli to regulate the level of its arginine biosynthetic enzymes in response to exogenous arginine is apparent when grown with glucose or lactate as a carbon source, but not when grown with glycerol, and only a small response is observed when grown with succinate (49).

In order to discuss the regulation or repression-derepression behavior of the various enzymes of the arginine system in such a way as to eliminate differences in the way some authors measure and define units of activity, it is useful to normalize and compare the results in terms of three ratios: (1) ratio A equals the specific activity of the argR$^-$ strain grown in arginine-supplemented media divided by the specific activity of the argR$^+$ strain grown in arginine-supplemented media and represents the total repression-derepression range; (2) ratio B is the specific activity of the argR$^-$ strain grown without exogenous arginine divided by that of the argR$^+$ strain grown without arginine and represents the fold increase above the partially derepressed rate of synthesis reflecting the intracellular, steady-state level of endogeneously synthesized arginine; (3) ratio C is equal to the specific activity of argR$^+$ strain grown without arginine divided by the specific activity of the argR$^+$ strain grown with arginine and represents the fold decrease below the partially derepressed rate of synthesis.

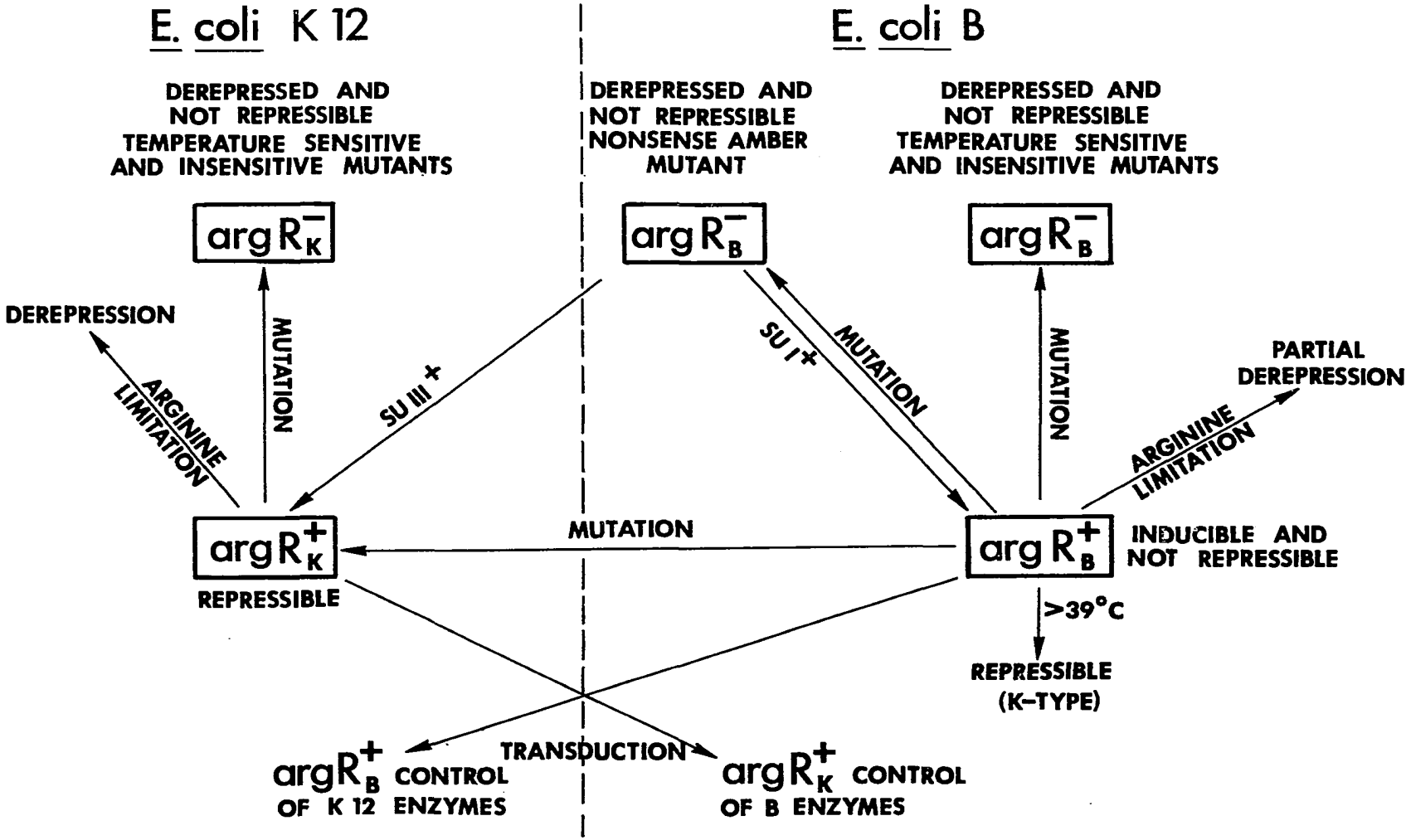


Figure 6. Summary of relationship between arg R_K and arg R_B.

The expression of the non-clustered genes will be discussed first, followed by a discussion of the expression of the clustered genes.

Information on the regulation of N-acetylglutamate synthetase, the first enzyme of the arginine biosynthetic pathway, which one would predict would be subject to both repression and feedback inhibition by arginine, has been sparse due to the inability to demonstrate significant activity in vitro. Ennis and Gorini (35) reported that in E. coli, B, which is not repressible by arginine, the incorporation of arginine-^{14}C into protein was decreased when unlabeled N-acetylglutamate was added but not when unlabeled glutamate was added. In 1963, it was reported (145) that exogenous arginine inhibits enzyme 1 activity by 95% and represses the formation of N-acetylglutamate synthetase using the resting cell assay.

Likewise, information on argininosuccinate synthetase is very limited due to the lack of a competent in vitro assay. There are two reports concerning its repression-derepression behavior (82, 83). For E. coli, K12, the authors report a ratio A of 10.0 ratio B of 2.4 and a ratio C of 3.3 (82) when the strains were grown in a synthetic rich medium (101) with and without arginine. Upon growth in minimal medium, ratio A is 4.3; ratios B and C cannot be calculated since the activities of argR$^+$ or argR$^-$ strains in minimal medium were not reported (83).

The regulation of acetylornithine δ-transaminase synthesis has been studied (3, 136) in E. coli, K12, W, and B (Table 1). Transaminase and acetylornithinase (see below) appear to be "coordinately" synthesized in K12; this is not the case in W or B. Thus, the repressor recognition sites of enzymes 4 and 5 (not closely linked on the E. coli chromosome, see Fig. 3) may be identical in

TABLE 1

REPRESSION-DEREPRESSION BEHAVIOR OF N-ACETYLORNITHINE δ-TRANSAMINASE IN ESCHERICHIA COLI

Strain	Ratio A	Ratio B	Ratio C	Reference
K12	10.3	2.96	3.50	136
W	33.3	———	11.1	136
B	———	———	1.0	3

K12 but appear to be somewhat different in W and B. Since the gene for enzyme 4 is not closely linked to any of the other arginine genes, argD should have its own repressor recognition site. If this is so, it should be possible to isolate mutants with altered repression-derepression behavior which is specific for enzyme 4 due to changes in its repressor recognition site. The isolation and characterization of such mutants has been reported (8, 38, 125, 140). "Revertants" of an E. coli, W, argD auxotroph were isolated and among these "revertants", some were found which have low levels of transaminase when grown on minimal medium. However, when grown with arginine the mutants have relatively high levels of transaminase, although they contained the normal repressed levels of enzymes 5, 6 and 8.

Enzyme 6, OTC, is the most thoroughly studied enzyme of the arginine system. Although reports of the range of synthetic capacity for OTC have been variable depending on the methodology (Table 2), OTC has the greatest range of synthetic rates of any of the arginine enzymes. Aside from this, there have been several interesting results and ideas come from OTC regulation studies.

If one grows a double arginine auxotroph (blocked both before and after enzyme 6) under conditions of arginine limitation (in a chemostat), the repressive effects of arginine can be reversed by exogenous ornithine (48). If full derepression has been effected by either extreme arginine limitation or by introduction of an argR$^-$ allele of the regulator gene, ornithine does not further increase the synthetic rate. However, partial repression or even full repression to a limited extent, can be reversed by ornithine (concentration of ornithine approximately the same as arginine for reversal of partial repression; concentration of ornithine 15 times that of arginine

TABLE 2

REPRESSION-DEREPRESSION BEHAVIOR OF ORNITHINE TRANSCARBAMYLASE IN ESCHERICHIA COLI

Strain	Ratio A	Ratio B	Ratio C	Reference
K10	736	34.7	21.0	68
K12	600	———	60.0	43
	———	———	19.2	50
	694	10.8	80.5	83
	———	———	12.0	71
	208	13.0	16.0	48
	———	———	20.0	68
	125	9.00	13.8	136
W	670	33.5	20.0	48
	1040	17.3	60.0	35
	70.6	5.71	9.00	38
	100	———	———	140
B	22.9	26.4	0.71	48
	———	———	0.50	68
	———	———	0.50	71

for reversal of full repression). The possible complication of competitive interaction between arginine and ornithine to get into the cells was eliminated. The author concluded "the inducing of ornithine is based on competition with arginine at some site within the cell: either the site of repressor action, if arginine is the direct repressor, or the site of repressor formation, if arginine is a precursor of the direct repressor." It can be seen that if a translational control model of regulation is assumed, the "ornithine effect" could be explained since ornithine has a binding tendency to OTC, and this binding could change the configuration of the nascent polypeptide chain such that the "active repressor" could not bind the growing polypeptide in order to repress its synthesis.

As mentioned above, OTC is coded for by two structural genes (argF and arg I) (45). Either of the wild type alleles of these loci confers to an OTC-less strain the ability to synthesize OTC under the control of arginine. It appears that argF accounts for approximately 20% of the OTC levels. Multiple OTC enzymes in other bacteria have been reported (17, 104).

As shown in Fig. 2, carbamyl phosphate is required in both arginine and uracil biosynthesis. Several investigations have been conducted to ascertain what, if any, relationship there is between RNA (uracil) synthesis and OTC synthesis (36, 46, 52). A uracil-requiring strain of E. coli, W, will not synthesize β-galactosidase when an adequate carbon source is available in the absence of added uracil; however, when arginine repression is relieved by removal of exogenous arginine, OTC synthesis (20 to 100 fold increase) occurs in the absence of exogenous uracil (52). This observation indicates the presence of a preformed OTC specific stable

mRNA whose translation can occur only in the absence of arginine; however, it does not suggest any specific regulatory relationship between uracil and arginine biosynthesis.

An indication of some sort of relationship between the two pathways first came from the observation that a mixture of 2-thiouracil and arginine prevents E. coli, W, WT (wild-type) colony formation on solid medium; however, neither arginine nor 2-thiouracil individually, ornithine plus 2-thiouracil, nor any other amino acid in combination with 2-thiouracil has this inhibitory effect (16). Arginine, though without effect by itself, increases the level of aspartate transcarbamylase (ATC) (the first enzyme of uracil biosynthesis, see Fig. 2), as much as 20-fold when present with 2-thiouracil. Uracil and 2-thiouracil added individually cause a two-to-three fold increase in OTC levels. However, neither the derepression of ATC by arginine nor the derepression of OTC by uracil is accompanied by changes in the level of carbamyl phosphokinase, the enzyme that catalyzes carbamyl phosphate synthesis (16, 52).

Recently a selective procedure (69) has been developed to allow the selection of a mutation in argF which relieves OTC synthesis of repression by arginine. In other words, it is a procedure for isolating enzyme-specific regulatory mutants for OTC. The mutant of E. coli, B, has "constitutive" levels of OTC while containing normal enzyme 5 and 8 activities. The mutation $arg0_F$ was shown to be cis-dominant and very closely linked to argF (at least 96% cotransducible), and hence appears to be an operator constitutive mutation. There was a mutant reported earlier in the literature (68, 49), which has a hyperinducible OTC which may

also be due to an altered operator region. Thus, for both argD and argF of the scattered genes, mutants have been reported which appear to have alterations in their repressor recognition sites.

There are few studies in the literature concerning the expression of the clustered arginine genes (argE, C, B, H) (Table 2). As pointed out earlier, enzymes 2 and 3 were first demonstrated in 1962 (9). However, since then, work on these two enzymes has been hampered due to the lack of assays sensitive enough to measure low levels accurately.

There are some conclusions which can be drawn about the regulation of enzymes 5 and 8. In every case, when the repression-derepression behavior (see Table 3) of enzyme 5 is compared to that of enzyme 8, the difference is such that one must conclude that they are regulated individually. Additional evidence for this conclusion comes from Unger, et al. (130) who showed that in a revertant of an acetylornithinase auxotroph with a 3% level of restoration of acetylornithinase, the intracellular arginine concentration and hence the concentration of functional repressor, is fully derepressive for acetylornithinase but partially repressive for argininosuccinase. It was also shown (20) that if a double auxotroph (argB, argG) is grown in 0.2 mM N-acetylarginine plus 3.0 mM N-acetylornithine, acetylornithinase is derepressed, whereas argininosuccinase is repressed. Gorini (69) reported a mutation in $argR_B$ which as growth temperature is increased shows differential derepression of acetylornithinase and argininosuccinase. All three of these studies are consistent with the view that enzymes 5 and 8 are regulated separately (noncoordinately). In one study (43), the synthesis of enzymes 3, 2 and 8 is said to

TABLE 3

REPORTS OF THE REGULATION OF ARGININE ENZYMES SPECIFIED BY THE CLUSTERED GENES IN ESCHERICHIA COLI

Strain	Enzyme 5			Enzyme 3			Enzyme 2			Enzyme 8			Reference
	Ratio A	Ratio B	Ratio C	Ratio A	Ratio B	Ratio C	Ratio A	Ratio B	Ratio C	Ratio A	Ratio B	Ratio C	
K12	6.7	2.6	2.6	—	—	—	—	—	—	33.	4.2	8.0	14
	8.0	—	—	6.0	—	—	25.	—	—	33.	—	—	141
	25.	—	5.0	50.	—	10.	50.	—	10.	50.	—	10.	43
	13.	2.3	5.7	30.	3.0	10.	25.	2.8	4.0	24.	2.4	10.	42
	—	—	4.1	—	—	4.6	—	—	4.2	—	—	4.9	44
W	9.1	2.4	3.6	—	—	—	—	—	—	—	3.4	—	130

be coordinate, although the experimental data is limited.

Plan of the Dissertation

The purpose of this dissertation was to gain information basic to defining the existence, number and location of promotors, initiators, and repressor recognition sites in the arginine cluster with a view towards understanding the control of the expression of the clustered arginine genes.

Rationale of Study

The following studies will be performed to fulfill these objectives. (1) The coordinacy relationships for the synthesis of the enzymes specified by the clustered genes will be determined. Coordinate synthesis of enzymes corresponding to contiguous genes is taken to be consistent with their sharing a common repressor recognition site and with the absence of an efficient internal promotor or initiator. Departure from coordinacy may result from the presence of individual repressor recognition sites, or of an efficient promotor or initiator. (2) If the genes of the cluster have individual repressor recognition sites, it should be possible to isolate relatively enzyme-specific regulatory mutants in which the information for repressor recognition has presumably been altered. (3) To implement mapping of mutations which alter the controlling elements governing the synthesis of arginine cluster enzymes, it is helpful to use deletion analysis. A large number of cluster auxotrophs will be isolated with a variety of mutagens known to induce point and deletion mutations and characterized with regard to genetic block and revertibility. These auxotrophs will be used to construct a deletion map of the cluster region.

CHAPTER II

MATERIALS AND METHODS

Bacterial Strains

All strains of E. coli not arising as a result of mutagenesis during the course of this project are listed in Table 4. Strains which were isolated during this work are numbered with a four digit number and are described in Chapter III. Stock cultures of each strain were maintained: (1) on nutrient agar slants at $4^{o}C$ and were transferred every three months; (2) in nutrient soft agar stabs at room temperature in small screw-capped vials sealed with paraffin.

Media and Reagents

Media

Unless otherwise noted, amino acids were added in a final concentration of 100 μg/ml to minimal media to supply the auxotrophic requirements of a particular strain. Solid media contained 15.0 g of agar per liter of media and soft agar had 7.5 grams per liter of media.

Minimal medium A (MMA). MMA (106) was prepared ten times concentrated (10X), stored over chloroform (2 ml/l), and diluted with H_2O just prior to sterilization by autoclaving. The 10X MMA contained in g/l: K_2HPO_4, 70.0,

TABLE 4

LIST OF STRAINS

Strain Number	Other Designation	Obtained From	Genotype
EcW-1	W, WT	———	prototroph
EcK-2	K12, WT	———	prototroph
EcK-101	Ra2	B. Low	prototrophic Hfr
EcK-111	P4 X 6	D. Ezekiel	metB 1, Hfr
EcK-112	P4 X 6R1	D. Ezekiel	metB 1, rna-1, Hfr
EcW-444	45-A-25	H. J. Vogel	argG
EcW-448	D2-18-0^{+}	H. J. Vogel	leu^{-}, pro^{-}, str^{r}
EcW-468	40/6/12 sm^{r} 22	———	argE, argR^{-}, str^{r}

KH_2PO_4, 30.0; sodium citrate • $2H_2O$, 5.0; $MgSO_4$ • $7H_2O$, 1.0; $(NH_4)_2SO_4$, 10.0. Glucose, added aspetically after autoclaving from a sterile stock solution of 25% was used as a carbon and energy source in a final concentration of 0.5%. Glucose was omitted when MMA was used to wash and resuspend cells. MMAarg refers to MMA supplemented with 0.3 mg L-arginine • HCl per liter. EMMA (enriched minimal medium A) designates MMA plus 1.25% nutrient broth.

Minimal medium E (MME). MME (142) was prepared as fifty times concentrated (50X), stored over chloroform (2 ml/l), and diluted with H_2O prior to sterilization by autoclaving. The 50X MME contained in g/l: $MgSO_4 \cdot 7\ H_2O$, 10.0; citric acid • H_2O, 100; K_2HPO_4, 500; $NaNH_4HPO_4$, 175. Glucose (0.5%, final concentration) was added separately after sterilization as above. Liquid stock cultures were prepared as MME + 0.2% glucose + 0.2% N-Z Case. N-Z Case is a vitamin-free enzymatic digest of casein (Sheffield Chemicals).

Minimal medium C (MMC). MMC (28) was prepared ten times strength (10X), stored over chloroform (2 ml/l) and diluted with H_2O just prior to sterilization by autoclaving. The 10X MMC contained in g/l: NH_4Cl, 50.0; NH_4NO_3, 10.0; Na_2SO_4, 20.0; K_2HPO_4, 90.0; KH_2PO_4, 30.0. Sterile glucose (final concentration, 0.5%) and $MgSO_4$ (final concentration, 4×10^{-4} M, added as 0.02 ml of 2 M $MgSO_4$ per 100 ml MMC) were added separately after autoclaving.

Minimal mating medium (MMM). MMM (29) was prepared ten times strength (10X), stored over chloroform (2 ml/l) and diluted with H_2O just prior to sterilization by autoclaving. The 10X MMM contained in g/l: NH_4Cl, 50.0; NH_4NO_3, 10.0; Na_2SO_4, 20.0; K_2HPO_4, 49.0; KH_2PO_4, 63.0. The pH of

MMM was adjusted to 6.3 prior to sterilization. Sterile glucose (final concentration, 0.5%) and $MgSO_4$ (final concentration, 4×10^{-4} M, added as 0.02 ml of 2M $MgSO_4$ per 100 ml MMM) were added separately after autoclaving.

LC broth. LC broth (80) contains in g/l: tryptone, 10.0; yeast extract, 5.0; NaCl, 10.0. The pH of LC broth was adjusted to 7.0 before autoclaving. LC + Ca^{+2} broth includes $CaCl_2$ (final concentration, 2.5×10^{-3} M) which was added aseptically by introducing 1.0 ml of a stock solution of 2.5×10^{-1} M (2.775 g/100 ml) into 100 ml of LC broth.

Arginine-free medium (AF). This was prepared by rehydrating Difco arginine assay medium with MMA (26.0 g/l MMA) and adding glucose (0.25%, final concentration) after autoclaving. The arginine assay medium was sterilized by filtration and added aseptically to MMA. AF + Can refers to arginine-free medium supplemented with L-canavanine (100 μg/ml, final concentration).

Peptone-beef extract broth (PBE). PBE (57) contained 1% peptone and 0.3% beef-extract dissolved in H_2O, and was adjusted with NaOH to a pH of 7.6.

Nutrient broth (NB). This is rehydrated Difco nutrient broth (8.0 g/l of H_2O). NBG refers to NB supplemented with glucose (0.5%, final concentration).

Brain-heart infusion broth (BH). This is rehydrated Difco brain-heart infusion broth (37.0 g/l of H_2O).

Limited enrichment medium. Limited enrichment medium refers to MMA agar plates to which an amino acid has been added at a concentration which will limit by more than 50% the colony size of a strain auxotrophic for that amino acid as compared to that of the prototrophic wild type after 48 hours incubation. The

growth limiting concentrations of amino acids determined and used were: (1) arginine, 4 μg/ml; (2) acetylornithine, 4 μg/ml; (3) leucine, 2 μg/ml; (4) proline, 2 μg/ml; (5) histidine, 2 μg/ml; (6) methionine, 1.5 μg/ml; (7) tryptophan, 4 μg/ml; (8) lysine, 4 μg/ml; and (9) threonine, 4 μg/ml.

Reagents

Tris-maleic buffer (TM buffer). TM buffer (111) consisted of the following in g/l: Tris HCl, 6.10; maleic acid, 5.80; $(NH_4)_2SO_4$, 1.00; $MgSO_4 \cdot 7H_2O$, 9.10; $Ca(NO_3)_2 \cdot 4H_2O$, 5.0×10^{-4}; $FeSO_4 \cdot 7H_2O$, 2.5×10^{-4}. The pH was adjusted to 6.0 with NaOH before autoclaving.

Acetate buffer. Acetate buffer (0.1M) (112) was prepared by mixing 51 parts of 0.1M acetic acid with 49 parts 0.1M sodium acetate (8.2 g/l). The pH was adjusted to 4.6 with 0.1M acetic acid.

Phage dilution buffer. This buffer (22) contains in g/l: Na_2HPO_4, 7.00; KH_2PO_4, 3.00; NH_4Cl, 1.00; $MgSO_4 \cdot 7H_2O$, 0.25; NaCl, 5.00; and gelatin, 0.02.

Growth of Bacterial Cultures

Bacteria from stock slants were inoculated into 3.0 ml of MME + 0.2% glucose + 0.2% N-Z Case (liquid stock cultures). These liquid stock cultures served as daily sources of cells. All cultures were incubated at 37°C. Liquid cultures of E. coli, W, were grown without aeration, whereas those of K12 were grown with aeration in a New Brunswick gyratory shaking water bath (Model G-77). Growth in liquid media was followed turbidimetrically by use of a Klett-Summerson

colorimeter fitted with a No. 66 filter.

Viable Cell Count

A standard curve relating viable cell concentration to colorimeter readings was constructed for logarithmic phase K12 cells growing in LC broth (Fig. 7). Samples were withdrawn periodically, diluted in 0.85% saline, and 0.1 ml aliquots of appropriate dilutions were spread in triplicate onto NBG agar plates. After 24 hours incubation, the number of colonies per plate were counted.

Genetic Methods

Isolation of Mutants

Mutagenesis. The following were used as mutagenic agents.

Ultraviolet light (UV). The bacteria were grown overnight in MMA supplemented with appropriate auxotrophic requirements. Two ml were transferred into 8 ml of fresh MMA plus auxotrophic requirements and incubated for two hours or until the cell density was 2-5 x 10^8/ml. Eight ml were then transferred to a sterile glass petri dish and irradiated in the dark for 40 seconds with a 15 watt germicidal UV lamp (GE15T8) positioned 40 cm above the level of the petri dish. During the UV exposure, the uncovered petri dish was rotated by means of an Eberbach rotator. This amount of irradiation gave a 99% kill (Fig. 8). Irradiation and the overnight incubation which followed were conducted in the dark in order to prevent light repair of UV-induced mutations. A yellow incandescent light was used as a source of subdued light when necessary for visibility during manipulations.

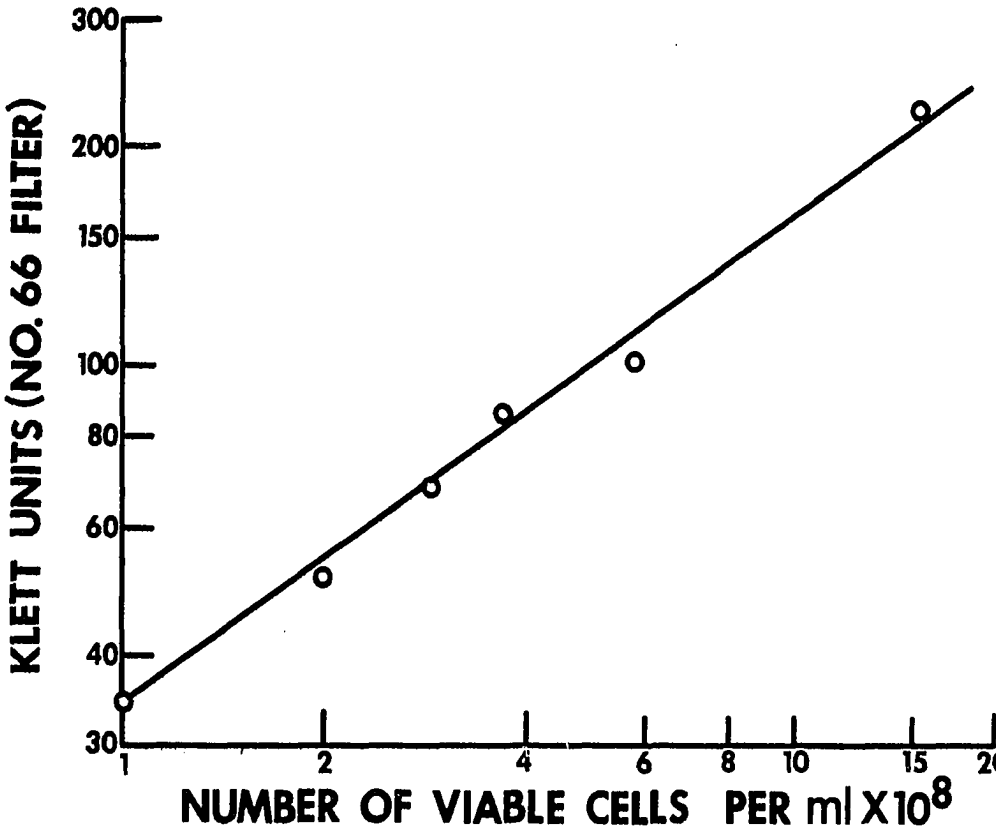


Figure 7. Standard curve relating number of viable cells of E. coli K 12 to Klett units.

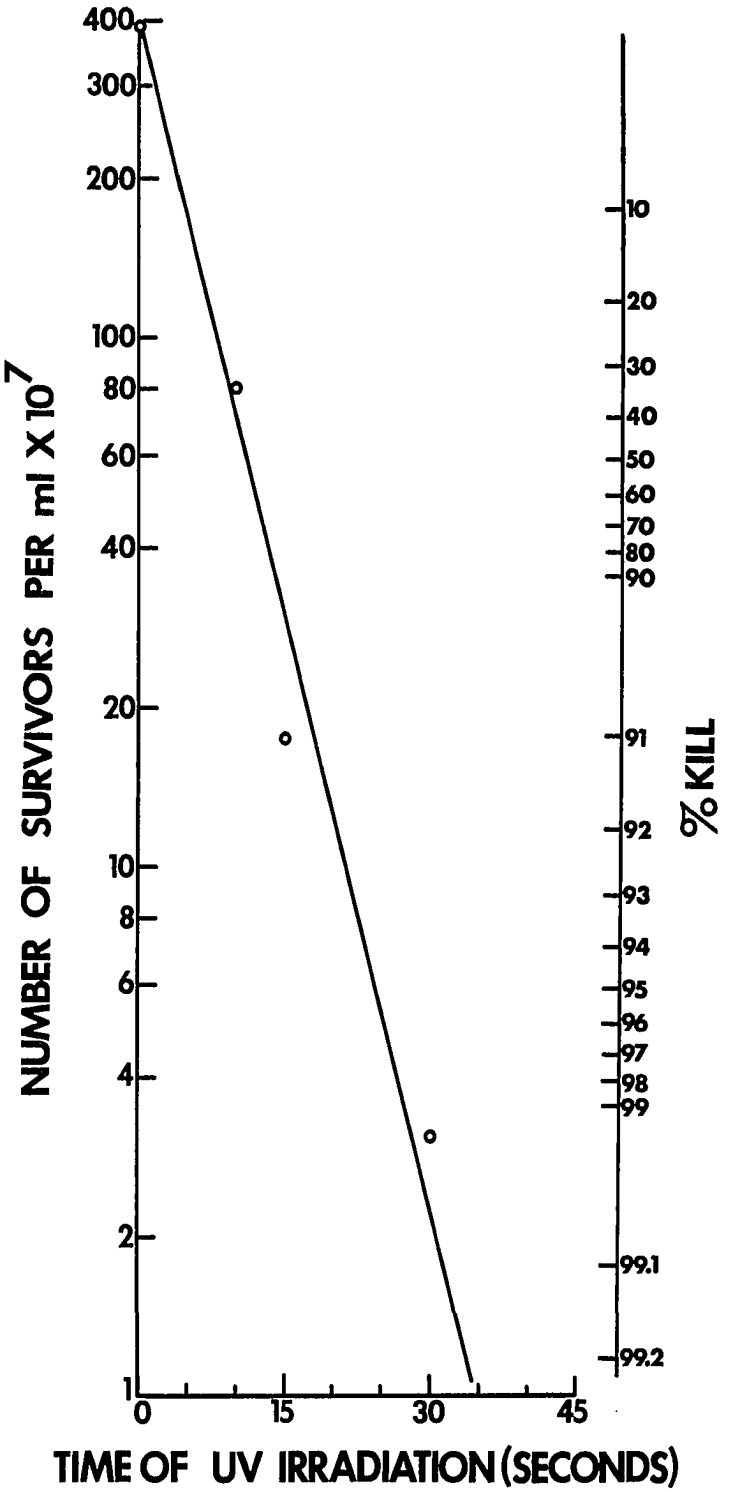


Figure 8. Survival of E. coli, K12, as a function of UV dosage.

