ISOLATION AND CHARACTERIZATION OF ARGININE MUTANTS OF SPONTANEOUS, NITROUS ACID- AND 2-AMINOPURINE-INDUCED ORIGIN IN SALMONELLA GALLINARUM - PULLORUM

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ABSTRACT

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by Mary Judith Robinson

The purpose of this study was to isolate and characterize a series of arginine auxotrophs of Salmonella gallinarum-pullorum 53W suitable for further genetic analysis.

Two mutagens, nitrous acid and 2-aminopurine, were employed for the induction of mutation to arginine auxotrophy. The penicillin selection method and the replica plating technique were utilized for the isolation of arginine mutants.

One hundred and eighteen arginine auxotrophs and bradytrophs of spontaneous, nitrous acid-and 2-aminopurine-induced origin representing blocks at all steps of the arginine cycle tested were isolated and were characterized on the basis of precursor utilization. The two mutagens were found to differ in effectiveness of inducing particular blocks to arginine synthesis. Evidence is presented which suggests that 53W contains a partial genetic block for the ornithine to citrulline conversion.

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M.J.R.

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INTRODUCTION

Studies of arginine auxotrophs have been of considerable importance in the development of three areas of genetic research: intermediary metabolism, linearity of biochemically related genes, and cell regulatory mechanisms.

The biosynthetic pathway to arginine in Escherichia coli differs from that in fungi by the use of acetylated intermediates in ornithine synthesis. It may be of taxonomic and evolutionary significance to determine whether the use of acetylated precursors is specific to the biosynthesis of arginine in E. coli or whether this alteration is generally a characteristic of arginine biosynthesis in bacteria. One aim of the isolation and characterization of arginine auxotrophs of Salmonella gallinarum-pullorum was to provide mutants for the determination of the metabolic pathway to arginine in this organism.

Chromosome maps of bacteria demonstrate a linkage of genes controlling the synthesis of enzymes in the same biosynthetic pathway for many enzyme systems. The order of genes in a linkage map coincides with the order of individual steps in the sequence of reactions they control.

In the case of arginine synthesis a linkage of genes controlling metabolically related enzymes has been observed

only over small regions of the chromosome consisting of two or three gene sites.

The trend in microorganisms appears to be a non-linked or only partially linked arrangement of genes controlling enzymes of the arginine pathway. Acceptance of a trend in genetic analysis rests upon its confirmation in other strains of bacteria. Well characterized arginine auxotrophs of S. gallinarum-pullorum may serve to determine the relation of gene linkage to the order of reactions in the pathway to arginine biosynthesis.

Complete and partially blocked arginine mutants (bradytrophs) have been of primary importance in the study of parallel enzyme repression in E. coli. Arginine auxotrophs of S. gallinarum-pullorum may be of value in similar studies.

In the past attempts to isolate arginine dependent mutants of spontaneous or induced origin in S. gallinarum-pullorum have rarely succeeded.

The following study was undertaken to isolate and characterize a series of arginine auxotrophs of S. gallinarum-pullorum suitable for further genetic analysis, using as inducing agents two mutagens not previously applied to Salmonella spp. for this purpose.

HISTORICAL REVIEW

Occurrence of Arginine Auxotrophs

Arginine auxotrophs have been readily isolated in E. coli (Vogel, 1955; Maas, 1961; Gorini, 1961), Micrococcus glutamicus (Udaka and Kinoshita, 1958), Torulopsis utilis, and Neurospora crassa (Vogel, 1955). Only two arginine mutants of Salmonella spp. have been reported (Clarke,1962). Of these mutants one was spontaneous (Iseki and Kashiwagi, 1957; cited by Clarke,1962) and the other nitrogen mustard induced (Stokes and Bayne, 1958). Clarke (1962) has recently attempted to isolate arginine mutants of spontaneous, ultraviolet--and $MnCl_2$-induced origin in Salmonella typhimurium. One arginine auxotroph was isolated. The rarity of arginine mutants in Salmonellae suggests a genus-wide refractoriness to the occurrence of this particular class of mutants.

Arginine Biosynthesis in Microorganisms

Intermediary metabolism occupies a prominent place in current biochemistry and microbial genetics. It is the aim of intermediary metabolism to discover individual steps in the synthesis of metabolites and to determine their arrangement in biosynthetic pathways.