N-Methyl-N'-nitro-N-nitrosoguanidine (NTG). The bacteria were cultivated overnight in MMA plus any auxotrophic requirements. Two ml were transferred into 8 ml of fresh MMA plus auxotrophic requirements and incubated for two hours or until cell density was 2-5 x 10^8/ml. Five ml of these log phase cells were centrifuged, washed with two ml of sterile TM buffer and resuspended in 1.0 ml of TM buffer. One ml of NTG solution (60 μg per ml, made fresh in H_2O) was added to the cell suspension, followed by incubation without aeration for 30 minutes. An aliquot (0.4 ml) of the treated cells was added to 5.0 ml of sterile saline (0.85% NaCl), centrifuged and resuspended in 2.0 ml of saline. This is essentially the procedure of Adelberg, et al. (121) except that NTG was used in a concentration of 30 μg per ml (instead of 100 μg per ml) and incubated for 30 minutes (instead of 10 minutes).

Nitrous acid (NA). The bacteria were grown overnight in MMA containing auxotrophic requirements. Five ml of this culture were centrifuged, washed with 5.0 ml of 0.1M acetate buffer, pH 4.6, centrifuged and resuspended in 0.3 ml of nitrous acid (prepared no more than one hour before use by adding 3.45 mg of sodium nitrite per ml of 0.1M acetate buffer, pH 4.6). After incubation for 10 minutes without aeration, 5.0 ml of MMA were added to stop mutagenesis and the cells were centrifuged and resuspended in 2.0 ml of MMA (116).

2-Aminopurine (2-AP). The bacteria were grown overnight in LC broth plus 2-AP (600 μg/ml) in 10 ml quantities with a starting inoculum of approximately 10^2 cells per tube. 2-AP (0.6 ml per 10 ml LC broth) was added from a stock solution of 10 mg/ml (116).

incubated 3 hours in 18 mm tubes containing 10 ml MMA supplemented with parental growth requirements plus requirements for which auxotrophy was being sought. Cells were centrifuged, washed with 2.0 ml MMA, centrifuged and resuspended in 0.5 ml MMA which was added directly to 10 ml of MMA containing parental growth requirements but not containing the requirements for which auxotrophy was being sought. After incubation for 3 hours, ampicillin (0.1 ml of a 2.0 mg/ml solution) was added (final concentration, 20 g/ml) and the cultures reincubated for 60 minutes. The ampicillin-treated cells were centrifuged, washed with 5.0 ml MMA, centrifuged, and resuspended in 0.3 ml MMA which was added directly to 10 ml of MMA supplemented with parental growth requirements for which auxotrophy was being sought.

Following overnight incubation, 1.0 ml was removed, centrifuged, washed with 5.0 ml MMA, centrifuged and resuspended in 0.5 ml of MMA. A 0.1 ml aliquot of this suspension was added to 10 ml of MMA with parental growth requirements but without requirements for which auxotrophy was being sought. These cultures were incubated for 3 hours. The cells were again treated for one hour with ampicillin as described above. Following the second ampicillin treatment, cells were centrifuged, washed with 5.0 ml of MMA, centrifuged, and resuspended in 2.0 ml saline. Samples (0.1 ml) of 10^2, 10^3, 10^4 saline dilutions were spread onto limited enrichment media.

Characterization of Mutants

Growth response on arginine intermediates. All arginine auxotrophs

isolated in the course of this study were characterized, after purification, as to their ability to grow on MMA plus acetylornithine (argA, B, C or D mutants), ornithine (argE mutants), citrulline (argF or I mutants) or arginine (argG or H mutants) (all intermediates added to media to give final concentration of 100 μg/ml).

Reversion studies. The methods used to characterize the revertibility of mutations were those of Whitfield, et al. (146) except as adapted for E. coli.

Spontaneous. The bacteria were grown overnight in 5.0 ml of NB. Fresh NB (5.0 ml) was added, and the cultures incubated for two hours. The turbidity of a 5.0 ml sample of the culture was measured, the remaining 5.0 ml was centrifuged and resuspended in NB to a cell density of 1.0×10^9/ml (Fig. 7). To 2.5 ml of MMAarg softagar (kept molten at 48^0C) was added 0.1 ml of cell suspension (1×10^8 cells/plate). The soft agar was poured over a plate of MMAarg. The number of colonies arising after five days of incubation is a direct indication of the spontaneous reversion rate. Duplicate plates were prepared for each strain in order that the reversion rate per 2.0×10^8 cells could be calculated.

NTG. The cells were prepared exactly as for spontaneous reversion. After the cells were overlayed, a drop of NTG (300 μg/ml) sterilized by filtration was placed on top of the soft agar layer. Plates were then incubated for 5 days and scored for colony growth peripheral to a small zone of inhibition due to too high a concentration of NTG to allow growth. Alternatively, NTG crystals were used.

Diethylsulfate (DES). The cells were prepared exactly as for spontaneous reversion. After the cells were overlayed, a drop of undiluted DES was placed on the surface of the soft agar layer, the plates incubated for 5 days and scored for

colony growth peripheral to a zone of inhibition due to too high a concentration of DES to allow growth.

Bacteriophage Techniques

Lysate preparation. Lysates of P1kc were prepared by the soft-agar overlay technique. The bacteria used as the donor were grown overnight in 3.0 ml LC broth. Fresh LC broth plus Ca^{+2} (3.0 ml) was added and the cultures incubated for two hours. To 2.5 ml of melted LC plus Ca^{+2} soft agar, were added 10^6 phage and 0.2 ml of donor bacteria and the entire mixture poured over an LC + Ca^{+2} agar plate. The plate was incubated for 6-8 hours until confluent lysis of the bacteria had occurred, then 4.0 ml of LC broth was pipetted onto the surface of the soft agar and allowed to stand overnight. The broth was removed, treated with chloroform to kill remaining bacteria, centrifuged to remove cell debris and bits of soft agar, and stored at $4^{\circ}C$ in a screw-capped tube. The lysate was titered to determine the number of phage (plaque forming units - pfu) per ml. This was done exactly as above except that approximately 10^2 pfu per plate were added instead of 10^6 pfu per plate and any phage sensitive strain could serve as an indicator. At the end of an overnight incubation, the number of plaques was counted (no confluent lysis).

Transduction. The recipient bacteria were grown overnight in 5.0 ml of LC broth. After addition of 5.0 ml of LC broth + Ca^{+2}, the culture was incubated for two hours. The turbidity of a 5.0 ml sample, withdrawn aseptically, was determined in a Klett-Summerson colorimeter with a No. 66 filter. The remaining 5.0 ml of culture were centrifuged and resuspended in LC + Ca^{+2} broth to give 1.0×10^9

cells/ml (Fig. 7). Bacteria and phage were then mixed gently in a total volume of 1.0 ml of LC + Ca^{+2} broth and incubated for 30 minutes without shaking. The exact quantity of each is dependent upon the ratio of phage to bacteria desired. This ratio is referred to as the multiplicity of infection (MOI). Infected cells were then centrifuged and resuspended in 1.0 ml MMA. Appropriate dilutions (dependent upon the number of bacteria infected and the MOI) were spread onto selective media. Transductants formed good-sized colonies after 48 hours incubation.

Auxotrophy to prototrophy. In the case of transduction from auxotrophy to prototrophy, selective media consisted of MMA agar minus the requirement for which prototrophy was sought plus any other auxotrophic requirements of the recipient strain.

Prototrophy to auxotrophy. If a transduction from prototrophy to auxotrophy was performed, the infected cells (suspended in MMA) were put through an ampicillin enrichment procedure in order to detect the rare auxotrophic transductants.

Dominant allele to recessive allele. If the donor marker is recessive to the recipient allele, then it is necessary to allow segregation of the two alleles in the "transductant" before placing the bacteria on selective media. For example, in the case of transfer of a $\underline{str^r}$ allele into a recipient which contains a $\underline{str^s}$ allele, the infected cells were plated onto MMA agar plus any auxotrophic requirements of the recipient. After six hours of incubation, streptomycin was added to the agar by spraying 0.3 ml of a stock solution of 10 mg/ml streptomycin onto the agar surface using a 10 ml glass atomizer (Kensington Scientific). Only $\underline{str^r}$ transductants formed colonies upon reincubation for an additional 42 hours.

colony growth peripheral to a zone of inhibition due to too high a concentration of DES to allow growth.

Bacteriophage Techniques

Lysate preparation. Lysates of P1kc were prepared by the soft-agar overlay technique. The bacteria used as the donor were grown overnight in 3.0 ml LC broth. Fresh LC broth plus Ca^{+2} (3.0 ml) was added and the cultures incubated for two hours. To 2.5 ml of melted LC plus Ca^{+2} soft agar, were added 10^6 phage and 0.2 ml of donor bacteria and the entire mixture poured over an LC + Ca^{+2} agar plate. The plate was incubated for 6-8 hours until confluent lysis of the bacteria had occurred, then 4.0 ml of LC broth was pipetted onto the surface of the soft agar and allowed to stand overnight. The broth was removed, treated with chloroform to kill remaining bacteria, centrifuged to remove cell debris and bits of soft agar, and stored at $4^{\circ}C$ in a screw-capped tube. The lysate was titered to determine the number of phage (plaque forming units - pfu) per ml. This was done exactly as above except that approximately 10^2 pfu per plate were added instead of 10^6 pfu per plate and any phage sensitive strain could serve as an indicator. At the end of an overnight incubation, the number of plaques was counted (no confluent lysis).

Transduction. The recipient bacteria were grown overnight in 5.0 ml of LC broth. After addition of 5.0 ml of LC broth + Ca^{+2}, the culture was incubated for two hours. The turbidity of a 5.0 ml sample, withdrawn aseptically, was determined in a Klett-Summerson colorimeter with a No. 66 filter. The remaining 5.0 ml of culture were centrifuged and resuspended in LC + Ca^{+2} broth to give 1.0×10^9

cells/ml (Fig. 7). Bacteria and phage were then mixed gently in a total volume of 1.0 ml of LC + Ca^{+2} broth and incubated for 30 minutes without shaking. The exact quantity of each is dependent upon the ratio of phage to bacteria desired. This ratio is referred to as the multiplicity of infection (MOI). Infected cells were then centrifuged and resuspended in 1.0 ml MMA. Appropriate dilutions (dependent upon the number of bacteria infected and the MOI) were spread onto selective media. Transductants formed good-sized colonies after 48 hours incubation.

Auxotrophy to prototrophy. In the case of transduction from auxotrophy to prototrophy, selective media consisted of MMA agar minus the requirement for which prototrophy was sought plus any other auxotrophic requirements of the recipient strain.

Prototrophy to aux[illegible] prototrophy to auxotrophy was performed, the i[illegible] were put through an ampicillin enrichment proce[illegible]otrophic transductants.

Dominant allele t[illegible] marker is recessive to the recipient allele, then it is n[illegible]gation of the two alleles in the "transductant" before placing the bacteria on selective media. For example, in the case of transfer of a $\underline{str^r}$ allele into a recipient which contains a $\underline{str^s}$ allele, the infected cells were plated onto MMA agar plus any auxotrophic requirements of the recipient. After six hours of incubation, streptomycin was added to the agar by spraying 0.3 ml of a stock solution of 10 mg/ml streptomycin onto the agar surface using a 10 ml glass atomizer (Kensington Scientific). Only $\underline{str^r}$ transductants formed colonies upon reincubation for an additional 42 hours.

On the other hand, if an argR$^+$ recipient is transduced to argR$^-$ and the recipient is prototrophic for arginine, the addition of 0.1 mg/liter of arginine to the AF + Can agar is sufficient to allow expression of the recessive argR$^-$ phenotype without allowing significant growth of the argR$^+$ background. If the recipient is auxotrophic for arginine and the block occurs before ornithine, the addition of ornithine (100 µg/ml) to AF + Can agar media is sufficient to allow segregation and to fulfill the auxotrophic requirement. The addition of the same concentration (100 µg/ml) of arginine or citrulline to AF + Can allows growth of the argR$^+$ strains.

It should be recalled that the ability to excrete arginine together with resistance to canavanine are the two criteria for distinguishing an argR$^-$ genotype on solid media. It is necessary to check the ability of canavanine-resistant, presumptive argR$^-$ transductants to crossfeed an arginine auxotroph. To an overnight 3.0 ml BH culture of EcK-444, were added 3.0 ml of fresh BH. Following a two hour incubation, cells were centrifuged, washed with 2.0 ml saline, centrifuged, resuspended in 2.0 ml of saline, and 0.1 ml of a 2×10^2 saline dilution spread as a lawn of cells onto MMA + orn (200 µg/ml) agar containing any growth requirements of the presumptive argR$^-$ transductants other than arginine. The "argR$^-$ strains" were inoculated onto the plates as stabs with a straight-wire inoculating needle. Crossfeeding (detectable as a halo of growth around a stab) is apparent after 24-48 hours incubation.

Deletion mapping. The method used in constructing a deletion map was modified slightly from those of Blume and Balbinder (18).

Spot plate test. Recipient cells were grown overnight in 3.0 ml of LC broth.

To this culture, 3.0 ml of fresh LC + Ca^{+2} broth were added; the cells were incubated for two hours. These "log phase" cells (0.1 ml) were spread as a lawn onto EMMA agar. A drop of phage which had been propagated twice on the donor bacteria was spotted onto the recipient lawn. In this way, 16 donors could be tested on the same plate. The donor phage were prepared by making a 1:10 dilution in phage dilution buffer of the stock lysate and irradiating with UV light for 34 seconds in an open sterile glass petri dish. This degree of irradiation gives 10% survival of phage (Fig. 9) and increases the frequency of transduction and hence the sensitivity of the spot plate test. The number of transductants arising after 48 hours incubation was scored. Any cross which yielded 6 or fewer transductants was repeated using the half plate test.

Half plate test. The recipient lawn and the donor phage were prepared as above. This test, however, consisted of spreading 0.1 ml of irradiated phage over half a plate, thus increasing by 10-20 fold the number of phage-infected bacteria. Consequently, the sensitivity of detection of $\underline{arg}^+$ transductants was enhanced 10-20 fold.

Whole plate test. This was a standard transduction (see above). The MOI used was 5.0 and the number of bacteria infected was 10^8. Infected cells (0.1 ml) were suspended in 1.0 ml MMA and spread onto MMA + met plates.

Conjugation Techniques

Interrupted mating. The procedure followed was that of Curtiss (29). Recipient (F^-) and donor (Hfr) cells were grown overnight in 5.0 ml of MMC + 0.1% N-Z Case. The Hfr was grown without shaking whereas the F^- recipient was

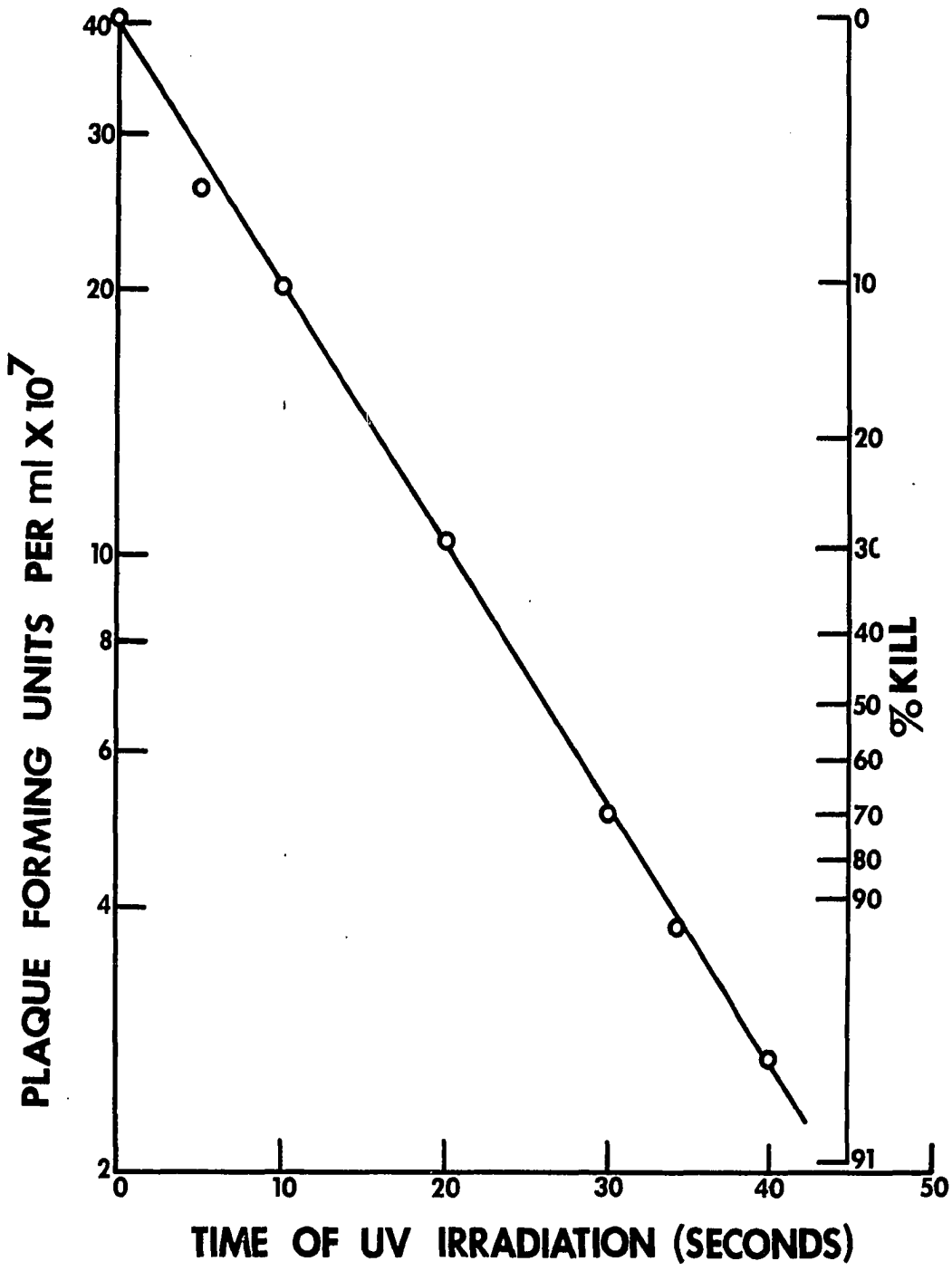


Figure 9. Survival of bacteriophage Plkc as a function of UV dosage.

grown with shaking. Both donor and recipient were brought into "log phase" by addition of 5.0 ml of fresh MMC + 0.1% N-Z Case and reincubated for two hours. The F^- recipient was centrifuged, washed with 5.0 ml MMC + 0.5% glucose, centrifuged, resuspended in 5.0 ml MMC + 0.5% glucose and incubated for 1 hour to effect amino acid starvation. The "starved" F^- cells and the "log" Hfr cells were centrifuged, washed with 3.0 ml MMM, centrifuged and resuspended in MMM (10 ml for Hfr, 1.0 ml for F^-). To the "mating flask" (125 ml Micro-Fernbach flask, deLong neck-Bellco) containing 10 ml MMM + 0.1% N-Z Case were added 0.1 ml of Hfr and 0.1 ml of F^- cells. The "mating flask" was then incubated for the desired time. Mating was interrupted by addition of 0.2 ml of the "mating culture" to 1.8 ml of MMC + 0.5% glucose plus any requirements of the recombinants sought followed by vigorous vortexing for 60 seconds. If the F^- is str^r and the Hfr is str^s streptomycin (final concentration of 100 μg/ml) can be added to the "interruption media" to select against the Hfr.

Hfr purification. Periodically, Hfr strains were purified to eliminate that portion of the population in which the sex factor had become dissociated from the bacterial chromosome (21). Bacteria were grown overnight in 1 ml of PBE broth, a small loopful transferred into 1 ml of fresh PBE broth, and mixed. A loopful of this culture was inoculated into: (1) 1.0 ml of fresh PBE broth and (2) 1.0 ml of PBE broth plus acridine orange (50 μg/ml) and incubated for 24 hours. Dilutions of 10^4 and 10^5 were spread onto NBG agar plates and incubated for 24 hours. Small colonies were picked for the transfer spot check (see below) as Hfr strains generally grow a bit more slowly than do the corresponding F^- strains. Acridine orange solution (1 mg/ml) was prepared in H_2O and diluted 1:20 with PBE broth prior to use.

Transfer spot check. In order to check the ability of an Hfr or F' strain to transfer genetic material, a spot test was performed as follows. The Hfr or F' and the recipient (F^-) were grown overnight in 3.0 ml of BH broth. Alternatively, if many donors (more than 50) were being checked, they were grown overnight on BH agar (16 strains per plate). Three ml of fresh BH broth were added to overnight cultures and incubated for two hours. The recipient (0.1 ml of a 2×10^2 saline dilution of the "log phase" BH culture) was spread as a lawn onto MMA plus recipient growth requirements but without the requirement for which prototrophy was being sought. The "log phase" donors were centrifuged, resuspended in 5.0 ml of MMA, and a loopful of cells spotted on the recipient lawn (16 spots per plate). Alternatively, if donors were grown on BH agar, a loopful of cells was spotted directly from the agar plate onto the recipient lawn. The spot plates were incubated for 24-48 hours and scored for colony formation. Auxotrophic requirements of the donor strain were omitted as a method of selecting against growth of donor cells.

Biochemical Methods

Preparation of Cell-Free Extracts

The following procedure was used to prepare cell-free extracts unless otherwise noted. The bacteria were grown overnight in a flask containing 50 ml of the designated medium to a cell density corresponding to 40 Klett units (mid-log phase). Eighteen ml were transferred into 300 ml of the same medium and reincubated. When the cell density again reached 40 Klett units, the culture was chilled and collected by centrifugation at $4^\circ C$ in a Sorvall RC2-B centrifuge (GSA head) at 10,000 rpm for thirty minutes. The cells were resuspended in 4.0 ml of 0.1M

PO_4 buffer, pH 7.0, containing 1×10^{-2}M GSH (3.08 mg/ml buffer), and sonified for 90 seconds in a 10 ml beaker with a Branson sonifier (Model S125) at a power setting of 4.0. Cell debris and unbroken cells were removed by centrifugation at 4^{0}C in a Sorvall RC2-B centrifuge (SS-1 head) at 17,000 rpm for 10 minutes. Portions of the cell-free extract were placed into four tubes and frozen until assayed.

Lowry Determination of Total Protein

The procedure used is a modification (151) of the Lowry method. Appropriately diluted extract (0.1 ml) was added to 0.9 ml H_2O. Five ml of a copper-EDTA reagent (containing in g/l: $Na_2CuEDTA$, 0.25; Na_2CO_3, 20; NaOH, 4.0) were added and the mixture vortexed vigorously. After 15.0 minutes, 0.50 ml of Folin and Ciocalteu phenol reagent (diluted 1:3 with H_2O just prior to use) was added followed by vigorous vortexing. After 30.0 minutes the amount of color was read in the Klett-Summerson colorimeter (No. 66 filter) which had been zeroed against a reagent blank. The addition of each reagent was individually timed. Varying amounts of bovine serum albumin were used to prepare a standard curve relating protein concentration to Klett units. A typical standard curve is shown in Fig. 10. All standard reaction mixtures contained 0.1 ml phosphate buffer plus GSH.

N-Acetyl-γ-Glutamokinase Assay

Principle. This assay is a modification of the assay of Sadasiv (109). The reaction yields as a by-product, ADP, which in the presence of pyruvate kinase and 2-phosphoenolpyruvate, leads to formation of pyruvate and subsequent regeneration of ATP. The pyruvate produced was determined colorimetrically as its 2,4-dinitrophenylhydrazone.

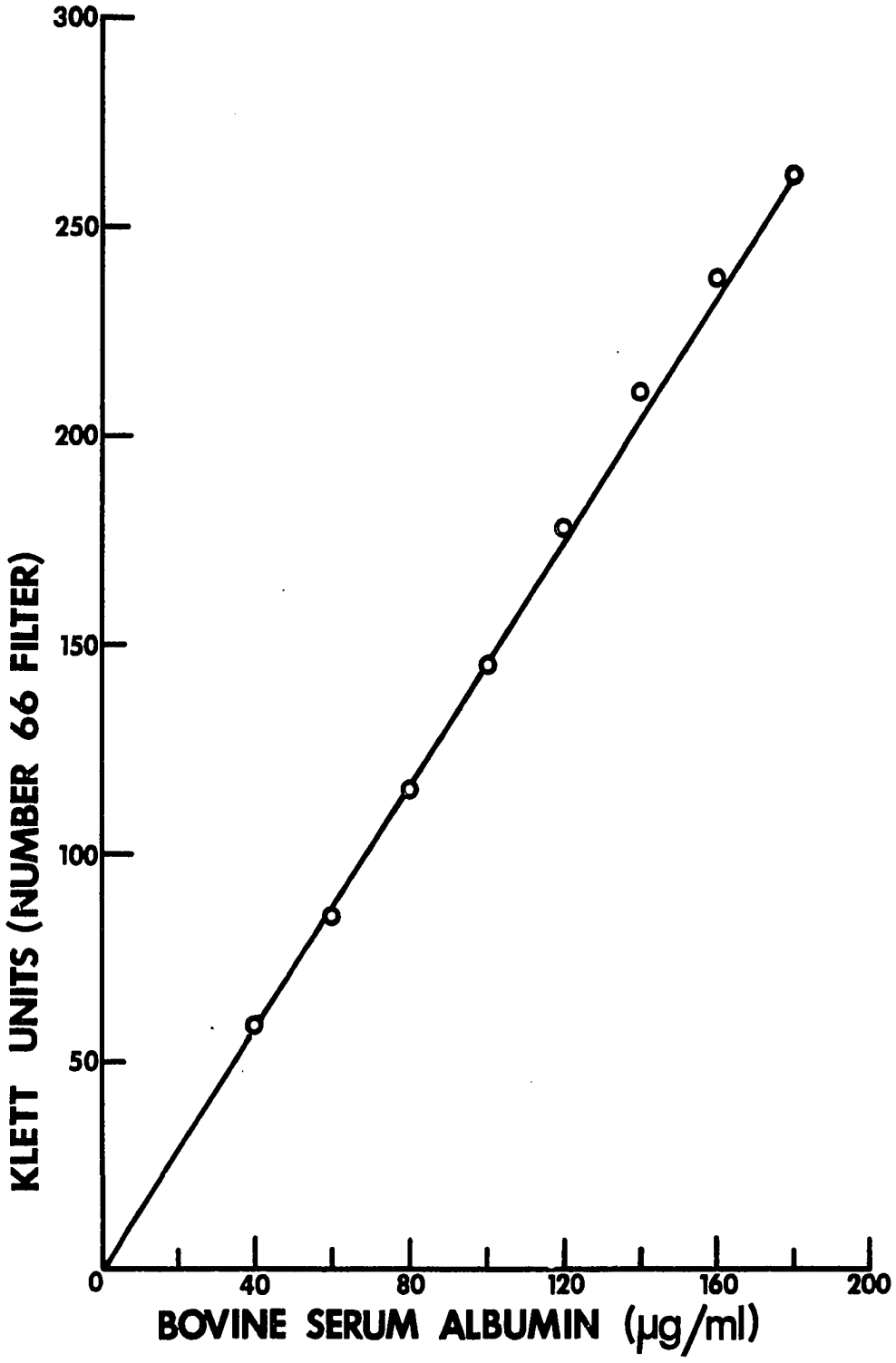


Figure 10. Standard curve relating bovine serum albumin concentration to Klett units.

Reagents. See Table 5.

Procedure. The assay was performed at 37°C in Wasserman test tubes containing the above reagents. The reaction was started by the addition of N-acetyl-L-glutamate and was stopped by addition of 0.5 ml of 0.1% 2,4-dinitrophenylhydrazine in 4N HCl. The reaction mixture was allowed to remain at room temperature for 15.0 minutes, 5.0 ml of 1.5 N NaOH was added and the contents subjected to vigorous agitation on a Vortex mixer. Fifteen minutes after NaOH addition, the color intensity was read in a Klett-Summerson colorimeter fitted with a No. 54 filter. The colorimeter was zeroed with a reagent blank. The blank for the enzyme reaction consisted of omitting the substrate (N-acetyl-L-glutamate) until after the addition of 2,4-dinitrophenylhydrazine. Sodium pyruvate was used as a standard.

Unit of Enzyme Activity. One unit of N-acetyl-γ-glutamokinase activity is defined as that amount of enzyme which catalyzes the formation of 0.1 μmole of pyruvate in 15 minutes at 37°C.

N-Acetylglutamic-γ-Semialdehyde Dehydrogenase Assay

Principle. The assay is a modification of the procedure of Marshek (87) and measures the increased absorbance at 340 mμ due to the reduction of NADP which occurs when N-acetylglutamic-γ-semialdehyde is converted enzymatically to N-acetylglutamyl phosphate (the reverse reaction).

Reagents. See Table 6.

Procedure. After the addition of NADP, timing was started and the increase in optical density at 340 mμ followed on a Gilford spectrophotometer

TABLE 5

REACTION MIXTURE FOR N-ACETYL-γ-GLUTAMOKINASE ASSAY

Reagent	Molarity	Made Up In	Vol. (ml)	Amount
[1] K-phosphate buffer, pH 7.4	0.500	H_2O	0.12	60 μmoles
ATP, $Na_2 \cdot 4H_2O$	0.040	0.1M K_2HPO_4	0.03	1.2 μmoles
2-Phosphoenolpyruvate, $Na_3 \cdot 3H_2O$	0.030	H_2O	0.03	9.9 μmoles
[2] Pyruvate kinase solution	50 E.U./ml	0.1M K-phosphate, pH 7.4, containing 1×10^{-3}M $MgCl_2$	0.06	3.0 E.U.
$MgCl_2 \cdot 6H_2O$	0.050	H_2O	0.06	3.0 μmoles
N-Acetyl-γ-glutamokinase	———	0.1M K-phosphate, pH 7.4, containing 2×10^{-4}M Cleland's reagent	0.10	as indicated
[3] Potassium flouride	0.040	H_2O	0.10	4.0 μmoles
N-Acetyl-L-glutamate	0.20	H_2O, pH adjusted to 7.4 with NaOH	0.10	20 μmoles

[1] The first five reagents shown can be prepared as a mixture and dispensed in 0.30 ml portions.
[2] The pyruvate kinase solution was prepared by dissolving the commercial ammonium sulfate slurry (1500 enzyme units) in several volumes of 0.1M K-phosphate, pH 7.4, containing 1×10^{-3}M $MgCl_2$, dialyzing at 4° against same buffer and making dialyzed solution to 30 ml with the same buffer. The final solution is stored at 4°C.
[3] It is shown in "Results" that this concentration of flouride does not inhibit N-acetyl-γ-glutamokinase.

TABLE 6

REACTION MIXTURE FOR N-ACETYLGLUTAMIC-γ-SEMIALDEHYDE DEHYDROGENASE ASSAY

Reagent	Molarity	Made Up In	Vol. (ml)	Amount
N-acetylglutamic-γ semialdehyde dehydrogenase	———	0.1M K-PO_4, pH. 7.0 containing 0.1M GSH	0.1	as indicated
[1]N-acetylglutamic-γ-semialdehyde	0.012	H_2O	0.3	3.6μmoles
Glycine-NaOH buffer, pH 9.8	0.200	H_2O	0.5	100 μmoles
K_2HPO_4	0.200	H_2O	0.2	40 μmoles
H_2O	———	———	0.1	———
NADP	0.002	H_2O	0.3	6.0μmoles

[1] For preparation of N-acetylglutamic-γ-semialdehyde, see page 53.

(Model 2400). The pH of the reaction mixture was 9.3. The assay was performed at 37°C.

If the reaction is started by the addition of the semialdehyde rather than by NADP, the initial reaction velocity is one-half that obtained under standard conditions.

Unit of enzyme activity. One unit of N-acetylglutamic- γ-semialdehyde dehydrogenase activity is defined as that amount of enzyme which causes an increase in absorbance of 0.010 per minute at 37°C.

Preparation and purification of N-acetylglutamic- γ-semialdehyde. N-acetylglutamic- γ-semialdehyde was prepared enzymatically from N-acetylornithine via the action of the enzyme acetylornithine δ-transaminase.

Purification of N-acetylornithine δ-transaminase. This enzyme was partially purified (70) from EcW-468 which is acetylornithinase-less and produces acetylornithine δ-transaminase at derepressed rates. The purification procedure is summarized in Fig. 11. The cells were grown to stationary phase in 165 liters of MME + arginine (100 μg/ml), harvested (wet weight yield of 2 grams of cells per liter harvested), and suspended in ice cold 0.1M phosphate buffer, pH 7.0, containing 10^{-3}M β-mercaptoethanol (B-ME) and 10^{-6}M pyridoxal phosphate (B_6PO_4) (Buffer 1). All operations were conducted at 4°C unless otherwise indicated. Cells were suspended in 1 ml of buffer per gram of cells and disrupted with a French press (18,000 psi). The cell-free extract, obtained after centrifugation at 31,890 x g for 30 minutes, was dialyzed for 12 hours against 20 volumes of Buffer 1. (This dialysate is Fraction I).

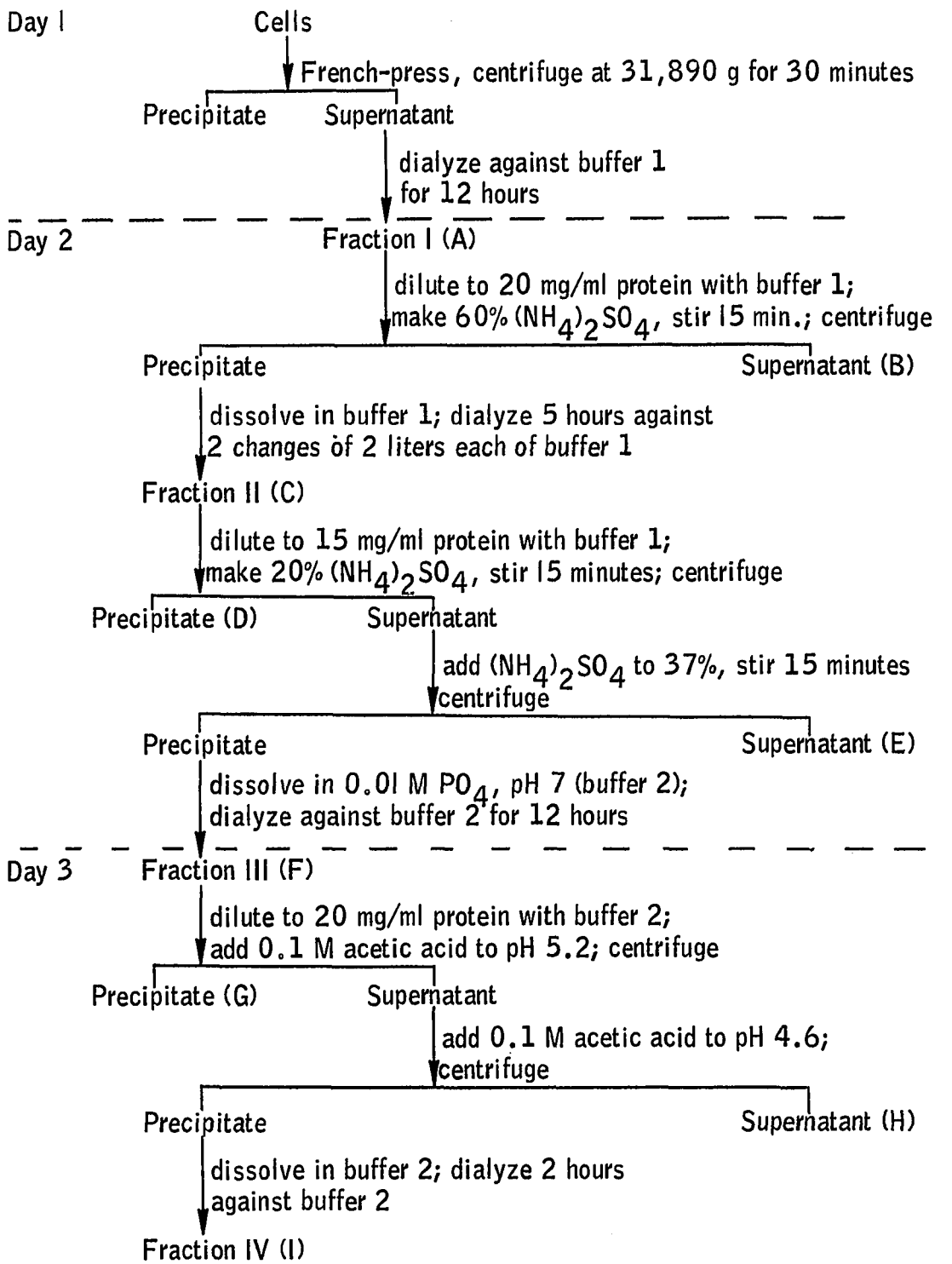


Figure 11. Flow diagram of N-acetylornithine δ-transaminase purification procedure.

Fraction I was diluted to 20 mg protein per ml with Buffer 1, made 60% saturated by addition of solid ammonium sulfate and stirred for 15 minutes. The precipitate was collected by centrifugation, dissolved in a minimum volume of Buffer 1 and dialyzed for five hours against 2 changes of 2 liters each of Buffer 1. (This dialysate is Fraction II).

Fraction II was diluted with Buffer 1 to a protein concentration of 15 mg/ml, followed by the addition of solid ammonium sulfate to bring the solution to 20% saturation. After stirring for 15 minutes, the precipitate was removed by centrifugation, and the supernatant fluid made 37% saturated with respect to ammonium sulfate (added as the solid form). The resulting precipitate was collected by centrifugation, dissolved in a minimum volume of 0.01M phosphate buffer, pH 7.0, containing 10^{-3}M B-ME and 10^{-6}M B_6PO_4 (Buffer 2), and dialyzed for 12 hours against 20 volumes of Buffer 2. (This dialysate is Fraction III).

Fraction III was diluted with Buffer 2 to a protein concentration of 20 mg/ml and 0.1M acetic acid was added dropwise while stirring to adjust the pH to 5.2. The resulting precipitate was removed by centrifugation. The pH of the supernatant was adjusted to 4.6 by further addition of 0.1M acetic acid, and the precipitate collected by centrifugation. The pH 4.6 pellet was dissolved in a minimum volume of Buffer 2 and dialyzed for 2 hours against 20 volumes of Buffer 2. This dialysate is Fraction IV. Fraction IV represents an approximately six-fold purification and a 57% recovery of the starting transaminase activity in the crude extract (Fraction I). There was a yield of approximately 5 mg "transaminase" protein per gram of cells harvested. For a summary of a typical purification, see

Table 7.

Preparation of N-acetylglutamic-γ-semialdehyde. The preparative reaction mixture contained approximately 100 mg of partially purified acetylornithine δ-transaminase (Fraction IV), 0.5 g of acetylornithine, 0.4 g of γ-ketoglutarate, and 1.0 mg of pyridoxal phosphate in a total volume of approximately 13 ml. The α-ketoglutarate was neutralized before Fraction IV was added, so as to avoid enzyme denaturation. The reaction mixture was incubated at 30^{0}C for at least 4 hours, after which time there was no further increase in semialdehyde production.

Purification of N-acetylglutamic-γ-semialdehyde. The reaction mixture was boiled for five minutes and centrifuged to remove denatured protein. The semialdehyde formed was purified by passage of the supernatant fluid through two ion-exchange resins. The N-acetylglutamic-γ-semialdehyde was measured by use of the N-acetylornithine δ-transaminase assay, from which the cofactors and extract were omitted. The semialdehyde was in the void volume from the first column (IR120-anionic exchange resin-Cl^{-} form) when eluted with H_2O (Fig. 12). The pooled semialdehyde-containing fractions were chromatographed from the second ion-exchange resin (IR4B-cationic resin-Na^{+} form) by elution with 0.05M K_2SO_4 (Fig. 13). The pooled fractions (tubes 53 through 90) were stored frozen until used.

N-Acetylornithine δ-Transaminase Assay

Principle. N-acetylornithine δ-transaminase was measured by the procedure of Albrecht and Vogel (3). This assay depends on the acid hydrolysis to glutamic-γ-semialdehyde of the enzymatically produced N-acetylglutamic-γ-semialde-

TABLE 7

PURIFICATION OF ACETYLORNITHINE δ-TRANSAMINASE

Fraction	*Assay	Total Protein (mg per ml)	Transaminase (units per ml)	Specific Activity (units/mg protein)	Total Vol. (ml)	Total Units
I	A	52.67	342.9	6.51	226	77,518
	B	———	2.4	———	643	1,543
II	C	37.16	344.8	9.28	330	113,768
	D	———	32.6	———	14.5	476
	E	———	5.8	———	870	5,046
III	F	22.55	422.8	18.75	168	71,022
	G	———	67.2	———	40	2,688
	H	———	0	———	226	0
IV	I	26.89	950.3	35.34	46.5	44,187

*The assay letters refer to fractions indicated in Figure 11.

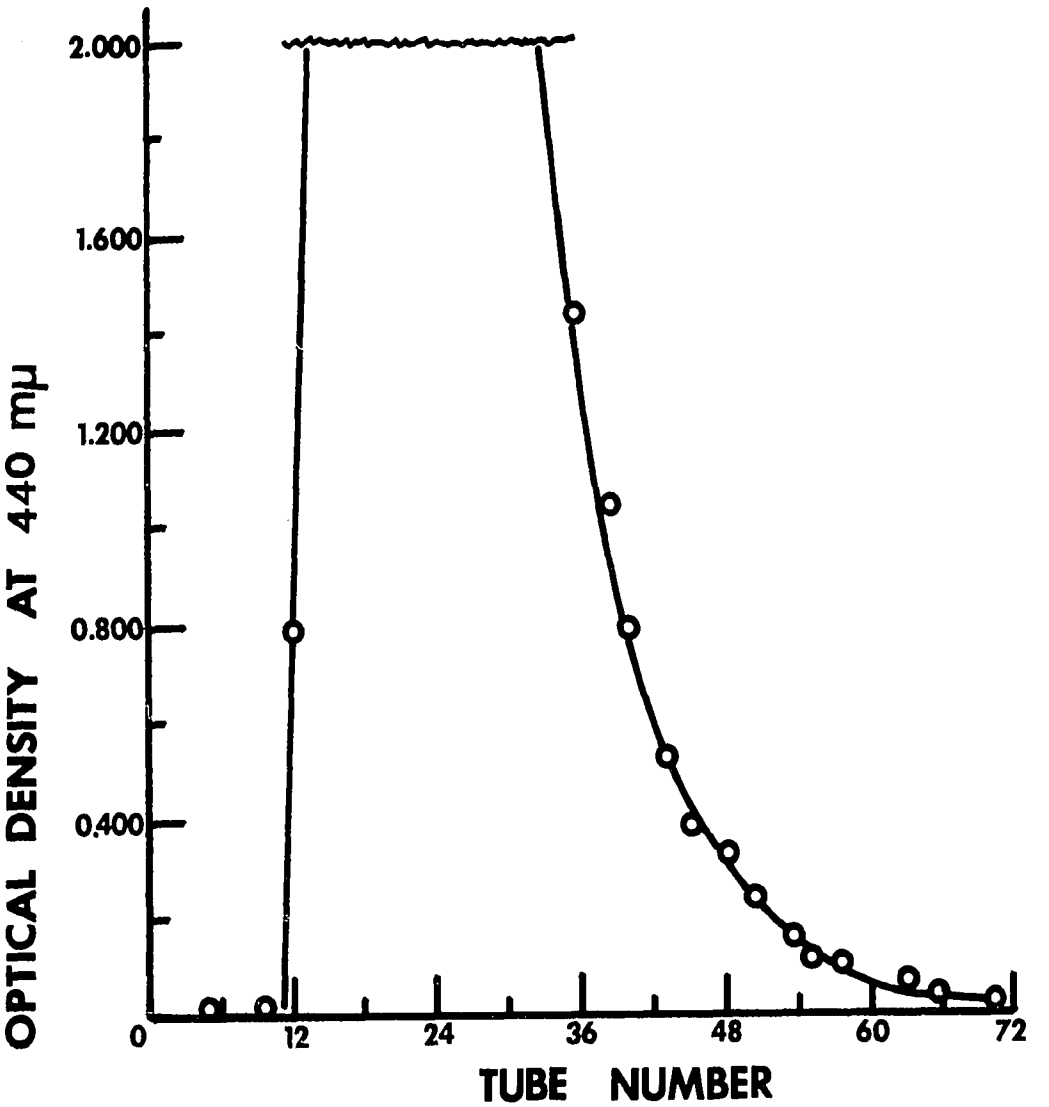


Figure 12. Elution profile of N-acetylglutamic-γ-semialdehyde from IR 120 ion exchange column.

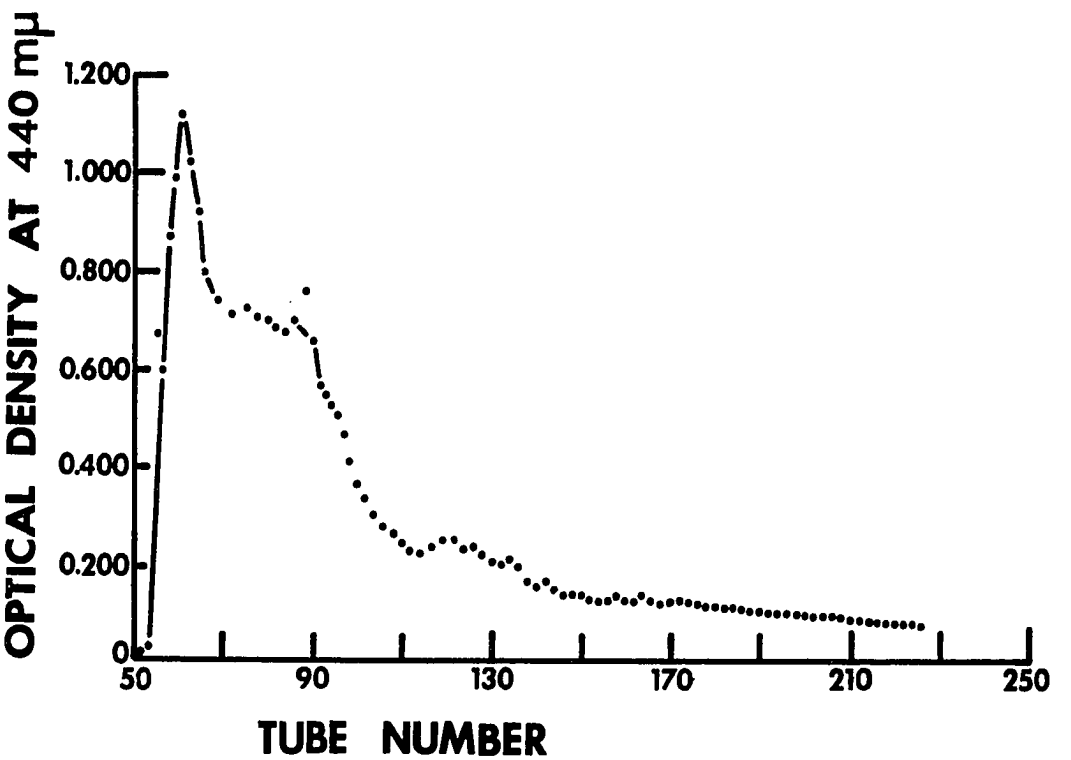


Figure 13. Elution profile of N-acetylglutamic-γ-semialdehyde from IR4B ion exchange column.

hyde. Glutamic- γ-semialdehyde cyclizes spontaneously to Δ^1-pyrroline-5-carboxylic acid which in turn reacts with o-aminobenzaldehyde to yield a yellow dihydroquinazolium compound.

Reagents. See Table 8.

Procedure. The reaction was started by the addition of extract, stopped by the addition of 0.3 ml of 6N HCl, and boiled for 30 minutes (to hydrolyze the N-acetylglutamic- γ-semialdehyde to glutamic- γ-semialdehyde). After addition of 1.0 ml of 3.6M sodium acetate (295 g/l H_2O) and 0.2 ml of 0.33M o-aminobenzaldehyde (oAB), the contents were vigorously agitated on a Vortex mixer. After 15 minutes, the absorbancy at 440 mμ was determined against a reagent blank.

Unit of enzyme activity. One unit of acetylornithine δ-transaminase activity is defined as that amount of enzyme which yields an absorbance of 0.100 at 440mμ per 15 minutes at 37°C. The value of 0.100 corresponds to 0.86 μmoles of Δ^1pyrroline-5-carboxylic acid per hydrolyzed reaction mixture.

General Notes. The commercial o-aminobenzaldehyde (K and K Chemicals) was not pure enough to use as purchased and therefore was redistilled with steam. Care had to be taken in water-flow rate so as to avoid solidification of the oAB in the condenser. The distillate was collected in a receiving flask chilled in an ice bath and allowed to remain in the cold for an hour after collection. The oAB crystallized in the form of water repellent platelets which were filtered on a chilled Buchner funnel, washed with ice-cold distilled water, and dried by vacuum. The residual oAB which did not crystallize in the cold was made to crystallize by the addition of 120 g NaCl/l distillate. The crystals were stored at -20°C.

TABLE 8

REACTION MIXTURE FOR ACETYLORNITHINE δ-TRANSAMINASE ASSAY

Reagent	Molarity	Made Up In	Vol. (ml)	Amount
K-phosphate buffer, pH 8.0	0.50	H_2O	0.10	50 μmoles
Pyridoxal phosphate	9.4×10^{-5}	H_2O	0.10	9.4 μmoles
α -Ketoglutarate, pH 6.0-6.5	0.017	H_2O, pH adjusted with 2N KOH	0.10	1.7 μmoles
Acetylornithine	0.014	H_2O	0.10	1.4 μmoles
Acetylornithine δ -transaminase	———	0.1M KPO_4, pH 7.0, containing 0.1M GSH	0.10	as indicated

Acetylornithinase Assay

Principle. Acetylornithinase was assayed by the method of Vogel and Bonner (142). This assay is based on the determination with ninhydrin of the ornithine produced in the enzymatic reaction.

Reagent. See Table 9.

Procedure. The reaction was started by the addition of substrate (acetylornithine) and stopped by the addition of 1.5 ml of ninhydrin reagent. After the reaction mixture was boiled for 10 minutes, 3.0 ml of 0.7 N NaOH were added, and the contents mixed vigorously on a Vortex mixer. After 20 minutes, the color was read in a Klett-Summerson colorimeter fitted with a No. 42 filter. Water was used as the blank.

Unit of enzyme activity. One unit of acetylornithinase activity is defined as that amount of enzyme which catalyzes the formation of 0.10 μmole of ornithine in 10 minutes at 37^{0}C.

Argininosuccinase Assay

Principle: Argininosuccinase was determined by a modification of the method of Ratner, et al. (105). Instead of the determination of the urea released, this assay is based on the determination with ninhydrin of ornithine formed. The ornithine is formed by added arginase (Worthington Biochemicals) from the arginine produced by argininosuccinase.

Reagents. See Table 10.

Procedure. The reaction was started by the addition of substrate (arginino-

TABLE 9

REACTION MIXTURE FOR ACETYLORNITHINASE ASSAY

Reagent	Molarity	Made Up In	Vol. (ml)	Amount
K-phosphate, pH 7.0	0.40	H_2O	0.10	40 µmoles
$CoCl_2 \cdot 6\ H_2O$	0.001	H_2O	0.10	0.1 µmoles
[1]Glutathione	0.004	H_2O	0.10	0.4 µmoles
Acetylornithinase	———	0.1M KPO_4, pH 7.0	0.10	as indicated
Acetylornithine	0.030	H_2O	0.10	3.0 µmoles

[1] This reagent must be made fresh every 72 hours.

The ninhydrin reagent used to stop the reaction is made up 1% in methyl cellosolve and diluted 2 parts ninhydrin with 1 part of 0.4M citric acid just prior to use.

TABLE 10

REACTION MIXTURE FOR ARGININOSUCCINASE ASSAY

Reagent	Molarity	Made Up In	Vol. (ml)	Amount
K-phosphate, pH 7.5	0.10	H_2O	0.10	10 μmoles
Arginase	40 E.U./ml.	H_2O	0.10	4.0 E.U.
Argininosuccinase	——	0.1M KPO4, pH 7.0, containing 0.1M GSH	0.10	as indicated
[1]Argininosuccinate	0.015	H_2O, pH 7.4	0.10	1.5 μmoles

[1]Argininosuccinate (Calbiochem) is sold as the barium salt. The barium was removed as Ba_2SO_4 by precipitation with 0.4M K_2SO_4 followed by centrifugation.

The ninhydrin reagent used to stop the reaction is the same as that used in the acetylornithinase assay (see Table 9).

succinate), stopped by the addition of 1.5 ml of ninhydrin reagent, and boiled for 10 minutes. After addition of 3.0 ml of 0.7 N NaOH, the reaction mixture was boiled for another 10 minutes and allowed to cool. Color was read against a water blank in a Klett-Summerson colorimeter fitted with a No. 42 filter.

Unit of enzyme activity. One unit of argininosuccinase activity is defined as that amount of enzyme which catalyzes the formation of 0.10 μmole of ornithine in 15 minutes at 37°C.

CHAPTER III

RESULTS

Assays

Before any studies into the regulation of expression of the four clustered arginine genes could be undertaken, it was necessary to show that the assays used for these enzymes gave reproducible results. It was especially important to validate the unpublished assays for enzymes 2 and 3.

N-Acetyl- γ-Glutamokinase (Enzyme 2)

The reaction catalyzed by N-acetyl- γ-glutamokinase is reaction 2, Fig. 1. As described in Chapter II, the assay used measures the pyruvate produced by added pyruvate kinase and is proportional to the amount of ADP which is formed as a by-product of the phosphorylation (109). Pyruvate is detectable as its 2,4-dinitrophenylhydrazone. Color intensity is proportional to pyruvate concentration, although the color fades slowly with time. That the fading is proportional to pyruvate concentration is shown in Fig. 14. In the standard assay, color intensity was read 15 minutes after the addition of NaOH. The amount of substrate used in the original assay (7.5 μmoles) was found not to be saturating (Fig. 15); therefore, a more nearly saturating amount of substrate (20 μmoles of N-acetylglutamate)

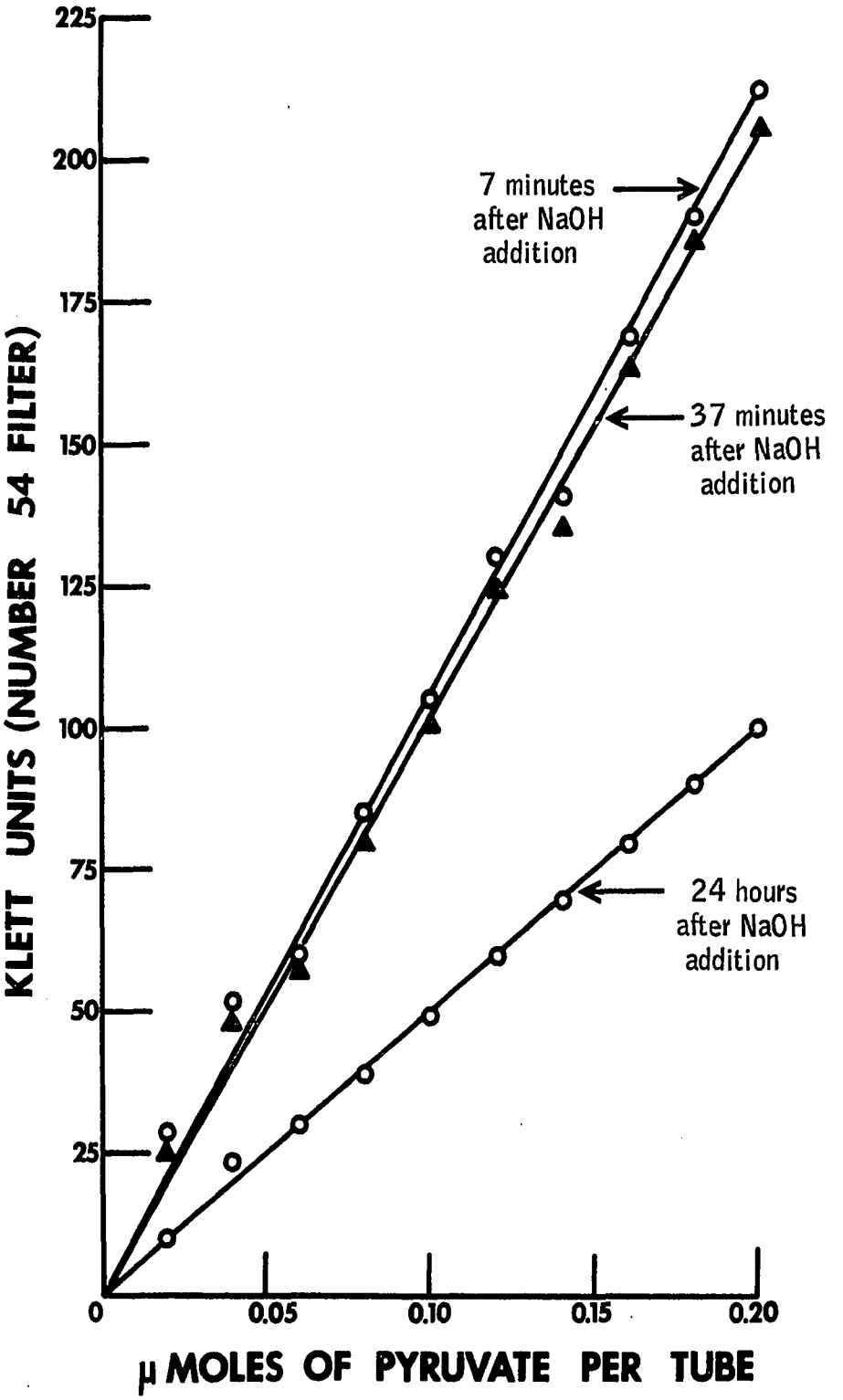


Figure 14. Stability of pyruvate-dinitrophenylhydrazone color as function of time.

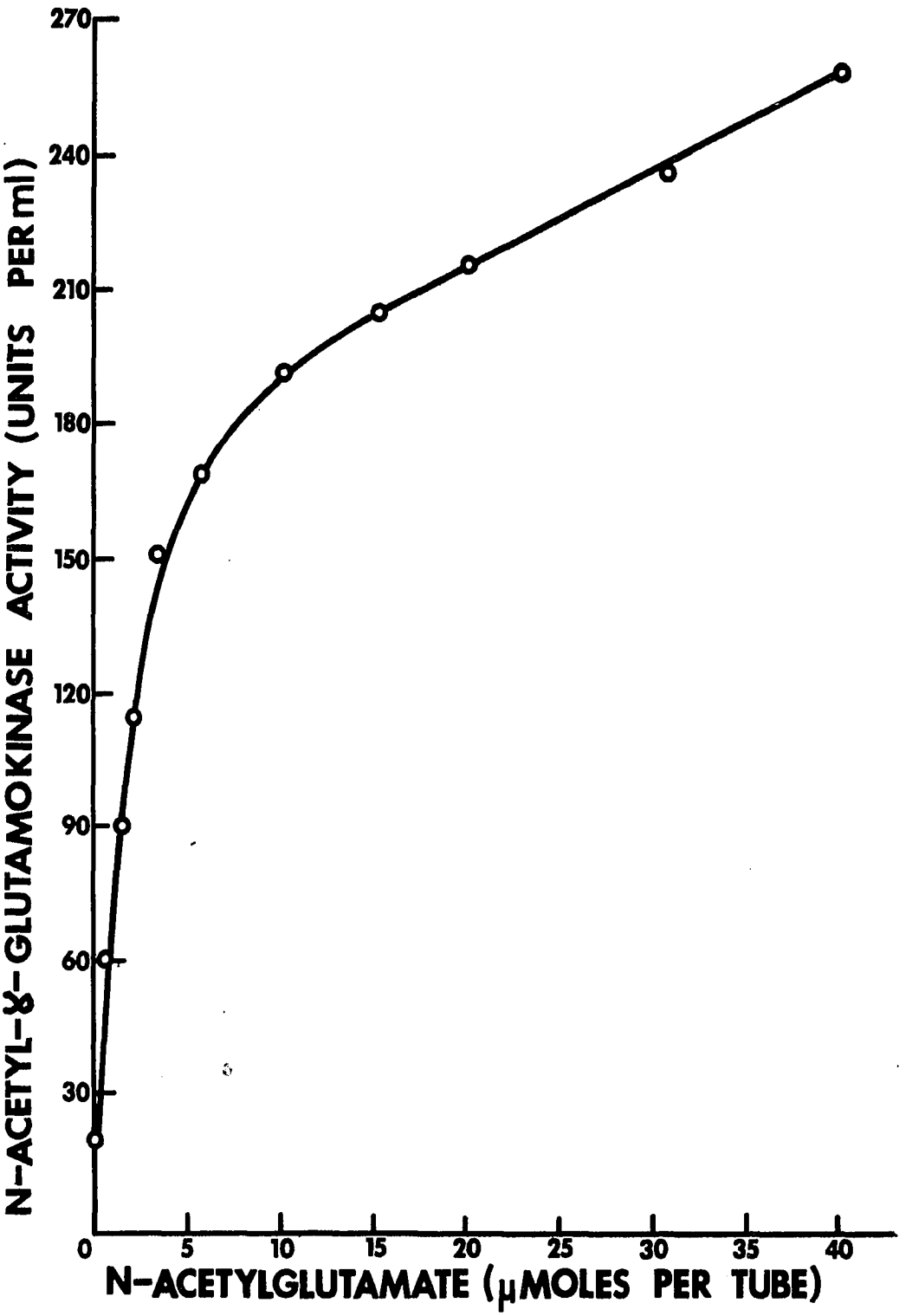


Figure 15. N-acetylglutamate concentration versus N-acetyl-γ-glutamokinase activity.

was added per reaction mixture.

In order to decrease the high enzyme blank (reagents and extract minus substrate) presumably due to the presence in the extract of an active ATP-ase, potassium fluoride (6.66×10^{-3}M, final concentration) was added. This amount of potassium fluoride reduces background activity by about 30% without inhibiting enzyme 2 activity (Table 11). Enzyme 2 activity as a function of protein concentration was determined (Fig. 16). Since a break in the linearity of activity occurred at 0.3 mg protein/ml extract, all extracts were diluted to a concentration of less than 0.3 mg protein/ml extract. The activity of N-acetyl-γ-glutamokinase as a function of time of incubation was determined to be linear for at least 120 minutes (Fig. 17). Due to loss of activity upon thawing, all K12 extracts were assayed immediately after sonication.

N-Acetylglutamic-γ-Semialdehyde Dehydrogenase (Enzyme 3)

The reaction catalyzed by N-acetylglutamic-γ-semialdehyde dehydrogenase is reaction 3, Fig. 1. The spectrophotometric assay measures the increase in optical density at 340 mμ due to the reduction of NADP (87). The amount of substrate used in the original assay (2.0 μmoles) was found not to be saturating (Fig. 18); hence, a more nearly saturating amount of substrate (3.6 μmoles of N-acetylglutamic-γ-semialdehyde) was added per reaction mixture. Enzyme 3 activity was determined to be directly proportional to the protein concentration of the crude extract from 0 to 12 mg/ml (Fig. 19).

TABLE 11

SELECTIVE REDUCTION BY POTASSIUM FLUORIDE OF BACKGROUND ACTIVITY IN N-ACETYL-γ-GLUTAMOKINASE ASSAY

Extract	Potassium Fluoride		Percent Reduction of Blank	Percent Inhibition of Enzyme 2 Activity
	μmoles added per reaction mixture	final concentration x 10^{-3}M		
W, *argR*$^-$	10	16.7	34	54
	8.0	13.3	28	45
	6.7	11.1	31	24
	5.0	8.33	25	0
	2.5	4.16	17	0
	1.0	1.67	10	0
W, *argR*$^+$	10	16.7	69	82
	5.0	8.33	33	18
	4.0	6.66	31	0
	3.0	5.00	28	0
	2.0	3.33	28	0

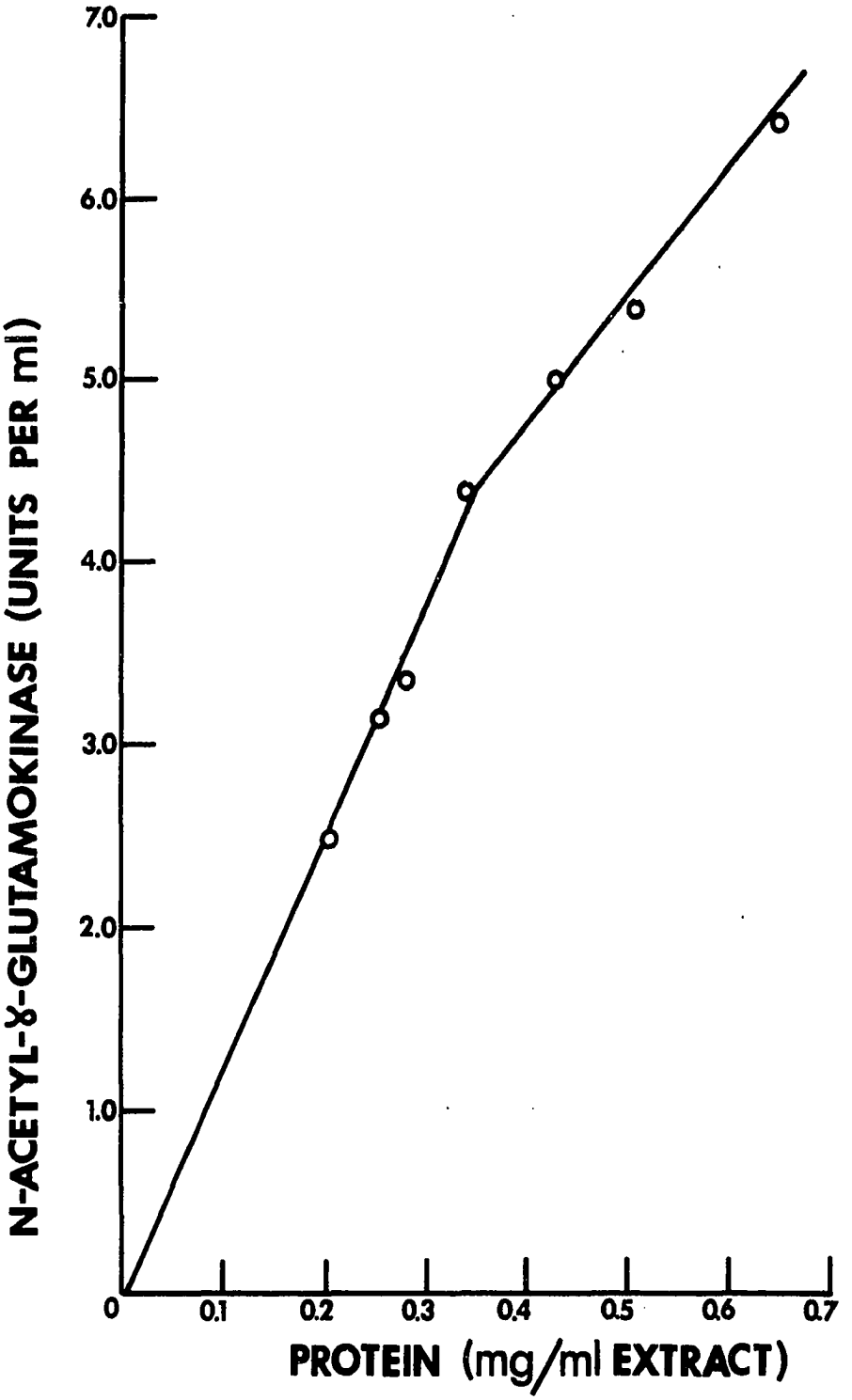


Figure 16. Activity of N-acetyl-γ-glutamokinase as a function of protein concentration.

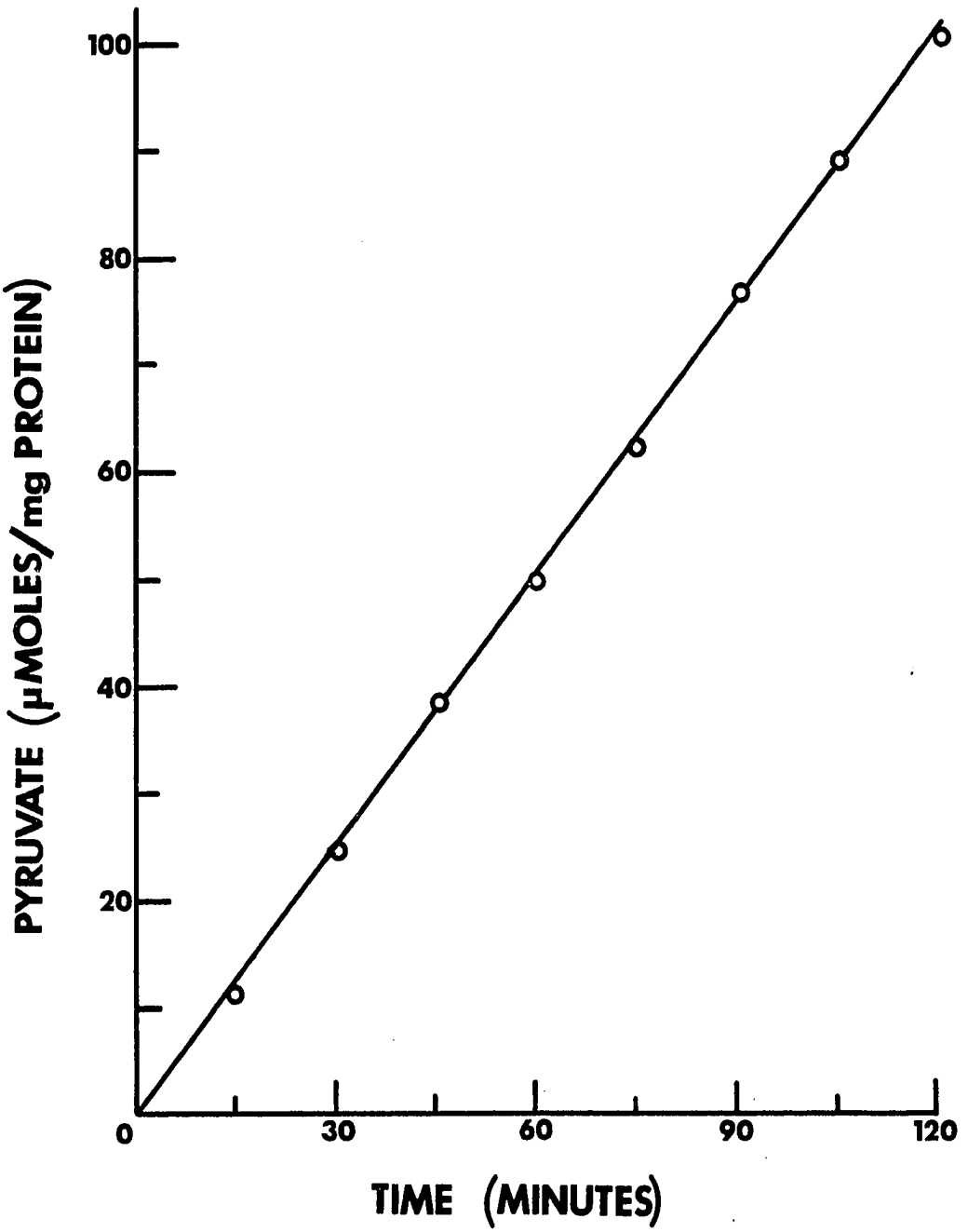


Figure 17. Proportionality of N-acetyl-γ-glutamokinase activity to time.

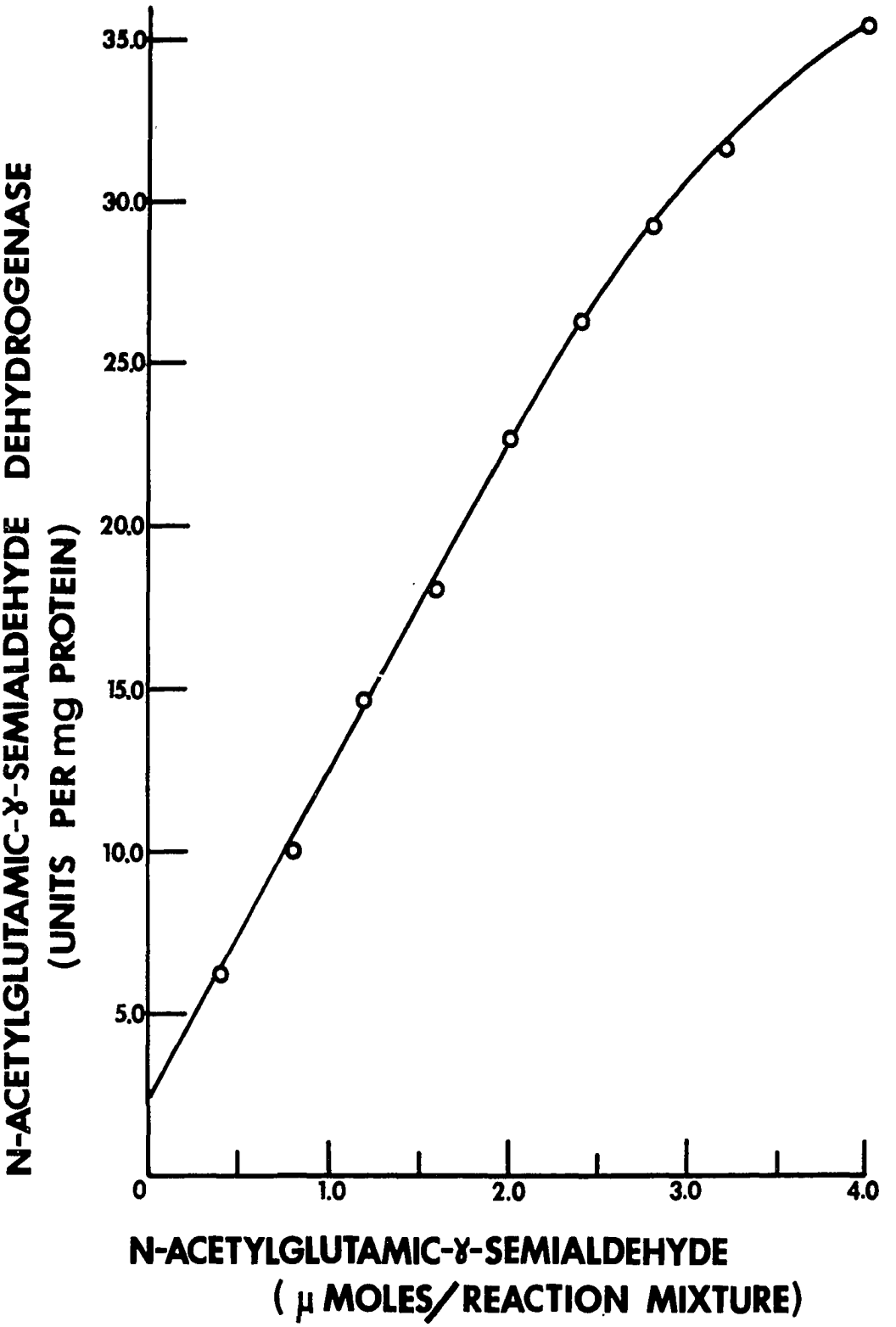


Figure 18. Activity of N-acetylglutamic-γ-semialdehyde dehydrogenase as a function of substrate concentration.

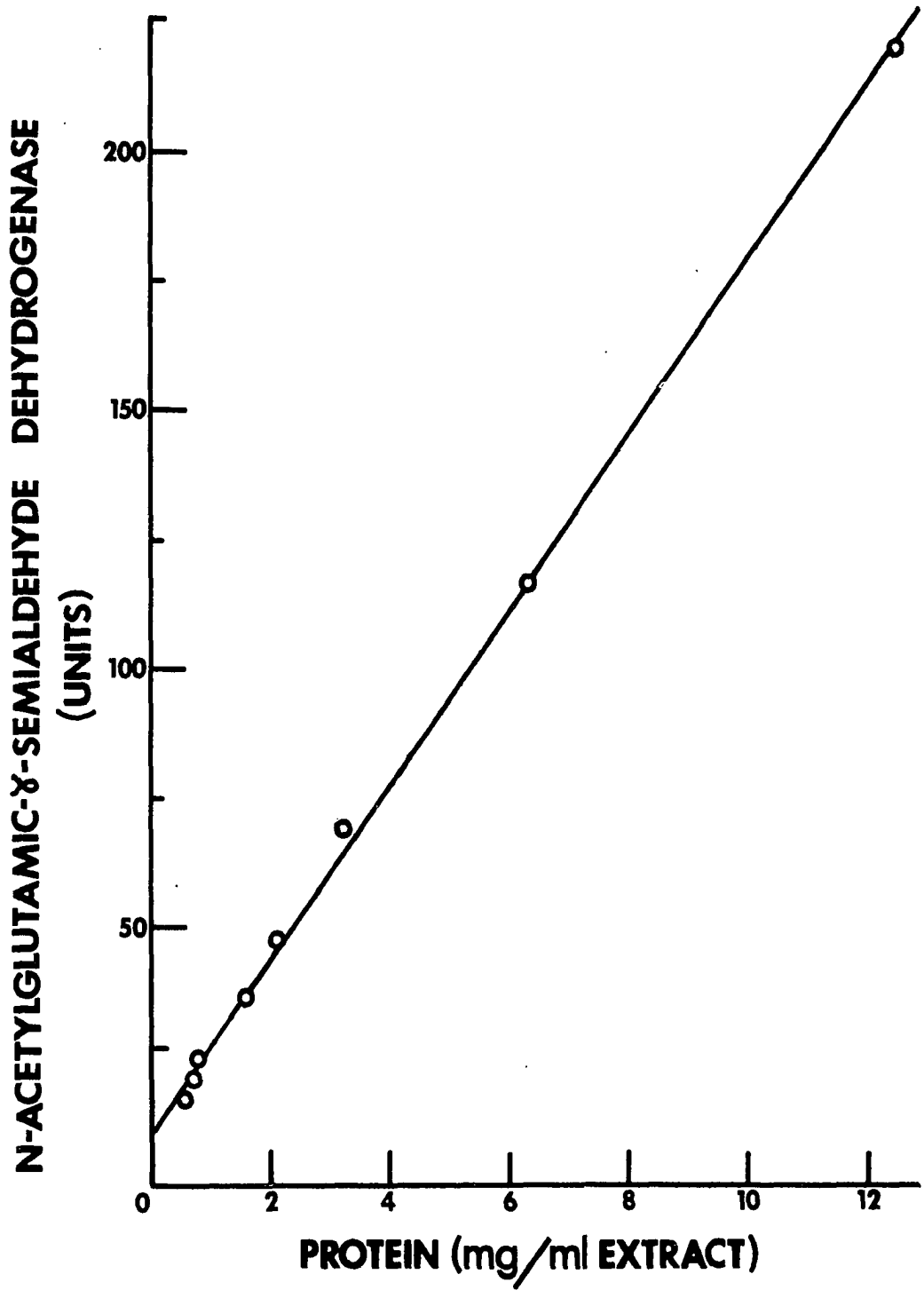


Figure 19. Activity of N-acetylglutamic-δ-semialdehyde dehydrogenase as a function of protein concentration.

N-Acetylornithine δ-Transaminase (Enzyme 4)

The reaction catalyzed by N-acetylornithine δ-transaminase is reaction 4, Fig. 1. The assay is based upon detection by o-aminobenzaldehyde (oAB) of the product of the reverse reaction (3). That enzyme 4 activity is directly proportional to time of incubation for at least 150 minutes is shown in Fig. 20. When transaminase activity was determined as a function of the protein concentration (Fig. 21), the linear relationship showed a break at about 1.0 mg protein/ml extract; therefore, all extracts were diluted below 1.0 mg protein/ml extract.

Acetylornithinase (Enzyme 5)

The reaction catalyzed by acetylornithinase is reaction 5, Fig. 1. Acetylornithinase is measured by detection with ninhydrin of the ornithine produced (142). Fig. 22 shows a linear relationship between acetylornithinase activity and time of incubation for at least 150 minutes. Enzyme 5 activity was determined to be directly proportional to the protein concentration up to 0.3 mg protein/ml extract (Fig. 23).

Argininosuccinase (Enzyme 8)

The reaction catalyzed by argininosuccinase is reaction 8, Fig. 1. Enzyme 8 activity, also measured by the ninhydrin detection of ornithine formed (105), was shown to be proportional to incubation time for at least 180 minutes (Fig. 24). When argininosuccinase activity was determined as a function of protein concentration (Fig. 25), the linear relationship showed a break at about 1.0 mg protein/ml

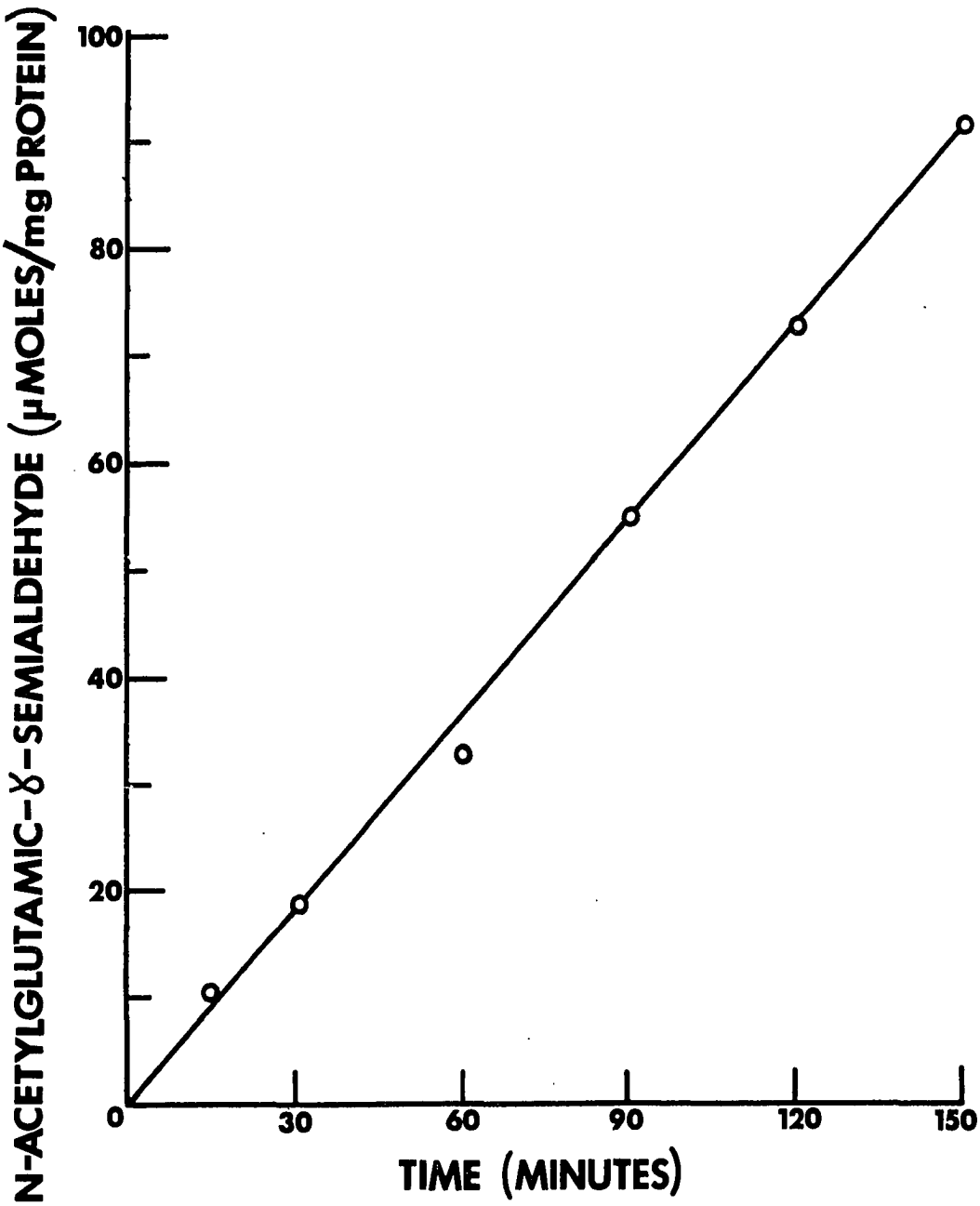


Figure 20. Proportionality of N-acetylornithine-δ-transaminase activity to time.

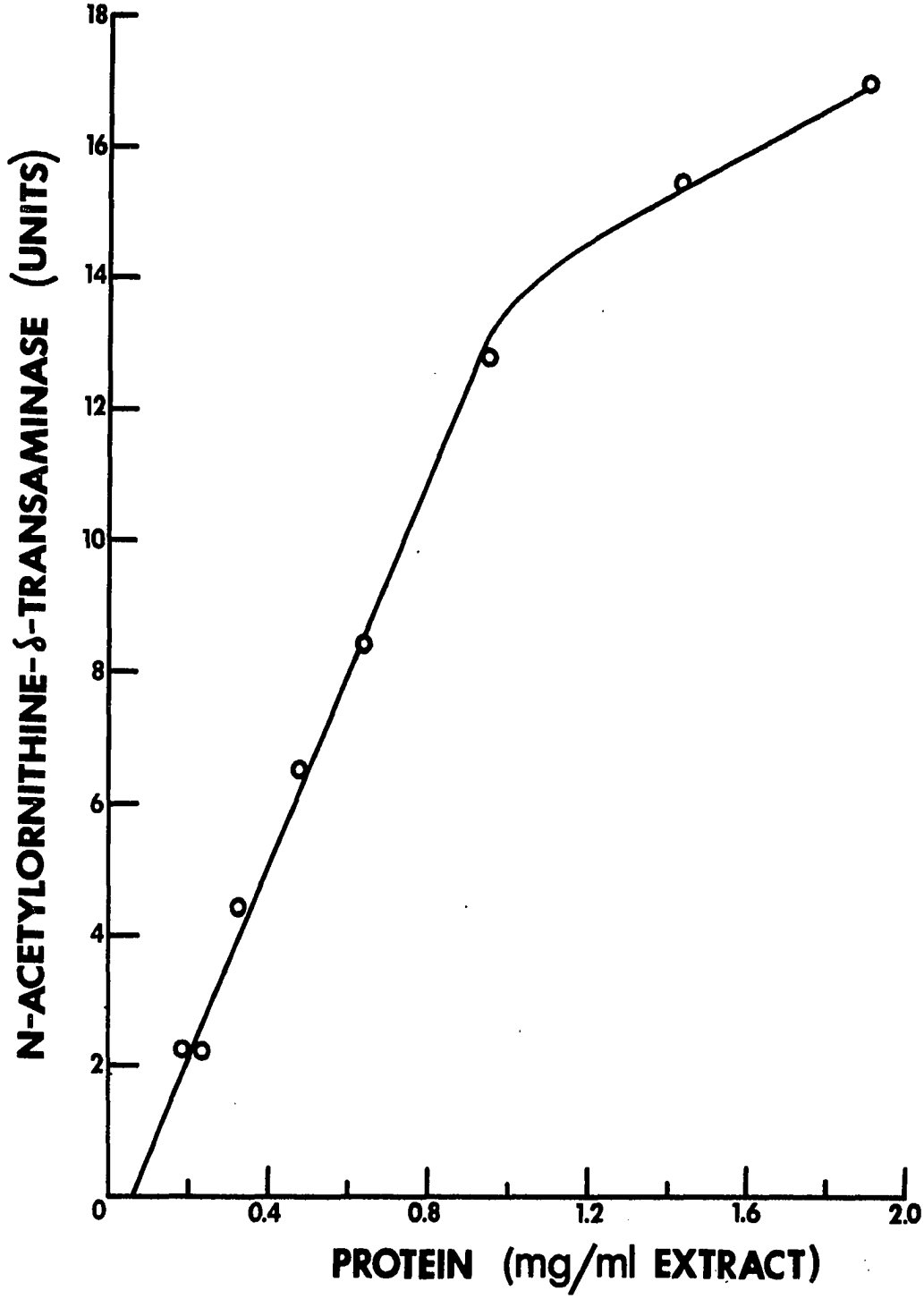


Figure 21. Activity of N-acetylornithine-δ-transaminase as a function of protein concentration.

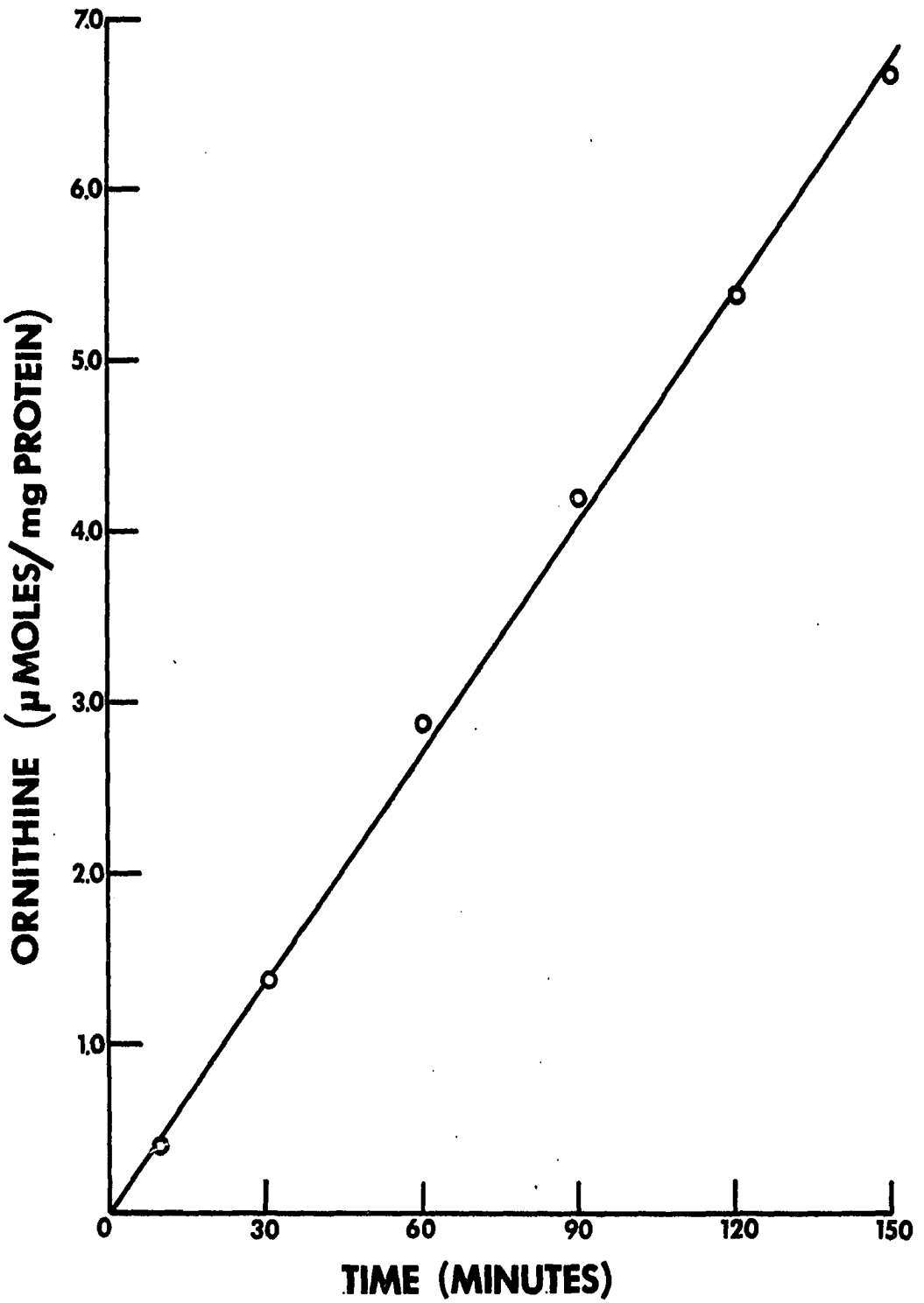


Figure 22. Proportionality of acetylornithinase activity to time.

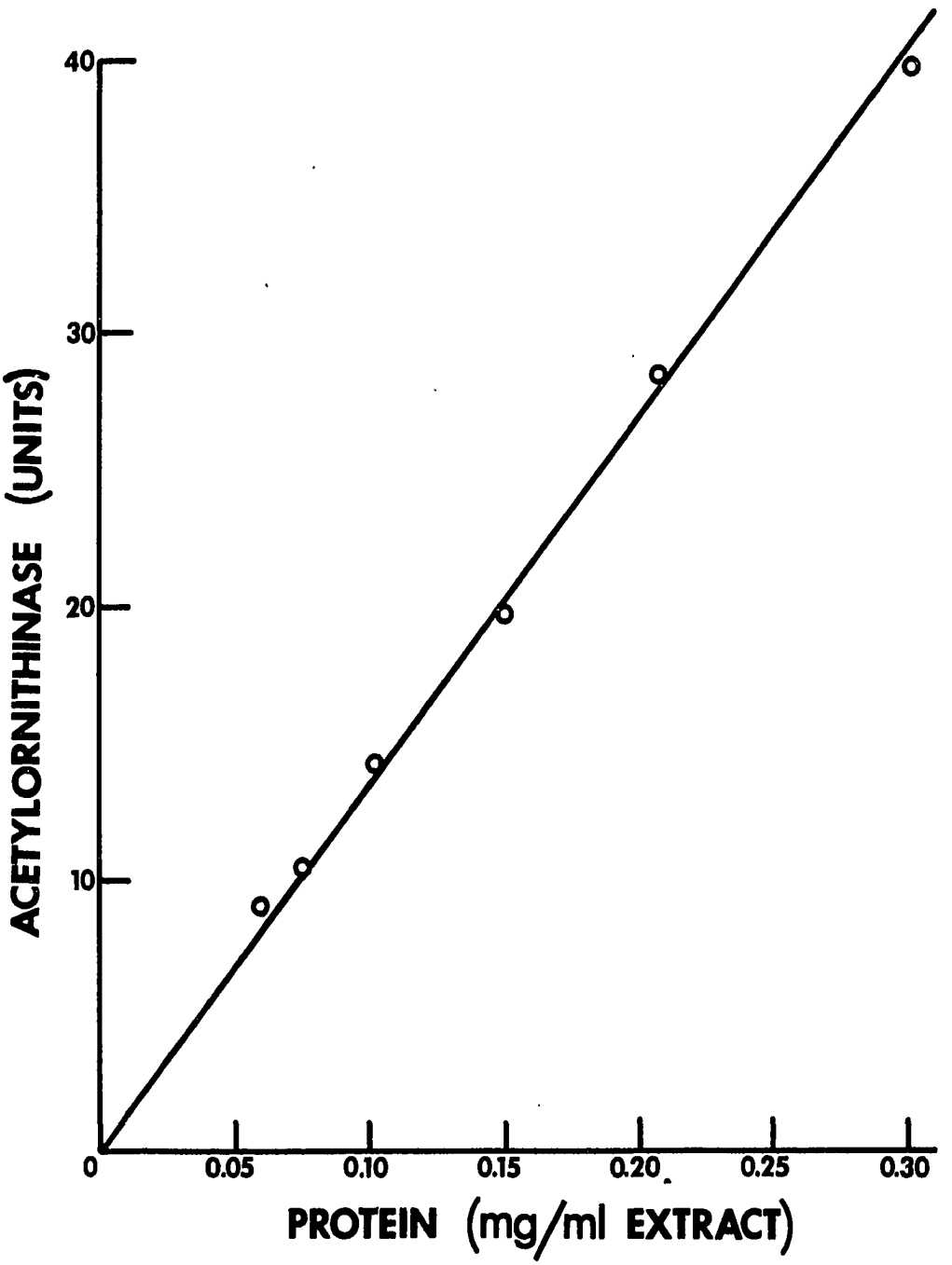


Figure 23. Activity of acetylornithinase as a function of protein concentration.

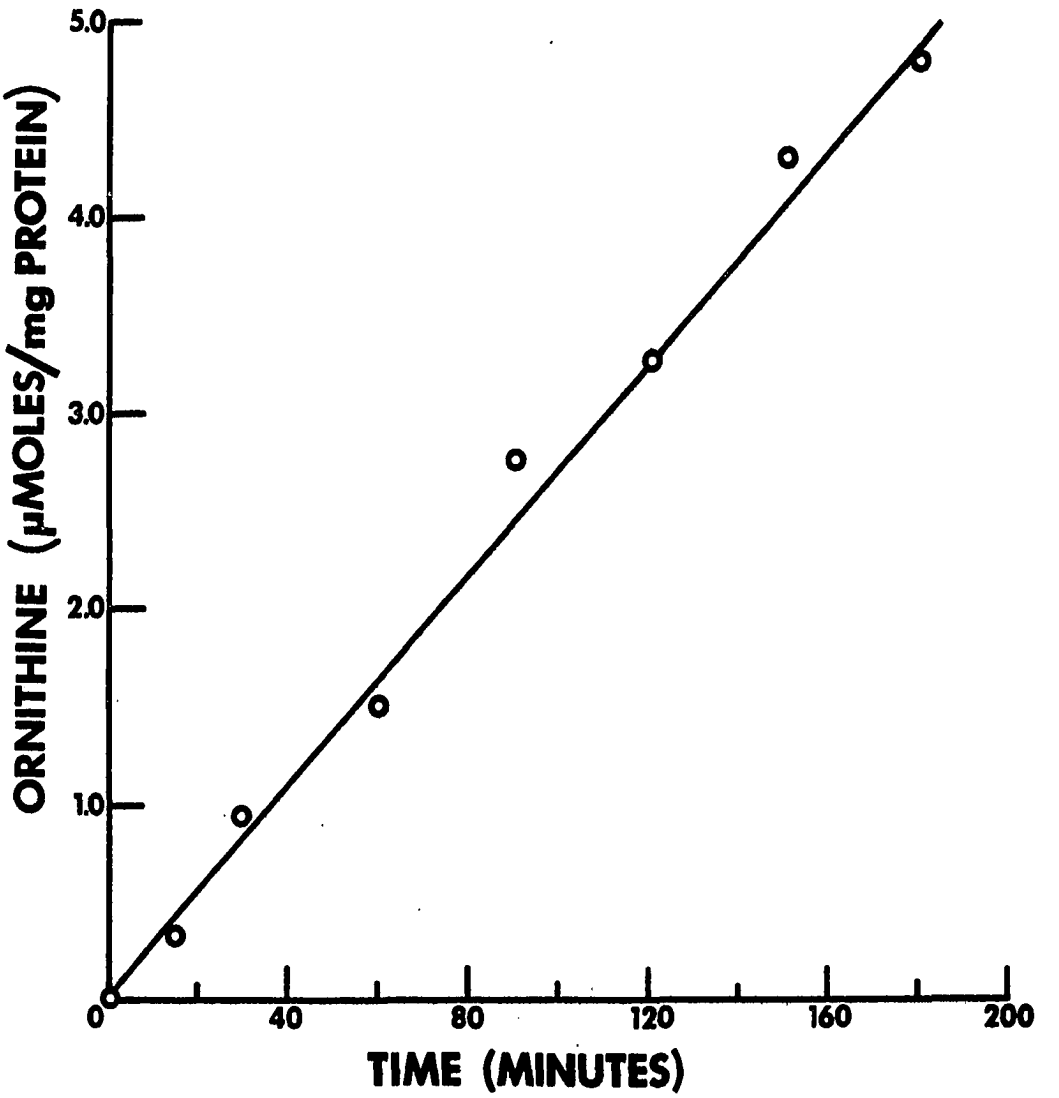


Figure 24. Proportionality of argininosuccinase activity to time.

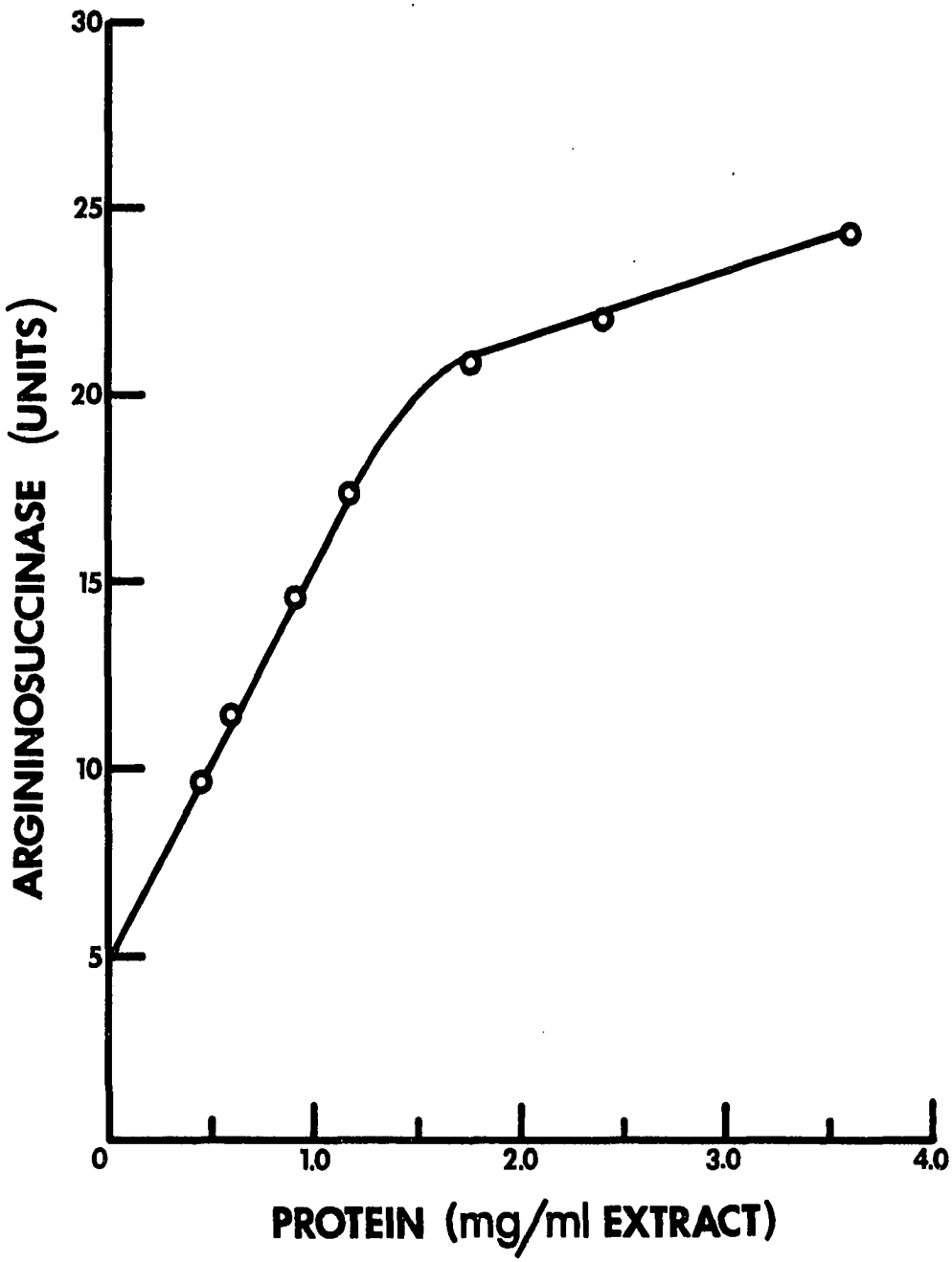


Figure 25. Activity of argininosuccinase as a function of protein concentration.

extract; therefore, all extracts were diluted below 1.0 mg protein/ml extract before assay.

Regulation of Arginine Enzymes Specified by Clustered Genes

E. coli, W, Wild Type

In view of the interest in the regulation of arginine biosynthesis in E. coli, W (130), it was felt that a study should be undertaken to determine the coordinacy relationships among the various enzymes encoded in the cluster of four arginine genes. In order to establish the coordinacy of synthesis of two enzymes, it is necessary to examine their intermediate rates of synthesis at points between the fully repressed and full derepressed rates (4, 5, 19, 46, 66, 67). In the present study, derepression was achieved in two ways: (1) genetically, by mutation of the argR gene and (2) physiologically, by limitation of endogenous arginine and hence of the concentration of functional arginine repressor.

Genetic derepression. The repression-derepression behavior in MMA with and without exogenous arginine of E. coli, W, $argR^+$ and $argR^-$ is given in Table 12. The results are expressed as percentages of the differential rates of enzyme synthesis with the $argR^-$ strain grown with arginine taken as 100. The corresponding specific activities are shown in parenthesis. These relative rates were used as a method of allowing the direct comparison of differential synthetic rates between two enzymes whose arbitrary units of activity are not equal. Alternatively, the ratios A, B and C, as defined in Chapter I, p. 19, also allow a direct comparison of two enzymes since these numbers represent the fold increase or de-

TABLE 12

REPRESSION-DEREPRESSION BEHAVIOR OF ARGININE ENZYMES IN E. COLI, W, GROWN IN MINIMAL MEDIUM A

Strain	Supplement	*Enzyme 5	3	2	8	4
W, argR+	arg	10.5 (21.3)	3.41 (1.74)	12.9 (6.42)	2.01 (0.26)	2.32 (0.20)
W, argR+	—	45.3 (92.1)	32.7 (16.7)	40.9 (20.3)	34.7 (4.42)	38.4 (3.32)
W, argR⁻	—	97.5 (198)	123 (62.9)	103 (51.3)	99.5 (12.7)	98.3 (8.48)
W, argR⁻	arg	100 (203)	100 (51.1)	100 (49.8)	100 (12.8)	100 (8.63)

*Enzyme: The first number to appear in each column is the percent activity calculated setting the argR⁻ grown with arginine to 100% and the number in parenthesis is the specific activity in units per milligram protein. Each number is average of 10 determinations.

Ratio	Enzyme 5	3	2	8	4
A	9.52	29.3	7.75	49.8	43.1
B	2.15	3.76	2.51	2.87	2.55
C	4.31	9.58	3.17	17.3	16.6

crease in the differential rates of synthesis and therefore eliminate differences in the methods of expressing specific activities. It can be seen from Table 12 that (1) enzymes 5 and 2 seem to be coordinately regulated since their fully repressed rates are 10.5% and 12.9%, respectively, and their partially derepressed rates are 45.3% and 40.9%, respectively; (2) enzymes 3 and 8 give the appearance of being coordinately regulated since their fully repressed rates are 3.41% and 2.01%, respectively, and their partially derepressed rates are 32.7% and 34.7%, respectively. However, it should be noted that the difference between 3.41% and 2.01% in relative rates of synthesis of enzymes 3 and 8, is reflected in a much larger difference if compared by the ratio method (particularly, ratios A and C). Enzyme 4 appears to be coordinately synthesized with enzyme 8 since their fully repressed rates are 2.32% and 2.01%, respectively, and their partially derepressed rates are 38.4% and 34.1%, respectively.

In order to show that the above relationships were not a property of MMA, the *argR*$^+$ and *argR*$^-$ strains of *E. coli*, W, were grown in MME with and without arginine. The results are given in Table 13. Again, it would appear that enzymes 5 and 2 are being regulated coordinately. There is a greater difference (nearly three-fold) in MME than in MMA between the fully repressed rates of synthesis of enzymes 3 and 8. This finding supports the view that synthesis of enzymes 3 and 8 is probably not coordinate in strain W.

The same type of study was also done in AF medium with and without arginine and the results are shown in Table 14. In this rich synthetic medium, there appears to be a significant difference in the repressed rates of synthesis

TABLE 13

REPRESSION-DEREPRESSION BEHAVIOR OF ARGININE ENZYMES IN E. COLI, W, GROWN IN MINIMAL MEDIUM E

Strain	Supplement	*Enzyme 5	3	2	8	4
W, argR+	arg	10.4 (19.8)	6.38 (2.97)	14.3 (5.96)	2.20 (0.17)	2.56 (0.73)
W, argR+	—	38.9 (7.39)	34.4 (16.0)	31.4 (13.1)	29.7 (2.30)	38.9 (3.58)
W, argR⁻	—	98.1 (186)	130 (60.3)	119 (45.8)	109 (8.45)	104 (9.58)
W, argR⁻	arg	100 (190)	100 (46.4)	100 (41.7)	100 (7.75)	100 (9.21)

*Enzyme: The first number to appear in each column is the percent activity calculated setting the argR⁻ grown with arginine to 100% and the number in parenthesis is the specific activity in units per milligram protein. Each number is the average of 8 determinations.

Ratio	Enzyme 5	3	2	8	4
A	9.61	15.7	6.49	45.5	39.1
B	2.52	3.77	3.50	3.67	2.67
C	3.74	5.39	2.19	13.5	15.2

TABLE 14

REPRESSION-DEREPRESSION BEHAVIOR OF ARGININE ENZYMES IN E. COLI, W, GROWN IN ARGININE-FREE MEDIUM

Strain	Supplement	*Enzyme 5	3	2	8	4
W, argR+	arg	4.79 (9.77)	1.11 (0.34)	13.4 (4.94)	1.62 (0.25)	1.62 (0.13)
W, argR+	—	58.2 (124)	59.8 (18.7)	54.9 (20.2)	59.8 (9.37)	63.4 (5.28)
W, argR⁻	—	105 (223)	125 (39.0)	102 (37.6)	100 (15.7)	104 (8.66)
W, argR⁻	arg	100 (212)	100 (31.2)	100 (36.9)	100 (15.7)	100 (8.33)

*Enzyme: The first number to appear in each column is the percent activity calculated setting the argR⁻ grown with arginine to 100% and the number in parenthesis is the specific activity in units per milligram protein. Each number is the average of 8 determinations.

Ratio	Enzyme 5	3	2	8	4
A	21.8	90.1	7.46	61.7	61.7
B	1.80	2.09	1.85	1.67	1.64
C	12.7	53.9	4.09	36.9	39.1

of enzyme 5 (4.79) and 2 (13.4). The fully repressed rate of synthesis for enzyme 2 remains essentially the same in AF medium (13.4) as in MMA (12.9) and MME (14.3); however, enzyme 5 was repressed by an additional 2.25 fold in AF (4.79) below that found in MMA (10.5) and MME (10.4). The reason for this has not been determined; however, it should be noted that the repressed rates of enzymes 3, 8 and 4 are also lower in AF medium (1.11, 1.62 and 1.62, respectively) than in either MMA (3.41, 2.01 and 2.32) or MME (6.38, 2.20 and 2.56).

In unsupplemented AF medium, the rates of synthesis of all the arginine enzymes are between 55% and 63%. This is significantly higher (almost two-fold) than in MMA or MME (Tables 12 and 13, respectively). Apparently, the derepression of the arginine enzymes is a result of a limitation in the arginine available for protein synthesis (physiological derepression). This is probably much the same as reported by Unger, et al. (130) in MMA when the endogenous arginine supply is limiting.

As additional evidence against the coordinate synthesis of enzymes 3 and 8 in E. coli, W, it should be pointed out that in all three media in contrast to enzyme 8, synthesis of enzyme 3 appears to be slightly repressible by arginine in the argR$^-$ derivative; for example, in MMA the argR$^-$ strain has a value of 123%, but when arginine is added to the medium the value of 100% is obtained (Table 12). In other words, the repressor recognition site associated with enzyme 3 appears to have some affinity for the altered protein product of an argR$^-$ gene (in the presence of arginine). In fact, this is the first suggestion that arginine (corepressor) can interact with (bind) the product of an argR$^-$ gene.

Physiological derepression. It has been shown by Vogel (135) that derepression of enzyme 5 occurs when a mutant of strain W blocked before acetylornithinase is grown in low levels of acetylornithine. It was demonstrated that concentration of acetylornithine into the cell is due to a specific permease which is not as efficient as arginine permease. It was shown that at low levels of acetylornithine, growth is restricted (as indicated by an increased doubling time) reflecting the limitation of the endogenous arginine supply. Since arginine synthesis from acetylornithine is limited, the steady state concentration of "active" repressor is presumably decreased causing derepression of the arginine biosynthetic enzymes. This was the method chosen to physiologically derepress the synthesis of the "cluster" enzymes. E. coli, W-528, which has a mutation in argC, and W-526, which has a mutation in argB, were grown in MMA plus various concentrations of acetylornithine (Tables 15 and 16). The doubling times in the different concentrations of acetylornithine for both strains illustrate the fact that at low concentrations of acetylornithine, the steady state concentration of endogenously synthesized arginine is limiting growth. The expected consequence is derepression of the arginine pathway.

In Table 15, the specific activities for EcW-528 are shown in the upper half of the table. For comparison purposes, the lower half represents the same data recalculated as the percentage of the specific activity of the argR$^-$ strain grown in MMA plus arginine taken as 100. It appears that enzymes 5 and 2 derepress coordinately with respect to one another, but noncoordinately with respect to enzyme 8. Although enzymes 4 and 8 derepress to the same extent

TABLE 15

REPRESSION-DEREPRESSION BEHAVIOR OF ARGININE ENZYMES AND GROWTH RATES OF E. COLI, W-528, AS A FUNCTION OF N-ACETYLORNITHINE CONCENTRATION

Supplement (mg/ml)	Doubling Time (minutes)	*Enzyme 5	3	2	8	4
Arg, 0.1	55	20.4	——	5.79	0.20	0.56
AcO, 5.0	56	30.5	——	6.64	0.98	0.94
AcO, 2.5	53	67.0	——	13.6	3.12	2.78
AcO, 1.5	54	79.3	——	18.2	4.38	4.22
AcO, 0.5	58	181	——	34.3	9.29	5.05
AcO, 0.05	72	281	——	70.6	14.5	8.71

*Enzyme: These numbers are specific activities in units per mg protein. Each number is the average of 2 determinations.

Supplement (mg/ml)	**Enzyme 5	3	2	8	4
Arg, 0.1	10.0	——	11.6	1.56	6.48
AcO, 5.0	14.9	——	13.3	7.65	10.9
AcO, 2.5	32.8	——	27.2	24.4	32.2
AcO, 1.5	38.9	——	36.4	34.2	48.9
AcO, 0.5	88.7	——	66.6	72.6	58.5
AcO, 0.05	138	——	141	114	101

**Enzyme: These numbers are percent activity calculated by setting the argR⁻ strain grown with arginine to 100 (Table 12). Each number is the average of 2 determinations.

TABLE 16

REPRESSION-DEREPRESSION BEHAVIOR OF ARGININE ENZYMES AND GROWTH RATES OF E. COLI, W-526, AS A FUNCTION OF N-ACETYLORNITHINE CONCENTRATION

Supplement (mg/ml)	Doubling Time (minutes)	*Enzyme 5	3	2	8	4
Arg, 0.1	57	17.2	0.76	—	0.29	0.40
AcO, 5.0	62	23.7	4.23	—	1.50	1.27
AcO, 4.0	68	28.7	5.95	—	2.31	1.89
AcO, 3.5	56	50.1	13.9	—	3.87	2.71
AcO, 2.5	58	78.4	21.3	—	6.12	5.00
AcO, 1.5	62	96.5	37.6	—	8.97	7.22
AcO, 0.5	72	132	49.2	—	12.1	8.36
AcO, 0.05	90	174	75.1	—	15.2	10.9

*Enzyme: These numbers are specific activities in units per mg protein. Each number is the average of 2 determinations.

Supplement (mg/ml)	**Enzyme 5	3	2	8	4
Arg, 0.1	8.43	1.48	—	2.26	4.63
AcO, 5.0	11.6	8.25	—	11.7	14.7
AcO, 4.0	14.1	11.6	—	18.0	21.9
AcO, 3.5	24.6	27.1	—	30.2	31.4
AcO, 2.5	38.4	41.5	—	47.8	57.9
AcO, 1.5	47.3	73.3	—	70.1	83.6
AcO, 0.5	64.7	95.9	—	94.5	96.8
AcO, 0.05	85.3	146	—	119	126

**Enzyme: These numbers are percent activity calculated by setting the argR⁻ strain grown with arginine to 100 (Table 12). Each number is the average of 2 determinations.

(101% and 114%, respectively), there is a significant lack of coordinacy of synthetic rates at the higher concentrations of acetylornithine.

In Table 16, the same type of data is presented for E. coli, W-526. It can be seen that enzymes 3 and 8 do not derepress coordinately with enzyme 5 or 4. In fact, enzymes 3 and 8 do not appear to derepress coordinately with one another (note enzyme 3 seems to derepress faster than enzyme 8 below an acetylornithine concentration of 0.5 mg/ml).

It has become fashionable to analyze coordinacy data by plotting the specific activity of one enzyme versus the specific activity of the other enzyme (5). However, by judicious selection of units and scale, it is possible to depict erroneously parallel regulation as coordinate synthesis. Parallel regulation means derepression and repression occur under the same conditions for both enzymes although not to the same magnitude, i.e., a twenty-five fold derepression of enzyme Y as compared to an hundred-fold derepression of enzyme Z. For this reason, this author has chosen rather to plot percent enzyme activity. When percent activity of one enzyme is plotted against percent activity of another enzyme, coordinate synthesis will demand that the points lie on a straight line at a 45^{0} angle passing through the origin. Anything else, i.e., straight line not at a 45^{0} angle or not passing through the origin, or a curved line, will not be considered as coordinate synthesis.

Figure 26 is a graphical presentation of the coordinacy plots of the data presented in Tables 12, 15 and 16. The dotted line is the 45^{0} line indicative of coordinate synthesis; the solid line is the best line which can be drawn through the actual points. The other coordinacy plots not shown (e.g., enzyme 5 versus

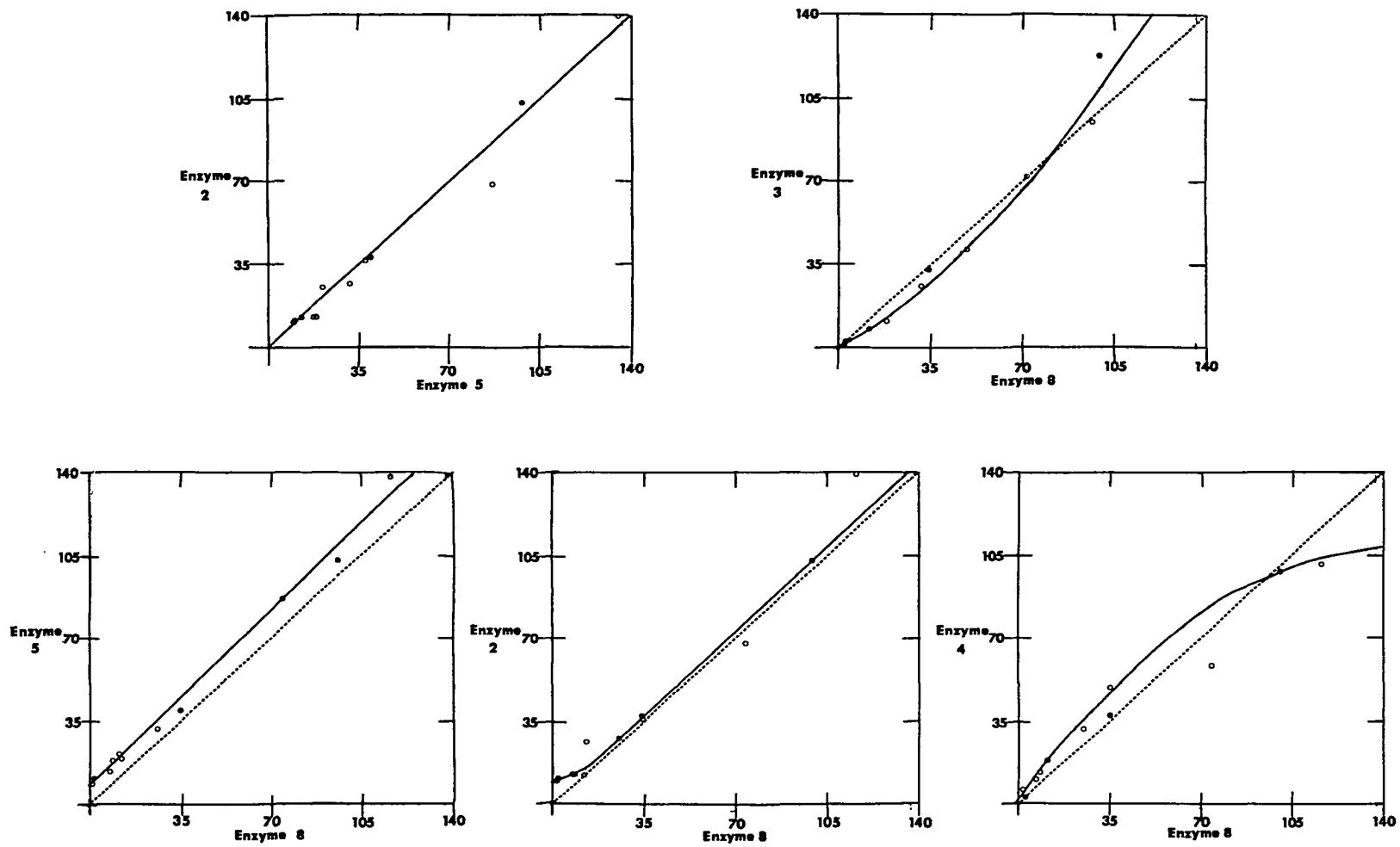


Figure 26. Coordinate derepression of two of the enzymes specified by the clustered arginine genes in E. coli, W.

enzyme 3, enzyme 3 versus enzyme 4, etc.) do not represent coordinate synthesis. That this is the case can be reasoned from the close similarity in the repression-derepression behavior of enzymes 5 and 2 and of enzymes 3 and 8. As the data is plotted, the only enzymes which meet the criteria for coordinacy are enzymes 5 and 2. This result was unexpected in view of the limited study (43) of E. coli, K12, in which enzymes 3, 2 and 8 were reported to be coordinately synthesized and the report (41) that the gene order in E. coli, K12 was argE-C-B-H. The reason the curved line on the plot of enzyme 3 activity versus enzyme 8 activity was drawn instead of a straight line is that the difference between 123% activity for enzyme 3 and 99.5% activity for enzyme 8 (Table 12) is considered to be significant. The reason the curve on the plot of enzyme 2 activity versus enzyme 8 activity is a significant departure from the 45^{0} line is that the fully repressed rate for enzyme 2 is 12.9% whereas the fully repressed rate for enzyme 8 is 2.01% (Table 12).

Homoarginine as a corepressor. As discussed in Chapter I, homoarginine can act as a corepressor in the synthesis of enzymes 5 and 6 in E. coli, W. However, the effect of this arginine analogue has not been studied in depth. We have extended these studies to include the ability of homoarginine to act as a corepressor in regulating the synthesis of all of the enzymes encoded by the cluster of four arginine genes (Table 17 and 18). Table 17 shows the specific activities of the arginine enzymes upon growth of E. coli, W, $argR^{+}$ and $argR^{-}$ with added homoarginine in MMA, MME and AF medium. These activities were determined as part of the experiments shown in Tables 12, 13 and 14.

The ability of homoarginine to act as a corepressor in E. coli, W, is

TABLE 17

SPECIFIC ACTIVITIES OF THE ARGININE ENZYMES IN E. COLI, W, GROWN IN VARIOUS MEDIA PLUS HOMOARGININE

Strain	Media	*Enzyme 5	3	2	8	4
W, argR$^+$	MMA	84.7	15.2	19.7	4.15	2.46
W, argR$^-$	MMA	251	65.4	59.5	15.0	9.92
W, argR$^+$	MME	76.1	13.1	14.4	2.37	3.29
W, argR$^-$	MME	195	62.7	40.3	9.55	9.87
W, argR$^+$	AF	93.0	14.8	17.8	7.50	2.96
W, argR$^-$	AF	230	41.3	39.9	15.5	7.97

*Enzyme: Numbers in this Table are specific activities - units per mg protein.

TABLE 18

EFFECT OF HOMOARGININE ON REPRESSION-DEREPRESSION BEHAVIOR OF ARGININE ENZYMES IN E. COLI, W

Strain	Medium	*Enzyme 5	3	2	8	4
W, *argR*$^+$	MMA	0.92	0.91	0.97	0.94	0.74
W, *argR*$^-$	MMA	1.27	1.04	1.16	1.18	1.17
W, *argR*$^+$	MME	1.03	0.82	1.10	1.03	0.92
W, *argR*$^-$	MME	1.05	1.04	0.88	1.13	1.03
W, *argR*$^+$	AF	0.75	0.79	0.88	0.80	0.56
W, *argR*$^-$	AF	1.03	1.06	1.06	0.99	0.92

*The numbers in each column are obtained by dividing the specific activity of a given strain grown with homoarginine by the specific activity of the same strain without any supplement in the medium.

perhaps best seen as represented in Table 18. The results are expressed as the ratio of the specific activity of the strain carrying the indicated argR allele grown with homoarginine divided by the specific activity of the same strain grown without any supplement (these activities are listed in Tables 12, 13 and 14). Thus, a number less than one represents a specific activity in homoarginine which is less than that obtained in unsupplemented medium; a number greater than one reflects a specific activity greater than that obtained in unsupplemented medium. It is apparent that homoarginine has some corepressor activity on the rate of enzyme 4 synthesis (note a ratio of 0.74 for MMA and 0.56 for AF). However, it is not possible to distinguish whether homoarginine is binding to the repressor or to the repressor recognition site. Additionally, in AF medium, it appears that homoarginine may have some repressive effects on the synthesis of the clustered enzymes. The synthesis of enzyme 5 is further derepressed by a factor of 1.27 when homoarginine is added to an MMA culture of an argR$^-$ derivative of strain W.

Acetylornithinase synthesis in a mutant of E. coli, W, was reported (32) to be repressible by the addition of homoarginine to MME (a factor of 0.69). It may be this repressibility is associated with the presence of the argM locus.

Enzyme-Specific Regulatory Mutants

In a further effort to determine whether the enzyme-forming systems corresponding to argE and argB share a common repressor recognition site or whether each has its own copy of an identical repressor recognition site, an attempt was made to isolate mutants which had an alteration in regulation of

enzyme 5 and/or enzyme 2 but with no effect on that of enzymes 3 or 8.

In order to isolate such enzyme-specific mutants, ornithine-independent, phenotypic revertants of acetylornithinase auxotrophs were screened by enzyme assay for alterations in repression-derepression behavior. This rather laborious screening procedure was necessitated by the following reasons: (1) an alteration in regulation of enzyme 5 would not be expected to lead to auxotrophy; therefore, the use of limiting ornithine as a selective medium was not possible, (2) there was no systematic way to select specifically for derepressed synthesis of enzyme 5 as opposed to pathway-wide derepression (i.e., canavanine-resistance). It was hypothesized that if the repressor recognition site for enzyme 5 were translated, an auxotrophic mutation affecting the repressor recognition site may "revert" either by an internal or external suppressor or true reversion. It was also known that certain revertants of argE mutations which produce low levels of enzyme 5, do not show alterations in the repression-derepression behavior of enzyme 5 presumably because the information for repressor recognition has not been affected (130).

Among the many screened, two revertants derived from different argE auxotrophs showed alterations in repression-derepression behavior. Both the auxotrophic mutations and the reversions were UV-induced. The repression-derepression behavior of one of these revertants EcW-1403 in MMA is given in Table 19. This revertant is carrying a suppressor not closely linked to argE since the argE mutation is easily separable from the suppressor by use of an Hfr derivative of 1403 which transfers the chromosome in a clockwise fashion with leu being transferred very early. Strain 1570 is an argE recombinant of such a cross [donor,

TABLE 19

ENZYME-SPECIFIC ALTERATIONS OF REPRESSION-DEREPRESSION BEHAVIOR OF ARGININE ENZYMES IN E. COLI, W-1403

Strain	Supplement	*Enzyme 5	3	2	8	4
1570 argR+	arg	0. (0)	1.63 (0.88)	23.0 (6.84)	4.33 (0.42)	6.19 (0.46)
1403 argR+	arg	17.2 (0.86)	2.21 (1.19)	24.9 (7.41)	2.54 (0.24)	5.35 (0.39)
1403 argR+	——	64.5 (3.16)	29.3 (15.8)	34.3 (10.2)	33.1 (3.21)	40.3 (2.99)
1403 argR⁻	——	103 (5.04)	114 (61.6)	96.0 (28.6)	104 (10.1)	97.3 (7.23)
1403 argR⁻	arg	100 (4.90)	100 (64.0)	100 (29.7)	100 (9.70)	100 (7.43)

*Enzyme: The first number to appear in each column is the percent activity calculated setting the argR⁻ grown with arginine to 100% and the number in parenthesis is the specific activity in units per milligram protein. Each number is the average of 10 determinations.

Ratio	Enzyme 5	3	2	8	4
A	5.81	45.2	4.01	39.4	18.7
B	1.59	3.89	2.79	3.14	2.41
C	3.75	13.2	1.37	13.0	7.53

EcW-"1403" (argE, su^+, Hfr); recipient, EcW-448 (pro^-, leu^-, str^r).

Enzymes 3 and 8 appear to be under normal regulation in 1403 and 1570. However, in 1403, enzyme 5 is not repressed to the normal extent (17.2% as opposed to the wild type rate of 10.5%). The repressibility of enzyme 2 is also changed (24.9% as compared to 12.9%). Therefore, in 1403, which has become $argE^+$ due to the presence of an external suppressor, the repressibility of enzymes 5 and 2 has been affected simultaneously.

When 1403 $argR^+$ is grown in MMA without arginine, enzyme 5 synthesis is poised at 64.5% as compared to 45.3% for wild type. This observation is consistent with the idea that the repressor recognition site for enzyme 5 synthesis has been altered in such a way that it is no longer as sensitive to the "active" repressor as that of the wild type. That this high value for enzyme 5 of 64.5% is due to the derepression of enzyme 5 because of limitation in arginine supply may be ruled out since it has been shown (130) that when the rate of synthesis of enzyme 5 is low enough to begin to restrict the arginine supply, synthesis of enzyme 8 derepresses. In the case of mutant 1403 synthesis of enzyme 8 is not derepressed. It should also be noted that the fully repressed rate of synthesis of enzyme 4 is 5.35% (Table 19) as compared to the normal of 2.32% (Table 12). The reason for this is not clear although the possibility exists that the external suppressor has altered the repressor recognition site governing enzyme 4 synthesis. In 1570, the parental argE auxotroph, enzyme 2 repressibility is altered by the mutation in argE, 23.0% (Table 19) as compared to the normal 12.9% (Table 12).

Table 20 shows the repression-derepression behavior of EcW-1406. EcW-1406 is carrying a "reversion" which is closely associated with argE, since the original mutation is not separable from the "reversion" by conjugation techniques. In contrast to 1403, synthesis of enzyme 5 in strain 1406 appears to be more sensitive to "active" repressor than it is in wild ype. This statement is based on the finding that the ratio C for enzyme 5 is 11.7 (Table 20) compared to a normal ratio C of 4.31 (Table 12). As in mutant 1403, enzyme 2 is also affected (a repressed rate of 6.80% as compared to 12.9% for the wild type). Again, it appears that the rates of synthesis of enzymes 5 and 2 have been modified whereas those of enzymes 3, 8 and 4 have not been markedly affected.

The observed alterations in repression-derepression behavior of enzymes 5 and 2 but not of enzymes 3 and 8, support the conclusion that the altered regulatory behavior is specific for enzymes 2 and 5 and is not a property of the argR gene, and appears consistent with reports of the involvement of individual repressor recognition sites in the synthesis of enzymes 5 and 8 (69, 130, 141).

E. coli, K12, Wild Type

The findings in E. coli, W, of coordinate control of enzymes 5 and 2 are of special interest because in E. coli, K12, argE (enzyme 5) is separated from argB (enzyme 2) by argC (enzyme 3) according to the gene order reported by Glansdorff (41). Therefore, it was decided to reinvestigate and extend the studies of the expression of the clustered arginine genes in E. coli, K12, a strain which is more amenable to genetic analysis than E. coli, W.

TABLE 20

ENZYME-SPECIFIC ALTERATIONS OF REPRESSION-DEREPRESSION BEHAVIOR OF ARGININE ENZYMES IN E. COLI, W-1406

Strain	Supplement	*Enzyme 5	3	2	8	4
1406 argR+	arg	3.91 (0.17)	2.50 (1.53)	6.80 (2.59)	2.78 (0.26)	2.50 (0.20)
1406 argR+	—	45.7 (2.01)	30.9 (19.0)	38.5 (14.7)	37.4 (3.55)	43.1 (3.55)
1406 argR-	—	98.0 (4.31)	96.6 (59.3)	105 (40.1)	104 (9.88)	95.0 (7.83)
1406 argR-	arg	100 (4.40)	100 (61.0)	100 (38.1)	100 (9.55)	100 (8.24)

*Enzyme: The first number to appear in each column is the percent activity calculated setting the argR- grown with arginine to 100% and the number in parenthesis is the specific activity in units per milligram protein. Each number is the average of 10 determinations.

Ratio	Enzyme 5	3	2	8	4
A	25.6	40.0	14.7	36.0	40.0
B	2.34	3.12	2.72	2.78	2.20
C	11.7	12.4	5.66	13.5	17.2

Genetic derepression. Shown in Table 21 are the rates of synthesis of the arginine enzymes in E. coli, K12, argR$^+$ and argR$^-$, grown in MMA with and without arginine. It would seem that synthesis of enzymes 5 and 2 is not coordinate in K12 (compare a repressed rate of 8.06% for enzyme 5 and 3.41% for enzyme 2 and a ratio A of 12.4 and 29.3, respectively). On the other hand, enzymes 3 and 8 appear to be coordinately synthesized (ratio A of 42.3 and 40.3, respectively). However, a small difference in the percent derepression was observed for the argR$^+$ strain grown without arginine: 18.1% for enzyme 3 compared to 24.7% for enzyme 8. Enzyme 4 had previously been reported to be coordinately synthesized with enzyme 5 in strain K12 (136). In the present study, as well, when derepression is achieved by mutation of the argR gene, the rates of synthesis of enzymes 4 and 5 are not markedly different.

Physiological derepression. In view of the results for enzymes 5 and 2 in E. coli, W, and in order to establish conclusively the apparent coordinacy between enzymes 3 and 8 in strain K12, E. coli, K12-1509 (argA) was grown on various concentrations of acetylornithine to achieve varying degrees of physiological limitation of endogenous arginine synthesis and thus varying degrees of derepression of the arginine biosynthetic enzymes. The results are shown in Table 22. As can be seen from the doubling times, acetylornithine (0.05 mg/ml) did not limit growth to the same extent as it did in E. coli W-528 and W-526 whose doubling times increased from 55 to 72 and 57 to 90 minutes, respectively (Tables 15 and 16). Nevertheless, this concentration of acetylornithine did result in a significant degree of derepression of the synthesis of all five of the enzymes measured.

TABLE 21

REPRESSION-DEREPRESSION BEHAVIOR OF ARGININE ENZYMES
IN E. COLI, K12, GROWN IN MINIMAL MEDIUM A

Strain	Supplement	*Enzyme 5	3	2	8	4
K12, argR+	arg	8.06 (12.5)	2.37 (1.74)	3.41 (1.34)	2.48 (0.25)	10.1 (0.91)
K12, argR+	—	25.3 (39.1)	18.1 (13.1)	19.2 (7.59)	24.7 (2.43)	37.7 (3.38)
K12, argR⁻	—	101. (156)	97.6 (70.9)	94.7 (37.4)	97.9 (9.64)	100. (8.97)
K12, argR⁻	arg	100. (155)	100. (72.6)	100. (39.5)	100. (9.85)	100. (8.97)

*Enzyme: The first number to appear in each column is the percent activity calculated setting the argR⁻ grown with arginine to 100% and the number in parenthesis is the specific activity in units per milligram protein. Each number is the average of 6 determinations.

Ratio	Enzyme 5	3	2	8	4
A	12.4	42.3	29.3	40.3	9.90
B	3.99	5.39	4.93	3.96	2.65
C	3.13	7.63	5.63	9.95	3.73

TABLE 22

REPRESSION-DEREPRESSION BEHAVIOR OF ARGININE ENZYMES AND GROWTH RATES OF E. COLI, K12-1509, AS A FUNCTION OF N-ACETYLORNITHINE CONCENTRATION

Supplement (mg/ml)	Doubling Time (minutes)	*Enzyme 5	3	2	8	4
Arg, 0.1	78	14.6	1.31	1.92	0.26	0.53
AcO, 7.0	82	29.2	6.76	4.39	1.53	2.02
AcO, 6.0	90	31.0	6.58	4.18	1.47	2.14
AcO, 2.0	84	68.6	20.8	9.79	4.35	4.70
AcO, 0.5	87	79.2	26.6	11.3	5.07	5.50
AcO, 0.2	85	86.0	27.8	10.7	4.48	5.84
AcO, 0.05	87	87.7	29.8	10.8	5.60	6.06

*Enzyme: These numbers are specific activities in units per mg protein. Each number is the average of 2 determinations.

Supplement (mg/ml)	**Enzyme 5	3	2	8	4
Arg, 0.1	1.00	1.00	1.00	1.00	1.00
AcO, 7.0	2.00	5.16	2.28	5.88	3.81
AcO, 6.0	2.12	5.02	2.17	5.65	4.03
AcO, 2.0	4.71	15.9	5.09	16.7	8.86
AcO, 0.5	5.44	20.3	5.89	19.5	10.4
AcO, 0.2	5.90	21.2	5.56	17.2	11.0
AcO, 0.05	6.02	22.8	5.64	21.5	11.4

**Enzyme: The numbers represent the fold increase above the fully repressed specific activities. Each number is the average of 2 determinations.

If the specific activities in the upper half of Table 22 are converted to the percent activity of the argR$^-$ strain grown with arginine taken as 100 (as was done for strain W in Tables 13 and 14), enzymes 3 and 8 appear to be coordinately synthesized (not shown). However, if the rates of synthesis of mutant 1509 grown with arginine are set equal to 1.00 and all other rates calculated as fold-increase above this fully repressed rate, a different picture emerges (see Table 22, bottom half). Whereas a coordinate relationship is again apparent between enzymes 3 and 8, enzymes 2 and 5 appear also to be derepressing coordinately. Although synthesis of enzyme 4 appears coordinate with that of enzyme 5 when derepression is achieved by mutation in the argR gene (Table 21), it is not coordinate with that of enzymes 5, 3, 2 or 8 when derepression is achieved by endogenous arginine limitation (Table 22). If the E. coli, W, data is calculated in this manner, there is no change in the conclusions reached since enzymes 5 and 2 taken as a pair have similar fully repressed rates.

In Figure 27, the fold increase above the basal rates for each enzyme has been plotted against those of the others. The dotted line is the 45^0 line indicative of coordinate synthesis; the solid line is the best line which can be drawn through the actual points. The other coordinacy plots not shown (e.g., enzyme 5 versus enzyme 3, enzyme 3 versus enzyme 4, etc.) do not represent coordinate synthesis. That this is the case can be reasoned from the close similarity in the repression-derepression behavior of enzymes 3 and 8. It is apparent from this analysis of the data that enzymes 3 and 8 are coordinately synthesized throughout the entire measurable repression-derepression range in E. coli, K12. Although

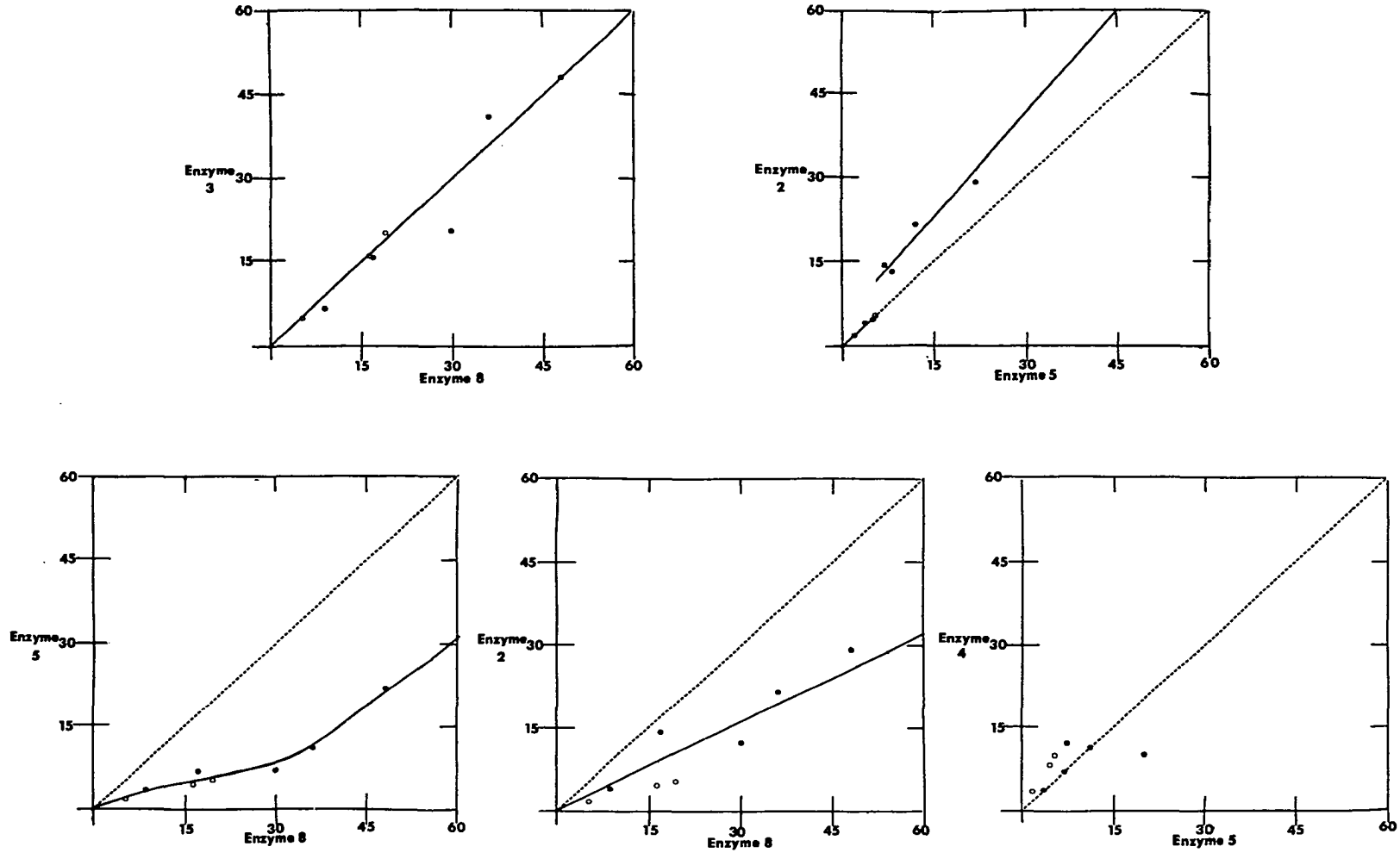


Figure 27. Coordinate derepression of two of the enzymes specified by the clustered arginine genes in E. coli, K12.

enzymes 5 and 2 derepress coordinately for at least a six-fold range above their different basal rates, the results of genetic derepression (Table 21 and Fig. 27) indicate they are not synthesized coordinately (genetic derepression effects a greater range of derepression). For a discussion of the differences between genetic and physiological derepression, see Chapter IV.

In view of the results obtained in E. coli, W, and K12, and the reported gene order in the arginine cluster in K12 (41), it was deemed necessary to construct a fine structure map of the arginine cluster (possible only in K12) so that the existence, number and location of internal promoters, initiators, and repressor recognition sites may be defined.

Genetic Analysis of the Arginine Cluster

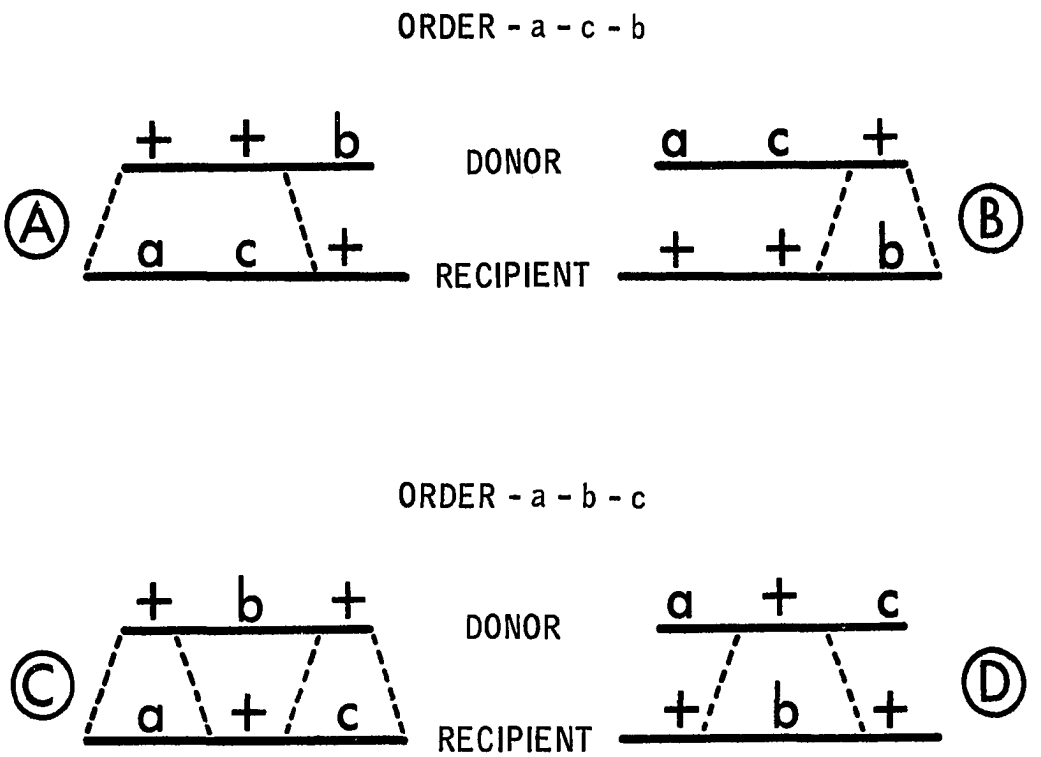
The first approximation to a fine-structure map of a cluster of genes can be obtained by constructing a deletion map, that is an ordering of the genes by a series of overlapping deletion mutations. Point mutations can then be located within the appropriate deletions. Point mutations which lie in the same region of a deletion map can be ordered with respect to one another by reciprocal three-point crosses with an outside marker. This outside marker should have a high cotransduction frequency with the clustered genes in order to be useful.

Mapping of the rna Locus

As a closely linked marker outside the arginine cluster, ppc has been used by Glansdorff (41, 42, 44). However, after repeated unsuccessful attempts

to isolate ppc mutants by this author, the rifamycin-resistance locus (rna-1) was chosen as the outside marker. Rifamycin-resistance has been shown to be due to an alteration in a subunit of RNA polymerase (126), hence the rna designation for the locus. Rifamycin-resistance is 60-70% cotransducible with argH (7). However, it was not shown whether the rna locus was within the arginine cluster, to the right, or to the left of the cluster. Therefore, it was necessary to determine the precise location of rna-1.

The only unambiguous method for determining the order of sites is by means of 3-factor reciprocal crosses. The principle of this method as applied to transduction is shown in Fig. 28. It is known that metB lies to the left of argE-C-B-H and rna-1 (124). Reciprocal crosses, in which each mutant is alternatively used as donor and recipient, were performed by propagating phage P1kc on each mutant strain (donor) and then using it to transduce the other strain (recipient), as represented in Fig. 28. Several different arginine cluster mutants were used so that if rna-1 were located within the cluster, its relative position could be determined. If the order were assumed to be a-c-b (metB-rna-arg) (crosses A and B, Fig. 28), two cross-overs would be required to yield wild-type (+++) recombinants in both cross A and cross B; therefore, the frequencies of wild type recombinants obtained in the reciprocal crosses A and B should be approximately equal. If the order were a-b-c (metB-arg-rna) (crosses C and D, Fig. 28), four cross-overs would be required to yield wild type recombinants in cross C whereas only two cross-overs would be necessary in cross D. Therefore, the frequency of wild type recombinants resulting from cross C would be significantly less than the



Dotted lines indicate cross-overs necessary to get +++ recombinants

If order is a - c - b, then ratio A/B recombinants = about 1

If order is a - b - c, then ratio C/D recombinants = low

a = metB1

b = argE, C, B, or H

c = rna-1

Figure 28. The mapping of sites by reciprocal, three factor crosses.

corresponding frequency from cross D. On the other hand, if the rna-1 locus (c) mapped within the arginine cluster (b), reciprocal crosses involving arginine loci (b) to the right of rna-1 (c) would yield approximately the same frequency of wild type recombinants (crosses A and B, Fig. 28). Reciprocal crosses involving arginine loci (b) to the left of rna-1 (c) would yield significantly different frequencies of wild type recombinants (crosses C and D, Fig. 28).

The results of the reciprocal crosses are given in Tables 23 and 24. For cross C (Table 23), the frequency of wild type recombinants was found to be zero with all seven arginine-requiring donors. For cross D (Table 24), the frequency of wild type recombinants was found to range from 0.22 to 0.30, with the exception of argC1417 which gave a frequency of 0.14. Thus the rna-1 locus lies outside of the arginine cluster to the right of argH and has an average cotransduction frequency to the arginine cluster of approximately 0.55 [(+c)/total number of recombinants, Table 24]. The cotransduction frequency between rna-1 and metB1 is approximately 0.10 [(a+c)/total number of recombinants, Table 24].

Construction of Deletion Map

Isolation of arginine auxotrophs. In order to construct a fine structure map of the arginine cluster by deletion mapping, it was first necessary to have many point mutations and several deletion mutations. To enhance the frequency of point mutations, the mutagens NTG and 2-AP were used. The 2-AP was used because it gives rise to a high percentage of nonsense mutations (147). Nitrous acid was used as the mutagen to induce deletion mutations (116). The procedures

TABLE 23

THREE POINT TRANSDUCTIONS BETWEEN ARG DONORS AND MET, RNA RECIPIENT

Donor Genotype	Recipient Genotype	Selected Marker	Cotransduction Frequency of (+b) total	(+++) total	(+b+) total
$+ b_1 +$	a + c	a^+	0.23 (52/223)	0 (0/223)	0.026 (6/223)
$+ b_2 +$	a + c	a^+	0.31 (71/228)	0 (0/228)	0.043 (10/228)
$+ b_3 +$	a + c	a^+	0.36 (82/225)	0 (0/225)	0.031 (7/225)
$+ b_4 +$	a + c	a^+	0.23 (52/226)	0 (0/226)	0.031 (7/226)
$+ b_5 +$	a + c	a^+	0.28 (65/232)	0 (0/232)	0.000 (0/232)
$+ b_6 +$	a + c	a^+	0.32 (75/228)	0 (0/228)	0.030 (7/228)
$+ b_7 +$	a + c	a^+	0.21 (47/225)	0 (0/225)	0.066 (15/225)

a = metB 1 b_2 = argC 1418 b_4 = argC 1417 b_6 = argE 1538 c = rna-1
b_1 = argB 1511 b_3 = argC 1508 b_5 = argE 1536 b_7 = argH 1542

TABLE 24

THREE POINT TRANSDUCTIONS BETWEEN MET, RNA, DONOR AND ARG RECIPIENT

Donor Genotype	Recipient Genotype	Selected Marker	Cotransduction Frequency of (a+) total	(+c) total	(+++) total	(a + c) total
a + c	+ b_1 +	b_1^+	0.36 (40/112)	0.46 (51/112)	0.22 (25/112)	0.045 (5/112)
a + c	+ b_2 +	b_2^+	0.29 (33/112)	0.55 (62/112)	0.25 (28/112)	0.098 (11/112)
a + c	+ b_3 +	b_3^+	0.24 (27/112)	0.58 (65/112)	0.29 (32/112)	0.11 (12/112)
a + c	+ b_4 +	b_4^+	0.21 (24/112)	0.77 (86/112)	0.14 (16/112)	0.13 (14/112)
a + c	+ b_5 +	b_5^+	0.41 (45/111)	0.44 (49/111)	0.30 (34/111)	0.14 (16/111)
a + c	+ b_6 +	b_6^+	0.35 (39/112)	0.46 (51/112)	0.29 (32/112)	0.089 (10/112)
a + c	+ b_7 +	b_7^+	0.30 (25/112)	0.60 (67/112)	0.27 (30/112)	0.089 (10/112)

a = metB 1 b_2 = argC 1418 b_4 = argC 1417 b_6 = argE 1538 c = rna-1
b_1 = argB 1511 b_3 = argC 1508 b_5 = argE 1536 b_7 = argH 1542

followed are described in Chapter II, and the auxotrophs isolated are listed in Table 25. Mutants 1415 through 1587 were selected on limiting acetylornithine, whereas mutants 1588 through 2092 were selected on limiting arginine. The parental strain for mutants 1415 through 1575 was EcK-101, and the parental strain for mutants 1576 through 2092 was EcK-111 (metB1).

Characterization of arginine auxotrophs. The auxotrophs were characterized as follows.

Growth response on arginine intermediates. Each auxotroph was purified by streaking twice on MMA + arginine and characterized by growth response on intermediates of the arginine pathway. The response for each mutant is shown in Table 25. The phenotype AcO$^-$ refers to the ability to grow on MMA+ AcO, MMA + Orn, MMA +Arg, but not on MMA. This is the expected phenotype of an argA, B, C or D strain, although the possibility of detecting argD mutants is virtually nonexistent because of the absence of a proA or B block in the parental strain (see Chapter I, also reference 64). The phenotype Orn$^-$ refers to the ability to grow on MMA + Orn, MMA + Cit, and MMA + Arg, but not on MMA or MMA + AcO. An Orn$^-$ phenotype is expected of an argE strain. A Cit$^-$ phenotype, i.e., growth on MMA + Cit and MMA + Arg, but not on MMA, MMA + AcO, or MMA + Orn, did not occur since the parental strains had the functional alleles of both argF and argI. It is necessary to mutate both genes before a requirement for citrulline is detectable (45). The phenotype Arg$^-$ refers to the ability to grow on MMA + Arg but not on MMA, MMA + AcO, MMA + Orn, or MMA + Cit. The Arg$^-$ phenotype would be expected of either an argG or H strain.

TABLE 25

ORIGIN AND CHARACTERIZATION OF ARGININE AUXOTROPHS

Strain Number	Origin		Phenotype	Linkage to *rna-1*	Revertibility		
	Expt-flask	Mutagen			Spontaneous	NTG	DES
1417	1-9	NTG	AcO^-	.40	3	+	+
1418	1-10			.58	8	+	+
1506	4-1			.40	6	+	+
1507	4-3			.52	121	+	+
1508	4-5			.62	1	+	+
1511	4-9			.70	1	+	+
1512	4-9			.66	4	+	+
1573	3-1			.51	0	-	-
1578	7-5			.39	32	+	+
1580	7-5			.50	HR	-	+
1581	7-6			.51	3	+	+
1582	7-6			.41	6	+	+
1583	7-7			.51	3	+	+
1584	7-7			.37	3	+	+
1585	7-8			.55	2	+	+
1586	7-9			.44	8	+	+
1643	14-1			.52	3	+	+
1645	14-1			.55	HR	+	+
1646	14-1			.58	4	+	+
1649	14-1			.60	2	+	+
1653	14-1			.49	44	+	+
1660	14-1			.51	6	+	+
1663	14-2			.57	2	+	+
1665	14-2			.40	7	+	+
1668	14-2			.60	0	+	+
1674	14-3			.61	4	+	+
1675	14-3			.58	HR	+	+
1688	14-4			.60	4	+	+
1694	14-5			.08	HR	-	-
1696	14-5			.56	HR	-	+
1713	14-7			.62	3	+	+
1717	14-7			.50	5	+	+
1722	14-8			.59	3	+	+

TABLE 25 continued

Strain Number	Origin		Phenotype	Linkage to rna-1	Revertibility		
	Expt-flask	Mutagen			Spontaneous	NTG	DES
1726	14-8	NTG	AcO^-	.54	HR	+	+
1727	14-8			ND	ND	ND	ND
1731	14-9			.66	0	+	-
1734	14-9			.54	1	+	+
1738	14-9			.70	0	-	-
1741	14-10			.43	82	+	+
1415	1-6			.00	ND	ND	ND
1419	1-10			.00	ND	ND	ND
1509	4-8			.00	ND	ND	ND
1510	4-9			.00	ND	ND	ND
1575	3-1			.00	ND	ND	ND
1576	7-3			.00	ND	ND	ND
1577	7-4			.00	ND	ND	ND
1579	7-5			.00	ND	ND	ND
1587	7-10			.00	ND	ND	ND
1656	14-1			.00	ND	ND	ND
1657	14-1			ND	ND	ND	ND
1664	14-2			.00	ND	ND	ND
1669	14-2			.00	ND	ND	ND
1678	14-3			.00	ND	ND	ND
1692	14-4			.00	ND	ND	ND
1697	14-5			.00	ND	ND	ND
1700	14-5			.00	ND	ND	ND
1716	14-7			.00	ND	ND	ND
1720	14-8			.00	ND	ND	ND
1735	14-9			.00	ND	ND	ND
1743	14-1			.00	ND	ND	ND
1637	13-9	2-AP	AcO^-	.63	63	+	+
1639	13-9			.63	58	+	+
1789	18-2			.60	3	-	-
1790	18-2			.58	3	-	-
1791	18-2			.41	128	+	+
1794	18-4			.48	3	+	+
1801	18-5			.49	25	+	+
1634	13-1			.00	ND	ND	ND

TABLE 25 continued

Strain Number	Origin		Phenotype	Linkage to rna-1	Revertibility		
	Exp't-flask	Mutagen			Spontaneous	NTG	DES
1750	15-4	2-AP	AcO^-	.00	ND	ND	ND
1756	16-4			.00	ND	ND	ND
1762	16-10			.00	ND	ND	ND
1803	18-6			.00	ND	ND	ND
1590	8-6	HNO_2	AcO^-	.44	HR	+	+
1593	8-6			.43	1	-	-
1598	10-5			.38	0	-	+
1599	10-6			.49	14	+	+
1601	10-8			.50	HR	+	+
1765	17-1			.54	1	+	+
1767	17-2			.57	HR	+	+
1782	17-8			.45	22	+	+
1838	21-1			.56	0	-	-
1847	21-1			.55	0	-	-
1860	21-2			.59	12	-	-
1878	21-3			.54	4	-	-
1883	21-3			.46	5	+	+
1885	21-3			.62	6	-	-
1889	21-3			.58	2	-	-
1892	21-3			.33	0	-	+
2018	21-9			.59	4	-	-
2041	21-9			.61	4	-	-
2058	21-10			.51	2	-	-
2082	22-9			.39	6	-	-
1773	17-5			.00	ND	ND	ND
1775	17-6			.00	ND	ND	ND
1821	20-1			.00	0	-	-
1825	21-1			.00	1	-	-
1828	21-1			.00	1	-	-
1829	21-1			.00	5	-	-
1845	21-1			.00	1	-	-
1855	21-2			.00	0	-	-
1880	21-3			.00	4	-	+
1888	21-3			.00	0	-	-
1904	21-4			.00	1	-	-
1926	21-5			.00	0	-	-

TABLE 25 continued

Strain Number	Origin		Phenotype	Linkage to rna-1	Revertibility		
	Expt-flask	Mutagen			Spontaneous	NTG	DES
1934	21-5	HNO_2	AcO^-	.00	0	-	-
1936	21-5			.00	0	-	-
1997	21-8			.00	0	-	-
2013	21-8			.00	1	-	-
2042	21-9			.00	2	-	-
2045	21-9			.00	0	-	-
2046	21-9			.00	0	-	-
2052	21-10			.00	1	-	-
2064	21-10			.00	2	-	+
2078	22-9			.00	15	-	-
2081	22-9			.00	0	-	-
2085	22-10			.00	1	-	-
1826	21-1			ND	HR	+	+
1830	21-1			ND	6	-	+
1831	21-1			ND	4	+	+
1832	21-1			ND	HR	+	+
1833	21-1			ND	HR	+	+
1834	21-1			ND	9	+	+
1835	21-1			ND	0	+	+
1836	21-1			ND	LKY	-	-
1837	21-1			ND	HR	+	+
1839	21-1			ND	HR	+	+
1840	21-1			ND	3	+	+
1842	21-1			ND	3	+	+
1846	21-1			ND	HR	+	+
1848	21-1			ND	HR	+	+
1849	21-1			ND	LKY	-	-
1859	21-2			ND	7	+	+
1861	21-2			ND	6	+	+
1863	21-2			ND	9	+	+
1865	21-2			ND	151	+	+
1868	21-2			ND	4	+	+
1870	21-2			ND	121	+	+
1871	21-2			ND	2	+	+
1874	21-2			ND	LKY	+	+
1876	21-3			ND	HR	+	+

TABLE 25 continued

Strain Number	Origin		Pheno-type	Linkage to rna-1	Revertibility		
	Exp't-flask	Mutagen			Spontaneous	NTG	DES
1886	21-3	HNO_2	AcO^-	ND	20	-	+
1891	21-3			ND	HR	-	+
1894	21-3			ND	8	+	+
1895	21-3			ND	10	+	+
1901	21-4			ND	LKY	+	+
1903	21-4			ND	HR	+	+
1906	21-4			ND	0	+	+
1907	21-4			ND	6	-	+
1912	21-4			ND	HR	-	-
1913	21-5			ND	HR	-	+
1914	21-5			ND	0	+	-
1915	21-5			ND	6	-	-
1916	21-5			ND	LKY	+	+
1917	21-5			ND	4	+	+
1918	21-5			ND	HR	+	+
1920	21-5			ND	HR	+	+
1922	21-5			ND	1	+	+
1923	21-5			ND	1	+	+
1924	21-5			ND	1	-	+
1925	21-5			ND	HR	-	-
1927	21-5			ND	HR	+	+
1928	21-5			ND	1	+	+
1929	21-5			ND	98	-	+
1931	21-5			ND	LKY	-	-
1932	21-5			ND	67	+	+
1935	21-5			ND	LKY	-	-
1937	21-5			ND	103	+	+
1943	21-6			ND	3	+	+
1950	21-6			ND	10	+	+
1952	21-6			ND	59	+	+
1953	21-6			ND	57	-	-
1954	21-6			ND	67	-	+
1955	21-6			ND	13	-	-
1956	21-6			ND	18	-	-
1958	21-6			ND	16	-	-

TABLE 25 continued

Strain Number	Origin		Pheno-type	Linkage to rna-1	Revertibility		
	Expt-flask	Mutagen			Spontaneous	NTG	DES
1959	21-6	HNO_2	AcO^-	ND	49	+	+
1961	21-6			ND	0	+	+
1962	21-6			ND	17	+	+
1963	21-6			ND	HR	-	-
1964	21-6			ND	53	-	-
1967	21-6			ND	HR	+	+
1972	21-7			ND	HR	+	+
1973	21-7			ND	2	+	+
1977	21-7			ND	HR	+	+
1980	21-7			ND	5	+	+
1985	21-7			ND	3	+	+
1986	21-7			ND	HR	-	+
1990	21-7			ND	11	-	-
1999	21-8			ND	7	+	+
2002	21-8			ND	LKY	-	-
2004	21-8			ND	18	+	+
2005	21-8			ND	0	+	+
2010	21-8			ND	LKY	-	-
2011	21-8			ND	LKY	-	-
2016	21-8			ND	5	-	-
2017	21-8			ND	10	-	-
2019	21-9			ND	HR	-	-
2021	21-9			ND	HR	+	+
2022	21-9			ND	2	-	+
2023	21-9			ND	1	-	+
2031	21-9			ND	2	+	+
2032	21-9			ND	HR	-	+
2033	21-9			ND	180	+	+
2043	21-9			ND	HR	+	+
2049	21-10			ND	3	+	+
2050	21-10			ND	15	+	+
2053	21-10			ND	16	+	+
2054	21-10			ND	100	-	-
2056	21-10			ND	3	-	+
2063	21-10			ND	HR	+	+

TABLE 25 continued

Strain Number	Origin		Pheno-type	Linkage to rna-1	Revertibility		
	Exp't-flask	Mutagen			Spontaneous	NTG	DES
2066	21-10	HNO_2	AcO^-	ND	HR	+	+
2067	21-10			ND	4	+	+
2071	22-1			ND	LKY	-	-
2072	22-1			ND	LKY	-	+
2074	22-3			ND	19	-	-
2077	22-4			ND	LKY	+	+
2080	22-9			ND	HR	+	+
2083	22-9			ND	HR	+	+
2090	22-10			ND	HR	+	+
2092	22-10			ND	HR	+	+
1641	14-1	NTG	Orn$^-$	.50	19	+	+
1644	14-1			.60	3	+	+
1662	14-1			.34	18	+	+
1666	14-2			.37	0	+	-
1685	14-4			.35	18	+	+
1689	14-4			.45	HR	-	-
1691	14-4			.40	29	+	+
1710	14-7			.32	5	-	-
1718	14-7			.46	4	+	+
1719	14-8			.34	6	+	+
1730	14-9			.47	2	+	+
1732	14-9			.57	4	+	+
1671	14-3			—	2	+	+
1715	1407			.00	ND	ND	ND
1751	15-7	2-AP	Orn$^-$	.34	HR	+	+
1757	16-4			.42	1	+	+
1786	18-1			.49	1	+	-
1792	18-2			.38	1	+	+
1798	18-4			.46	HR	+	+
1799	18-4			.37	HR	-	+
1802	18-6			.50	6	+	+
1591	8-7	HNO_2	Orn$^-$	.44	HR	-	-
1596	8-7			.39	43	+	+
1764	17-1			.39	7	-	+
1823	20-2			.42	2	-	-

TABLE 25 continued

Strain Number	Origin		Phenotype	Linkage to rna-1	Revertibility		
	Expt-flask	Mutagen			Spontaneous	NTG	DES
1862	21-2	HNO_2	Orn^-	.33	2	-	-
2084	22-10			.38	1	-	-
2091	22-10			.35	3	-	-
1594	8-9			.00	ND	ND	ND
1822	20-1			ND	HR	-	-
1854	21-2			ND	63	+	+
1960	21-6			ND	HR	+	+
2025	21-9			ND	16	+	+
2044	21-9			ND	HR	+	+
2059	21-10			ND	HR	+	+
2073	22-2			ND	HR	-	-
1642	14-1	NTG	Arg^-	.56	9	+	+
1650	14-1			.62	17	+	+
1651	14-1			.03	127	+	+
1655	14-1			.65	1	+	+
1659	14-1			.54	2	+	-
1661	14-1			.47	HR	+	+
1670	14-2			.57	HR	+	+
1672	14-3			.56	29	+	-
1673	14-3			.64	HR	-	-
1680	14-3			.53	48	+	+
1682	14-3			.54	61	+	+
1684	14-4			.69	12	+	+
1687	14-4			.41	0	-	-
1698	14-5			.46	1	+	+
1704	14-6			.40	7	+	+
1706	14-6			.61	28	+	+
1707	14-6			.37	0	+	+
1708	14-7			.68	1	+	-
1709	14-7			.70	138	+	+
1728	14-8			.56	1	+	-
1737	14-9			.65	4	+	+
1739	14-10			.55	173	+	+
1742	14-10			.62	HR	+	+
1744	14-10			.62	158	+	+
1647	14-1			.00	ND	ND	ND

TABLE 25 continued

Strain Number	Origin		Phenotype	Linkage to rna-1	Revertibility		
	Exp't-flask	Mutagen			Spontaneous	NTG	DES
1648	14-1	NTG	Arg$^-$	.00	ND	ND	ND
1654	14-1			.00	ND	ND	ND
1658	14-1			.00	ND	ND	ND
1667	14-2			.00	ND	ND	ND
1676	14-3			.00	ND	ND	ND
1677	14-3			.00	ND	ND	ND
1679	14-3			.00	ND	ND	ND
1681	14-3			.00	ND	ND	ND
1683	14-4			.00	ND	ND	ND
1686	14-4			.00	ND	ND	ND
1690	14-4			.00	ND	ND	ND
1693	14-4			.00	ND	ND	ND
1695	14-5			.00	ND	ND	ND
1699	14-5			.00	ND	ND	ND
1701	14-6			.00	ND	ND	ND
1702	14-6			.00	ND	ND	ND
1703	14-6			.00	ND	ND	ND
1705	14-6			.00	ND	ND	ND
1711	14-7			.00	ND	ND	ND
1712	14-7			.00	ND	ND	ND
1714	14-7			.00	ND	ND	ND
1721	14-8			.00	ND	ND	ND
1723	14-8			.00	ND	ND	ND
1724	14-8			.00	ND	ND	ND
1725	14-8			.00	ND	ND	ND
1729	14-9			.00	ND	ND	ND
1733	14-9			.00	ND	ND	ND
1736	14-9			.00	ND	ND	ND
1740	14-10			.00	ND	ND	ND
1745	14-10			.00	ND	ND	ND
1746	14-10			.00	ND	ND	ND
1747	14-3			.00	ND	ND	ND
1607	11-4	2-AP	Arg$^-$	.47	21	+	+
1636	13-5			.65	69	+	+
1640	13-9			.65	23	+	+

TABLE 25 continued

Strain Number	Origin		Phenotype	Linkage to rna-1	Revertibility		
	Expt-flask	Mutagen			Spontaneous	NTG	DES
1753	16-3	2-AP	Arg$^-$	.34	HR	+	-
1754	16-3			.44	177	-	+
1758	16-5			.44	4	+	+
1759	16-6			.55	HR	+	+
1760	16-8			.60	4	+	+
1761	16-10			.46	23	+	+
1763	16-9			.61	4	+	+
1787	18-2			.51	3	+	+
1788	18-2			.50	13	+	+
1793	18-4			.61	2	+	+
1797	18-4			.48	4	+	+
1804	18-6			.39	11	+	+
1805	18-6			.40	13	+	+
1638	13-9			.00	ND	ND	ND
1749	15-1			.00	ND	ND	ND
1755	16-3			.00	ND	ND	ND
1785	18-1			.00	ND	ND	ND
1795	18-4			.00	ND	ND	ND
1796	18-4			.00	ND	ND	ND
1800	18-5			.00	ND	ND	ND
1806	18-8			.00	ND	ND	ND
1807	18-10			.00	ND	ND	ND
1588	8-9	HNO_2	Arg$^-$	.41	10	-	-
1589	8-9			.48	7	+	+
1600	10-6			.47	9	+	+
1602	10-8			.51	HR	+	+
1604	10-9			.65	14	-	-
1766	17-2			.50	0	-	-
1768	17-3			.53	HR	+	+
1772	17-5			.37	17	+	+
1779	17-7			.57	7	-	+
1780	17-7			.45	0	-	-
1781	17-7			.49	2	-	-
1784	17-9			.50	13	+	+
1856	21-2			.45	0	-	-

TABLE 25 continued

Strain Number	Origin		Phenotype	Linkage to rna-1	Revertibility		
	Exp't-flask	Mutagen			Spontaneous	NTG	DES
1857	21-2	HNO_2	Arg^-	.52	1	-	-
1884	21-3			.35	1	-	-
1909	21-4			.50	12	-	-
1930	21-5			.62	0	-	-
1940	21-6			.41	HR	-	-
1969	21-6			.52	0	-	-
1982	21-7			.39	2	-	-
2007	21-8			.39	0	-	-
2009	21-8			.57	1	-	-
2075	22-4			.68	1	-	-
1592	8-6			.00	ND	ND	ND
1595	8-1			.00	ND	ND	ND
1597	10-1			.00	ND	ND	ND
1603	10-9			.00	ND	ND	ND
1605	10-10			.00	ND	ND	ND
1769	17-3			.00	ND	ND	ND
1770	17-4			.00	ND	ND	ND
1771	17-4			.00	ND	ND	ND
1774	17-6			.00	ND	ND	ND
1776	17-6			.00	ND	ND	ND
1777	17-6			.00	ND	ND	ND
1778	17-7			.00	ND	ND	ND
1783	17-9			.00	ND	ND	ND
1824	21-1			.00	4	-	-
1844	21-1			.00	0	-	-
1850	21-1			.00	5	-	-
1851	21-1			.00	0	+	+
1858	21-2			.00	0	+	+
1867	21-2			.00	1	-	-
1890	21-3			.00	0	-	-
1893	21-3			.00	0	-	-
1898	21-4			.00	0	-	-
1933	21-5			.00	1	-	-
1984	21-7			.00	0	-	-
1995	21-7			.00	0	-	-
1998	21-8			.00	2	-	-

TABLE 25 continued

Strain Number	Origin		Phenotype	Linkage to rna-1	Revertibility		
	Exp't-flask	Mutagen			Spontaneous	NTG	DES
2000	21-8	HNO_2	Arg^-	.00	0	-	-
2001	21-8			.00	1	-	-
2003	21-8			.00	0	-	-
2006	21-8			.00	0	-	-
2008	21-8			.00	1	-	-
2014	21-8			.00	1	-	-
2020	21-9			.00	1	-	-
2026	21-9			.00	1	-	-
2028	21-9			.00	0	-	-
2079	22-9			.00	1	-	-
1827	21-1			ND	3	-	+
1841	21-1			ND	7	+	+
1843	21-1			ND	8	+	+
1852	21-1			ND	11	+	-
1853	21-2			ND	0	+	+
1864	21-2			ND	0	+	+
1866	21-2			ND	1	+	+
1869	21-2			ND	HR	+	+
1872	21-2			ND	HR	-	-
1873	21-2			ND	HR	+	+
1875	21-3			ND	0	+	+
1877	21-3			ND	1	+	+
1879	21-3			ND	17	+	+
1881	21-3			ND	1	+	+
1882	21-3			ND	HR	+	+
1887	21-3			ND	8	+	+
1896	21-4			ND	HR	-	-
1897	21-4			ND	1	-	+
1899	21-4			ND	0	-	+
1900	21-4			ND	2	+	+
1902	21-4			ND	2	+	+
1905	21-4			ND	4	+	+
1908	21-4			ND	44	-	-
1910	21-4			ND	8	-	-
1911	21-4			ND	11	+	+

TABLE 25 continued

Strain Number	Origin		Pheno-type	Linkage to *rna*-1	Revertibility		
	Expt-flask	Mutagen			Spontaneous	NTG	DES
1919	21-5	HNO_2	Arg^-	ND	22	-	-
1921	21-5			ND	HR	-	-
1938	21-5			ND	3	-	+
1939	21-6			ND	2	-	+
1941	21-6			ND	1	-	+
1942	21-6			ND	1	+	+
1944	21-6			ND	HR	-	-
1945	21-6			ND	HR	+	+
1946	21-6			ND	HR	-	-
1947	21-6			ND	HR	-	-
1948	21-6			ND	HR	-	-
1949	21-6			ND	HR	+	+
1951	21-6			ND	HR	-	-
1957	21-6			ND	HR	+	+
1965	21-6			ND	64	-	-
1966	21-6			ND	18	-	-
1968	21-6			ND	HR	+	+
1970	21-7			ND	1	-	+
1971	21-7			ND	3	+	+
1974	21-7			ND	LKY	+	+
1975	21-7			ND	16	+	+
1976	21-7			ND	HR	+	+
1978	21-7			ND	41	+	+
1979	21-7			ND	8	+	+
1981	21-7			ND	0	+	+
1983	21-7			ND	1	-	+
1987	21-7			ND	7	+	+
1988	21-7			ND	21	+	+
1989	21-7			ND	33	+	+
1991	21-7			ND	HR	-	-
1992	21-7			ND	1	+	+
1993	21-7			ND	HR	+	+
1994	21-7			ND	13	+	+
1996	21-8			ND	5	-	+
2012	21-8			ND	19	+	+

TABLE 25 continued

Strain Number	Origin		Phenotype	Linkage to rna-1	Revertibility		
	Expt-flask	Mutagen			Spontaneous	NTG	DES
2015	21-8	HNO_2	Arg^-	ND	5	+	+
2024	21-9			ND	80	+	+
2027	21-9			ND	0	-	+
2029	21-9			ND	1	+	+
2030	21-9			ND	3	+	+
2034	21-9			ND	43	+	+
2035	21-9			ND	6	+	+
2036	21-9			ND	0	-	+
2037	21-9			ND	0	+	+
2038	21-9			ND	0	+	+
2039	21-9			ND	21	+	+
2040	21-9			ND	HR	+	+
2047	21-9			ND	HR	+	+
2048	21-10			ND	HR	+	+
2051	21-10			ND	1	+	+
2055	21-10			ND	1	+	+
2057	21-10			ND	6	-	+
2060	21-10			ND	52	-	-
2061	21-10			ND	113	-	+
2062	21-10			ND	HR	-	-
2065	21-10			ND	160	-	-
2068	21-10			ND	HR	-	-
2069	21-10			ND	LKY	+	+
2070	21-10			ND	HR	+	+
2076	22-4			ND	9	+	+
2086	22-10			ND	54	-	+
2087	22-10			ND	36	+	+
2088	22-10			ND	31	+	+
2089	22-10			ND	35	+	+

Linkage to rna-1. Since rna-1 gave an average cotransduction frequency to the arginine cluster of 0.55, linkage to rna-1 was used to distinguish cluster mutations from noncluster mutations, e.g., argB or C strains from argA strains and argH mutants from argG mutants. Cotransduction frequencies by Plkc were determined by using Eck112 (metB1, rna-1) as a donor and the arginine auxotroph as a recipient. Transductants prototrophic with respect to arginine were selected on MMA + met, and approximately 100 colonies were checked for rifamycin-resistance on NBG + rifamycin agar. The occurrence of a significant number of rifamycin-resistant colonies was taken as evidence that the arginine auxotroph was carrying a mutation in the arginine cluster. ArgA or argG (Fig. 3) is not cotransducible with rna-1. The results are shown in Table 25. Special note should be made of mutants 1715 and 1594 which had the Orn^- phenotype expected of an argE mutant but which showed no linkage to rna-1 (even though more than 200 prototrophic transductants of each were checked for rifamycin-resistance). Althought an explanation for this phenotype is not obvious, these strains are presumably carrying a genetic block leading to ornithine-dependence in a locus heretofore not reported.

Spontaneous reversion rate. The spontaneous reversion rate per 2×10^8 cells was determined as described in Chapter II. The findings are shown in Table 25. Any rate greater than 200 colonies/2×10^8 cells was designated as HR (high reverting). An LKY (leaky) designation was given to those strains, which after 5 days of incubation, gave a continuous film of growth such that any revertant colonies were masked.

NTG-induced reversion. The revertibility by NTG of arginine auxotrophs (Table 25) was determined as described in Chapter II. Revertibility was scored as + when 4 or more colonies above the spontaneous rate were observed and as - when 3 colonies or fewer were observed.

DES-induced reversion. The revertibility by DES of arginine auxotrophs (Table 25) was determined as described in Chapter II. Revertibility was scored as + when 4 or more colonies above the spontaneous rate were observed and as - when 3 colonies or fewer were observed.

Auxotrophs which showed a significant spontaneous and/or mutagen induced reverion rate were considered to be point mutants. Auxotrophs which were neither spontaneously nor mutagen revertible were considered to be deletion mutants.

Determination of enzyme activity. Extracts of cultures of selected arginine point and deletion mutants were assayed for the arginine enzymes 5, 3, 2, 8 and 4 (Table 26). All strains were grown on MMA + arginine (plus methionine when needed). Strains 111 and 1588-2091 were grown to a density of 70 Klett units in 100 ml quantities instead ofto 40 Klett units and 300 ml quantities as described in Chapter II. The specific activities found for mutants 1588-2091 were expressed as percents of the corresponding activities in Eck111 (parental strain) taken as 100. Derivatives of strains 1415-1512, carrying the argR 32 allele of the regulator gene were made by transduction. Assays were performed on extracts of cultures of these derivatives. The enzyme activities for these mutants were expressed as percent of the corresponding act-

TABLE 26

RELATIVE ACTIVITIES OF ARGININE ENZYMES IN SELECTED ARGININE AUXOTROPHS

Strain	Phenotype	*Enzyme 5	3	2	8	4
1416 argR⁻	AcO⁻	46	0	80	197	9.7
1417 argR⁻	AcO⁻	124	0	9.1	8.1	42
1418 argR⁻	AcO⁻	80	0	42	102	58
1506 argR⁻	AcO⁻	9.1	1.7	0	7.6	23
1507 argR⁻	AcO⁻	27	0.8	14	31	54
1508 argR⁻	AcO⁻	111	0	69	134	87
1511 argR⁻	AcO⁻	93	87	0	119	64
1512 argR⁻	AcO⁻	5.3	1.9	0	2.6	5.5
1884 argR⁺	Arg⁻	94	105	72	0	92
1909 argR⁺	Arg⁻	72	114	115	0	123
1930 argR⁺	Arg⁻	67	106	121	0	78
2075 argR⁺	Arg⁻	78	107	97	0	104
1593 argR⁺	AcO⁻	74	83	97	247	81
1738 argR⁺	AcO⁻	105	93	63	92	103
1789 argR⁺	AcO⁻	87	78	74	72	92
1790 argR⁺	AcO⁻	92	85	96	95	93
1838 argR⁺	AcO⁻	71	90	120	25	95
1847 argR⁺	AcO⁻	74	98	100	105	71
1860 argR⁺	AcO⁻	66	64	94	165	113
1878 argR⁺	AcO⁻	78	94	65	122	109
1885 argR⁺	AcO⁻	83	91	93	125	96
1889 argR⁺	AcO⁻	70	88	89	121	107
1892 argR⁺	AcO⁻	97	103	90	84	62
2018 argR⁺	AcO⁻	71	96	100	114	88
2041 argR⁺	AcO⁻	72	103	92	428	85
2058 argR⁺	AcO⁻	47	97	95	106	106
2082 argR⁺	AcO⁻	87	97	91	75	71
1710 argR⁺	Orn⁻	0	88	81	54	150
1823 argR⁺	Orn⁻	0	103	88	144	125
1862 argR⁺	Orn⁻	0	114	101	102	104
2084 argR⁺	Orn⁻	0	79	129	109	138
2091 argR⁺	Orn⁻	0	97	103	111	92
1588 argR⁺	Arg⁻	83	112	68	0	102
1604 argR⁻	Arg⁻	81	106	75	0	83

TABLE 26 continued

Strain	Phenotype	*Enzyme 5	3	2	8	4
1687 argR$^+_+$	Arg$^-$	187	150	46	0	169
1766 argR$^+_+$	Arg$^-$	94	102	97	0	92
1780 argR$^+_+$	Arg$^-$	102	98	101	0	107
1781 argR$^+_+$	Arg$^-$	77	88	59	0	84
1856 argR$^+_+$	Arg$^-$	79	114	123	42	96
1857 argR$^+_+$	Arg$^-$	79	94	124	11	153
1969 argR$^+_+$	Arg$^-$	61	80	85	16	101
1982 argR$^+_+$	Arg$^-$	98	113	61	0	65
2007 argR$^+_+$	Arg$^-$	84	107	79	0	107
2009 argR$^+_-$	Arg$^-$	65	90	90	0	107
1415 argR$^-_-$	Ac0$^-$	86	86	52	104	57
1419 argR$^-_-$	Ac0$^-$	165	62	66	110	56
1509 argR$^-_-$	Ac0$^-$	123	103	60	134	86
1510 argR$^-_-$	Ac0$^-$	91	73	54	113	79

*Enzyme: These numbers represent percent enzyme activity calculated by setting the corresponding prototrophic argR$^+$ or argR$^-$ strain grown with arginine equal to 100.

ivities found in K12, argR32. The first eleven strains listed in the table are point mutations which have been shown to be linked to rna-1. The next 37 strains are presumptive cluster deletion mutants. The last four strains are argA mutants.

The following strains show the absence of the enzyme corresponding to the gene indicated and exhibit the presence of the other enzymes specified by the cluster genes. These phenotypes are typical of a complete genetic block in the indicated gene: argB, 1511; argC, 1418 and 1508; argE, 1710, 1823, 1862, 2084, and 2091; argH, 1588, 1604, 1766, 1780, 1781, 1884, 1909, 1930, 1982, 2009 and 2075; argA, 1415, 1419, 1509 and 1510.

Whereas assays were performed on cell-free extracts of argR32 canavanine-resistant, arginine-excreting derivatives of argB 1506 and argB 1512, enzyme synthesis in these strains is repressible by arginine (argR$^+$-type). On the other hand, assays performed on a cell-free extract of an argR32, canavanine-resistant, arginine-excreting derivative of argC 1507 indicated that enzyme synthesis in this strain is partially repressible by arginine. It may be that in the process of recombination into the recipient chromosome which occurs following transduction, the "high level, nonrepressible" argR32 allele in the first case became a "low level, arginine repressible" argR allele and in the second case became a "high level, arginine repressible" argR allele. Both these classes of argR alleles have been isolated elsewhere by direct mutation (82, 121).

The results obtained with argC 1416 are not readily explainable (enzyme 5 - 46%, enzyme 3 - 0%, enzyme 2 - 80%, enzyme 8 - 197%, and

enzyme 4 - 9.7%). ArgC 1417 appears to be carrying a polar mutation in argC.

The results obtained with argH 1856, argH 1857, and argH 1969 indicate that these deletion mutants have retained some enzyme 8 activity. With the exception of that for enzyme 2, the enzyme levels in argH 1687, $argR^+$ are greater than 150% for some unknown reason.

Strains 1738, 1789, 1790, 1847, 1860, 1878, 1885, 1889, 1892, 2018, 2058 and 2082 are all phenotypically AcO^- and by linkage to rna-1 were found to be associated with the arginine cluster. However, none of these strains shows the absence of any of the enzymes specified by the clustered genes. This class of mutations have been tentatively assigned the symbol argX.

Additionally, argX strains cannot grow on MMA + glutamate or AF medium. These strains are unable to grow on MMA at 25, 37 or 41°C, eliminating the possibility of an in vivo temperature-sensitive mutation in argB or C. Whereas, argX 1593 and argX 2041 have a significant elevation in enzyme 8 activity (247% and 428%, respectively), argX 1838 has only 25% of the normal enzyme 8 activity. ArgH 2007 which has no enzyme 8 activity appears to harbor a considerable deletion of argX (see mapping below).

Deletion mapping. For the purpose of construction of a "fine structure" map of the clustered arginine genes, 99 point cluster mutants and 32 deletion cluster mutants were used as donors and crossed with the 32 deletion mutants as recipients (total of 4,192 crosses) as described in Chapter II. The results are given in Tables 27-31. If one deletion mutant is crossed with another deletion mutant, prototrophic recombinants will arise only if the two mutations do not over-

TABLE 27

DELETION MAPPING OF ORNITHINE REQUIRING MUTANTS

	Recipient				
	1710	1823	1862	2084	2091
Donor					
1710	-	-	+	-	-
1823	+	-	+	+	+
1862	+	+	-	+	+
2084	-	+	+	-	+
2091	-	+	+	+	
1641	+	+	+	-	+
1644	+	+	-	+	+
1662	-	+	+	+	+
1666	-	+	+	+	+
1685	-	+	+	+	+
1691	+	+	+	+	+
1718	+	+	+	+	+
1719	+	+	+	+	+
1730	+	+	+	+	+
1732	+	+	+	+	+
1757	+	-	+	+	+
1764	+	+	+	+	+
1786	+	+	+	+	+
1792	+	+	+	+	+
1802	+	+	+	+	+
1854	+	-	+	+	+
2025	+	+	+	+	+

TABLE 28

CROSSES AMONG ACETYLORNITHINE REQUIRING DELETION MUTANTS

D \ R	1593	1738	1789	1790	1838	1847	1860	1878	1885	1889	1892	2018	2041	2058	2082	2007
1593	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
1738	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-
1789	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
1790	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
1838	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-
1847	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-
1860	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
1878	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
1885	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-
1889	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-
1892	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-
2018	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-
2041	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-
2058	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
2082	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
2007	-	-	+	+	-	-	+	-	-	-	-	-	-	+	+	-

TABLE 29

CROSSES AMONG ACETYLORNITHINE REQUIRING POINT AND DELETION MUTANTS YIELDING NO ARG$^+$ TRANSDUCTANTS

Donor	Recipient
1581	2058, 2082
1585	2007
1586	2007, 2041
1598	2007
1637	2007
1639	2007
1649	2007
1660	1789
1665	1789, 1790
1668	1885, 1889, 2007
1674	1789
1734	2058, 2082
1765	2007
1794	1790, 2082
1801	2007

The following strains gave arg$^+$ tranductants with all recipients: 1417, 1418, 1506, 1507, 1508, 1511, 1512, 1573, 1582, 1584, 1599, 1643, 1646 1653, 1657, 1663, 1688, 1713, 1717, 1722, 1727, 1731, 1741, 1782 1791, 1854.

TABLE 30

CROSSES AMONG ARGININE REQUIRING DELETION MUTANTS

D \ R	1588	1604	1687	1766	1780	1781	1856	1857	1969	1982	2007	2009
1588	-	+	+	+	+	+	+	+	+	+	+	+
1604	+	-	+	+	+	+	+	+	+	+	+	+
1687	+	+	-	+	+	+	+	-	+	-	+	+
1766	+	+	+	-	+	+	+	+	+	+	-	+
1780	+	+	+	+	-	+	+	+	+	+	-	+
1781	+	+	+	+	+	-	+	-	+	+	+	+
1856	+	+	+	+	+	+	-	+	+	+	+	+
1857	+	+	-	+	+	-	+	-	+	-	+	-
1969	+	+	+	+	+	+	+	+	-	+	+	+
1982	+	+	-	+	+	+	+	-	+	-	-	-
2007	+	+	+	-	-	+	+	+	+	-	-	-
2009	+	+	+	+	+	+	+	-	+	-	-	-

TABLE 31

CROSSES AMONG ARGININE REQUIRING POINT AND DELETION MUTANTS YIELDING NO ARG$^+$ TRANSDUCTANTS

Donor	Recipient
1589	2007
1607	1604
1636	1856
1642	1780, 2007
1650	2007
1651	1857, 2009, 1982
1655	1604
1659	1766, 2007
1661	1604
1672	1604
1680	2007
1682	2007
1698	1856
1704	1780, 2007
1706	1857, 1982, 2009
1707	1856
1708	1982, 2009
1709	1969
1728	2007
1737	2007
1739	1604
1744	2007
1758	2007
1768	2007
1772	1969
1779	1969
1787	2007
1793	1687
1797	1857, 2007
1884	2007
1909	1856
1930	1857, 1892, 2009
2075	2007

The following strains gave arg$^+$ transductants with all recipients: 1600, 1640, 1699, 1761, 1763, 1784, 1788, 1805.

lap. The same is true of a point mutation crossed with a deletion mutation; that is, if a point lies outside of the deletion mutation, prototrophic recombinants will result.

In Tables 27, 28 and 30 a cross which yielded prototrophic recombinants is indicated by a +; a cross which did not yield prototrophic recombinants is indicated by a - . Table 27 gives the results for the crosses of the Orn$^-$ deletion and point mutants. The deletion donors are listed above the double line and the point donors are listed below the double line. When Orn$^-$ mutants (either point or deletion) were crossed with argX or argH recipients, prototrophic recombinants resulted in every case (not shown). Table 28 gives the results of the AcO$^-$ deletion mutants and argH 2007 crossed with themselves and each with the others. The donors (D) are listed vertically and recipients (R) are listed horizontally. When AcO$^-$ deletions were crossed with Orn$^-$ mutants or arg$^-$ mutants, prototrophic recombinants resulted (not shown). Table 29 lists the crosses between AcO$^-$ point mutants and AcO$^-$ deletion mutants (plus argH 2007) which yielded no prototrophic recombinants. All other recipients yielded prototrophic recombinants when crossed with these point mutants (not shown). Table 30 gives the results of the Arg$^-$ deletion mutants crossed with themselves and each with the others. The donors (D) are listed vertically and recipients (R) are listed horizontally. When Arg$^-$ deletions were crossed with Orn$^-$ or AcO$^-$ mutants (except argH 2007, see Tables 28 and 29), prototrophic recombinants resulted (not shown). Table 31 lists the crosses between Arg$^-$ point mutants and Arg$^-$ deletion mutants which yielded no prototrophic recombinants. All other recipients yielded prototrophic

prototrophic recombinants when crossed with these point mutants (not shown).

Figure 29 is the best representation of the data in Tables 27-31. Certainly some of the relationships will change upon analysis of further crosses. The deletion recipients are shown below the line representing the chromosome and the point donors are shown above the line.

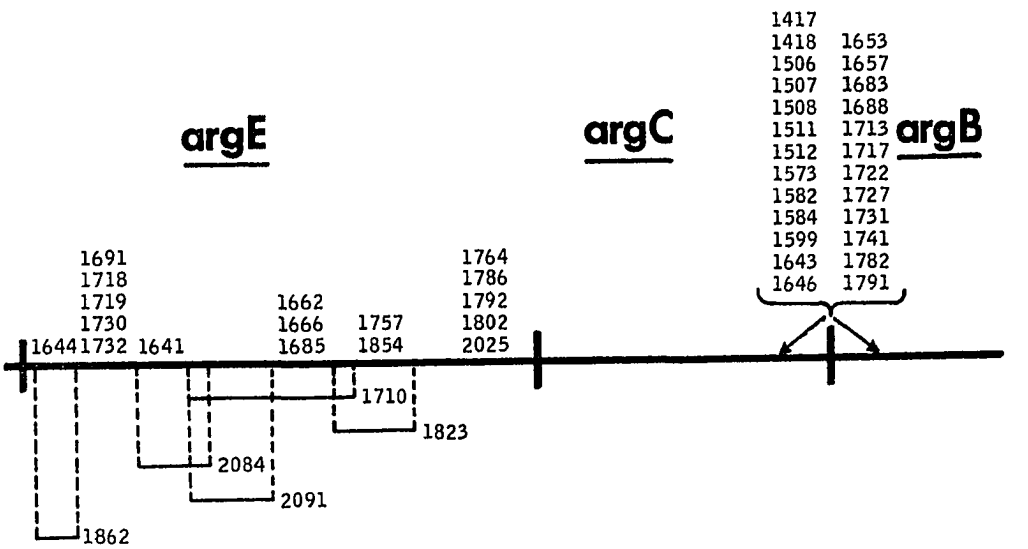
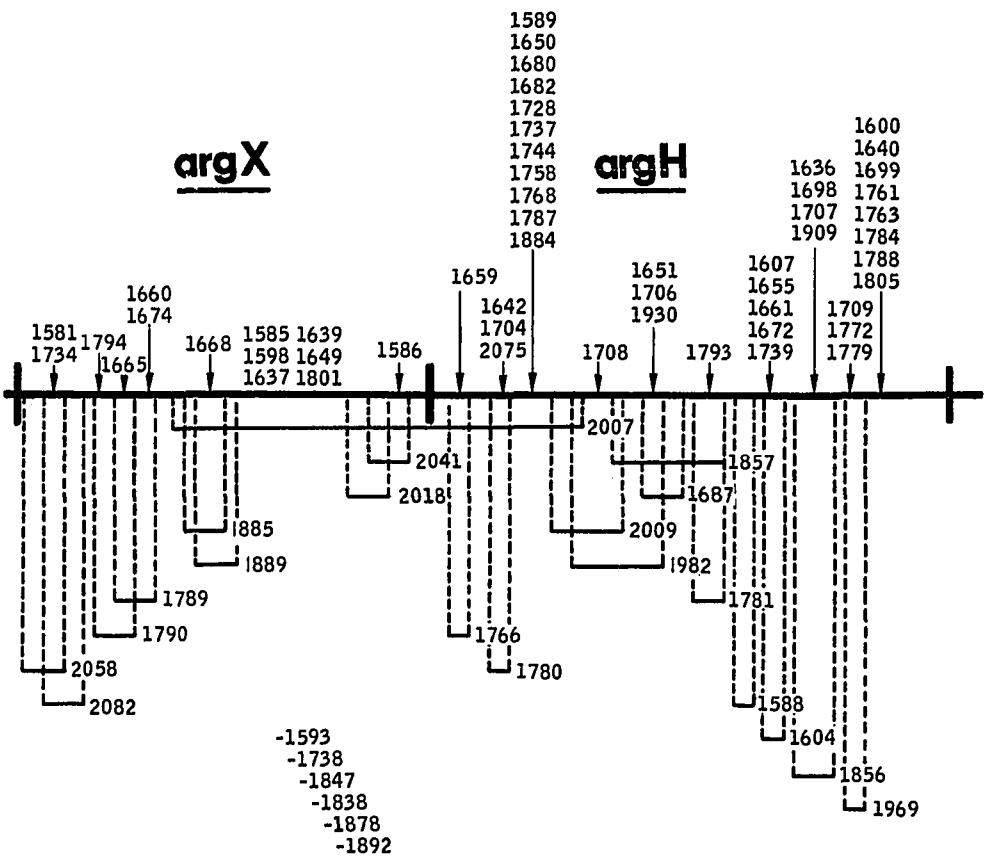


Figure 29. Partial deletion map of the clustered arginine genes.


argX
argH
1589
1650
1680
1682
1728
1737
1744
1758
1768
1787
1884
1600
1640
1699
1761
1763
1784
1788
1805
1636
1698
1707
1909
1607
1655
1661
1672
1739
1709
1772
1779
1659
1642
1704
2075
1651
1706
1930
1660
1674
1581
1734
1794
1665
1668
1585
1598
1637
1639
1649
1801
1586
1708
1793
2007
2041
2018
1857
1687
885
889
2009
1982
1789
1790
1781
1766
1780
2058
2082
1588
1604
1856
1969
-1593
-1738
-1847
-1838
-1878
-1892

CHAPTER IV

DISCUSSION

The regulation of enzyme synthesis can perhaps best be appreciated against the background of present knowledge of protein synthesis in general. Several excellent reviews have recently appeared in the literature (77, 78). There are also several recent reviews of the regulation of enzyme synthesis (15, 88, 129, 143). It is beyond the scope and nature of this discussion to attempt a comprehensive review of these active fields with their voluminous literatures. However, the regulation of expression of the clustered arginine genes will be discussed in the context of recent findings in other fields.

Expression of the clustered arginine genes was studied in strains W and K12 of E. coli. In E. coli, W, synthesis of enzymes 5 and 2 is coordinate whether studied under conditions of genetic derepression in MMA or MME (argR$^+$ and argR$^-$ grown with and without added arginine, see Tables 12 and 13) or physiological derepression (limiting the endogenous supply of arginine, see Tables 15 and 16).

An inconsistency with the observed coordinacy of synthesis of enzymes 5 and 2 occurred in AF medium (Table 14). Whereas the fully repressed rate for enzyme 5 is reduced by two-fold in AF medium as compared to that in MMA or MME, the rate of enzyme 2 synthesis remains unchanged. The reason for this

anomaly is not clear, although the possibility cannot be eliminated that a component of AF medium causes a pathway-wide increase in the binding affinity of "active" repressor for the respective recognition sites (fully repressed rates of enzymes 5, 3, 8 and 4 are lowered, Table 14). The other unexplainable discrepancy between the synthesis of enzymes 5 and 2 occurred in MMA and homoarginine (Table 18). Whereas the synthesis of enzyme 2 is unaffected by the addition of homoarginine to a culture of an argR$^-$ derivative of E. coli, W, the synthesis of enzyme 5 appears to be further derepressed by a factor of 1.27.

The synthesis of enzymes 3 and 8 is not considered to be coordinate in E. coli, W, for the following reasons: (1) when endogenous arginine is limited, synthesis of enzyme 3 proceeds at a greater rate than that of enzyme 8 (Table 16); (2) in all three media, synthesis of enzyme 3 is to a small but significant degree, repressible by arginine in an argR$^-$ derivative, whereas synthesis of enzyme 8 is not affected. Homoarginine does not have any corepressor activity on the synthesis of either enzymes 3 or 8.

Two enzyme-specific regulatory mutants of strain W were isolated which showed alterations in the rate of synthesis of enzymes 5 and 2, but not of enzymes 3 and 8. In one strain, 1403, synthesis is not as repressible as in the wild type parental strain. On the other hand, in mutant 1406 synthesis of enzymes 5 and 2 is "superrepressible." These findings are consistent with the view that a repressor recognition site can be changed by mutation in such a way as to increase or decrease its affinity for "active" repressor.

The observation of noncordinacy in the synthesis of enzymes 5 and 8 has

been reported for both W (130, 141) and K12 (43, 27). In view of the results obtained for strain W, it was of interest to extend the reported studies on the expression of the clustered arginine genes in K12. As in W, varying rates of derepression were achieved by (1) genetic derepression (*argR*$^-$) and (2) physiological derepression.

In K12, synthesis of 5 and 2 is not coordinate when studied by genetic derepression. Their fully repressed rates of synthesis are different (Table 21). However, when endogenous limitation of the arginine supply was used to study derepression, the synthesis of enzymes 5 and 2 was coordinate above their different basal levels for at least a six-fold range of derepression. The synthesis of enzymes 3 and 8 appears to be coordinate whether studied by genetic or physiological derepression. A very recent report agrees with our findings concerning the expression of the clustered arginine genes (27) when studied by genetic derepression; however, the observation of coordinacy of 5 and 2 above different basal rates has never been reported.

An explanation of the above results is made difficult by the following: (1) the gene order of *argE-C-B-H* has been reported (27, 41), thus making this the first example of coordinate synthesis of two enzymes specified by genes which are not immediately adjacent but very closely linked and perhaps encoded in the same polygenic mRNA. (2) The results obtained by genetic derepression differed from those obtained by physiological derepression for enzymes 5 and 2 in K12 and the degree of derepression obtained physiologically was greater than that obtained genetically in W.

There are several recent findings which strongly suggest that the translation of polygenic mRNA need not be coordinate. It has been demonstrated (94) that ribosomes can attach with high efficiency to start signals within a messenger. Thus, polarity cannot be explained simply on the basis of the detachment of ribosomes. Since ribosomes can reattach to the messenger, their failure to translate distal genes must reflect the unavailability of distal messenger. A decrease in messenger distal to nonsense codons has been detected in the trp (62) and lac (23) operons. This decrease presumably reflects either a decrease in transcription or increased degradation of messenger regions beyond the nonsense codon.

Further evidence indicating attachment of ribosomes at internal start signals comes from work with the single-stranded RNA bacteriophage f2. This RNA is known to specify the formation in vivo and in vitro of three proteins. Initiation of the translation of two f2 genes is independent and simultaneous, while that of the third gene is dependent on the translation of at least the first part of the coat gene on f2 RNA (79). The proportions of the three proteins formed are approximately 100:30:5 in the in vitro system with f2 RNA as a messenger. This suggests that there can be vast differences among the amounts of the various proteins translated from the same polygenic mRNA. When the secondary and tertiary structure of the f2 RNA is changed (e.g., by running the incorporation experiment at a higher temperature or by treating the f2 RNA with formaldehyde) then the absolute rate of the translation of the two minor products is increased and so is the relative rate of their synthesis (79).

In view of the above findings concerning the expression of polygenic mRNA

and since coordinate synthesis of enzymes 5 and 2 in E. coli, W, of enzymes 3 and 8 in strain K12, and of enzymes 5 and 2 above different basal levels in K12 was observed, the possible existence of a polygenic mRNA for the clustered arginine genes cannot be eliminated. It is thought that the cluster is read in a clockwise fashion since "polar" mutants exist in argC which greatly reduce the amounts of enzymes 2 and 8 formed (27, and strain 1417, Chapter III). However, if the cluster is transcribed as a polygenic mRNA, then according to presently accepted postulations, it should be possible to isolate "polar" mutations in argE. None of the few nonsense mutations so far examined in argE are polar (27). If a polygenic mRNA does exist for the cluster, its expression probably is, at least in part, regulated by internal start signals and/or the secondary and tertiary structure of the mRNA. Since the coordinate synthesis of two genes within the same cluster but not immediately adjacent has been observed, the possibility of an heretofore unnoticed type of regulation cannot be eliminated.

The discrepancies between physiological and genetic derepression should be considered in light of some observations in the trp system. Derepression of the trp operon leads to the synthesis of polycistronic trp mRNA (10, 61). Coordinate synthesis of the five enzymes has been demonstrated (25, 65, 66); however, this synthesis is semi-coordinate when the operon is repressed due to a low-efficiency promotor located between the D and C genes (95); this is also the case in Salmonella typhimurium (11, 12). When cultures are severely starved for tryptophan, synthesis of the trp operon enzymes is noncoordinate (120, 149).

When tryptophan is added to the culture medium to establish repression

of the trp operon, it has been observed that the rate of trp mRNA degradation increases (92). Mosteller and Yanofsky (97) have shown that rifamycin will rapidly block transcription-initiation while allowing the polymerase molecules which had already initiated to complete normal transcription of the trp operon. Using rifamycin to block further transcription-initiations, the degradation of trp mRNA was observed to proceed somewhat more slowly in the absence of tryptophan than when tryptophan was present (94). These authors demonstrated that the presence of tryptophan per se does not promote more rapid degradation, but rather starvation for an amino acid increases the lifetime of trp mRNA by about two-fold. This phenomenon explains why cells which have been starved for tryptophan (physiological derepression) produce approximately 2-3 times more of the trp enzyme than cells which are genetically derepressed (trpR$^-$ amber strain) (94).

In view of the fact that the rate of trp mRNA degradation is slowed under conditions of tryptophan starvation, it is not at all surprising that in E. coli, W, a greater extent of derepression was achieved by physiological derepression (arginine limitation) than by genetic derepression (argR$^-$) (Tables 15 and 16).

The expression of the non-linked argD gene (enzyme 4) was also studied in E. coli, W. Although enzyme 4 appears to be coordinately synthesized with enzyme 8 when studied by genetic derepression, when endogenous arginine is limited (physiologically) the coordinacy of synthesis of these two enzymes is not apparent. Additionally, whereas homoarginine does not have corepressor activity on the synthesis of enzyme 8, it does have corepressor activity on enzyme 4 synthesis in MMA and AF medium (Tables 17 and 18).

The most unexpected results of this study came as a result of the construction of a fine structure map of the clustered arginine genes. Two ornithine requiring strains were isolated whose auxotrophic mutation is not cotransducible with rna-1. Also, a class of mutations (argX) was discovered which map within the arginine cluster. The mutants were AcO^-, but had significant levels of all the "cluster" enzymes. These mutants cannot grow on MMA + glutamate or on AF medium. They do not grow on MMA at 25^0, 37^0, or 41^0C, eliminating the possibility of an in vivo temperature-sensitive mutation. Several possibilities exist as to the product of this gene. ArgX may code for (1) a part of enzyme 1; (2) a second arginyl-tRNA synthetase - recent reports indicate that synthetase mutations can confer auxotrophy for their cognate amino acid (37); (3) an arginyl-tRNA.

The construction of a fine structure map for the arginine cluster is, of course, incomplete since neither argB, argC, nor extensive cluster deletions were isolated. It would appear that something is preventing the occurrence (or detection) of deletion mutations in argB or argC as well as of multigenic deletions in the arginine cluster. Nitrous acid gave a significant increase as compared to NTG or 2-AP in the frequency of occurrence of deletion mutants among arginine auxotrophs (0.20 (59/295), 0.05 (4/74), 0.07 (2/30), respectively). The deletions occurred within the clustered arginine genes with a frequency of only 0.36 (21/59) compared to a frequency of occurrence of cluster auxotrophs among arginine auxotrophs for NTG of 0.57 (74/129) and 2-AP of 0.68 (30/44). Although generally low, the frequency of occurrence of deletion mutations for different loci in Salmonella can vary from zero to as high as 0.40 for cysC (31, 32). The reason

for this variation in frequency of occurrence of deletions is not known. The deletions induced by nitrous acid in the cluster had the following frequency: argE, 0.19 (4/21); argC, 0.00 (0/21); argB, 0.00 (0/21); argX, 0.24 (5/21); argH, 0.57 (12/21). In contrast, point mutations induced by NTG have the following frequencies: argE, 0.16 (12/74); argC-B-X, 0.51 (38/74); argH, 0.32 (24/74). Point mutations induced by 2-AP have the following frequencies: argE, 0.23 (7/30); argC-B-X, 0.23 (7/30); argH, 0.53 (16/30). When either of the three mutagens was used the occurrence of argE mutations was significantly less than argH mutations.

CHAPTER V

SUMMARY

The differential rates of synthesis of the arginine enzymes whose corresponding genes are clustered were determined in strains W and K12 of E. coli. In order to accomplish this, new assays for enzymes 2 and 3 had to be improved. Synthesis of enzymes 5 and 2 is coordinate in strain W and appears coordinate in strain K12 above different basal rates. Synthesis of enzymes 3 and 8 is coordinate in E. coli, K12, but not in E. coli, W. The rates of enzyme synthesis were varied (1) by genetic derepression achieved by mutation in argR and, (2) by physiological derepression effected by limitation of the endogenous arginine supply in suitable arginine auxotrophs.

Two relative enzyme-specific regulatory mutants of E. coli, W, were isolated as ornithine-independent, phenotypic revertants of acetylornithinase auxotrophs. These mutants show a concomitant alteration in repression-derepression behavior of enzyme 2 as well as that of enzyme 5, whereas synthesis of enzymes 3 and 8 is not altered. This behavior is consistent with the view that in E. coli, W, enzymes 5 and 2 share a common repressor recognition site.

The locus which is responsible for rifamycin-resistance (rna) was mapped

by reciprocal three-factor crosses. It was demonstrated to lie outside of the arginine cluster to the right of argH.

A partial deletion map of the arginine cluster was constructed by crossing 131 donors with 32 recipients. Among the 487 arginine auxotrophs isolated for the fine structure mapping, a new class (argX) of mutants was found. This class shows a specific growth response to acetylornithine, or any subsequent arginine intermediate or arginine but not to glutamate or any of the amino acids, vitamins, or nucleotid contained in arginine free assay medium. All of the enzymes specified by the clustered genes are present. It is shown that this is not an in vivo temperature-sensitive mutation. ArgX mutations map within the arginine cluster apparently between argB and argH.

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