Two techniques are commonly employed for the study of metabolic pathways with mutants: intermediate accumulations and precursor utilization.

Use of these techniques had led to the observation that requirements of certain microorganisms for pyrimidines and proline as well as a requirement for arginine can be overcome by the addition of arginine to a growth medium (Davis, 1962). The pathway of arginine synthesis was presumed to be intermediate in the synthesis of proline and pryimidines.

The biosynthetic pathways to arginine, proline, and pyrimidines have been described in Neurospora. The pathways were found to include the sequence of reactions given in Table I (Vogel and Kopac, 1959;Srb and Horowitz, 1944; Srb et al., 1950; Davis, 1962). Glutamic semialdehyde is shown twice to emphasize the probability that the semialdehyde as proline precursor is distinct from the semialdehyde as ornithine precursor. The gultamic-proline-ornithine interrelation in Torulopsis utilis is identical to that of Neurosopora (Abelson and Vogel, 1955).

The interrelated biosynthetic pathways to proline, arginine, and pyrimidines in bacteria have been described for E. coli. The sequence of reactions given in Table II was determined (Maas, 1961). Of particular significance is the utilization of acetylated compounds in ornithine synthesis.

Vogel (1955) has postulated a possible taxonomic and evolutionary significance in the difference between the pathway to arginine in fungi and bacteria.

TABLE I.--The proline, arginine, and pyrimidine biosynthetic pathways in Neurospora.

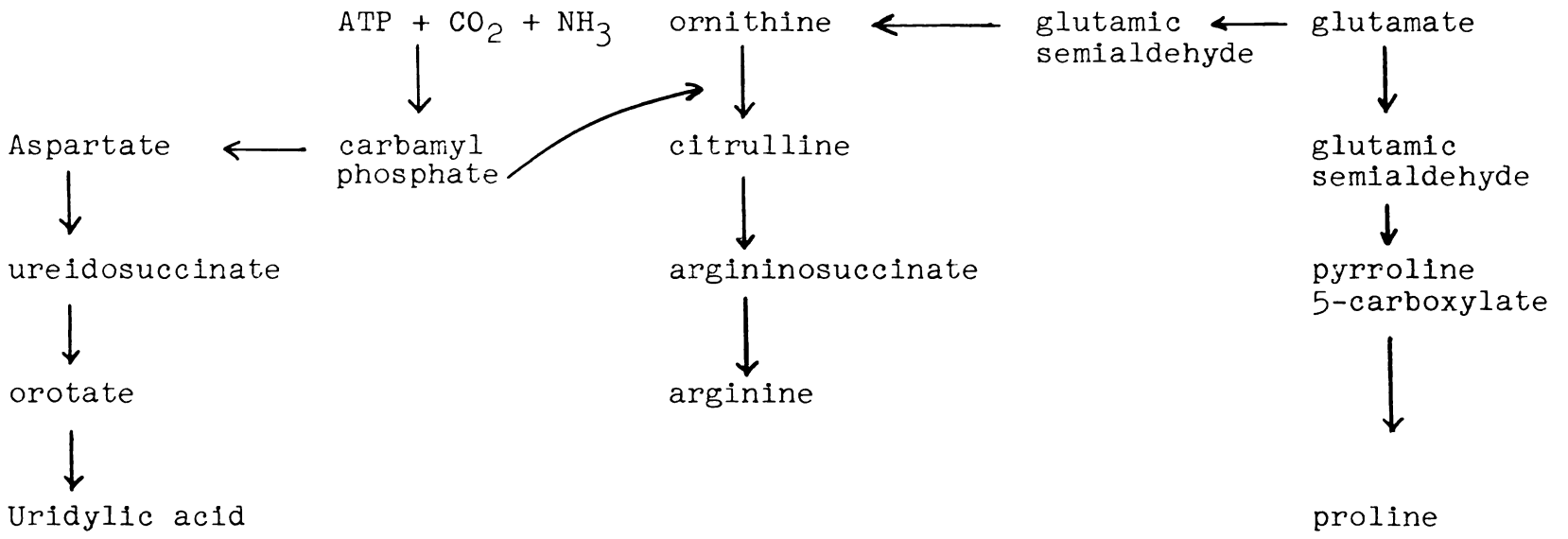
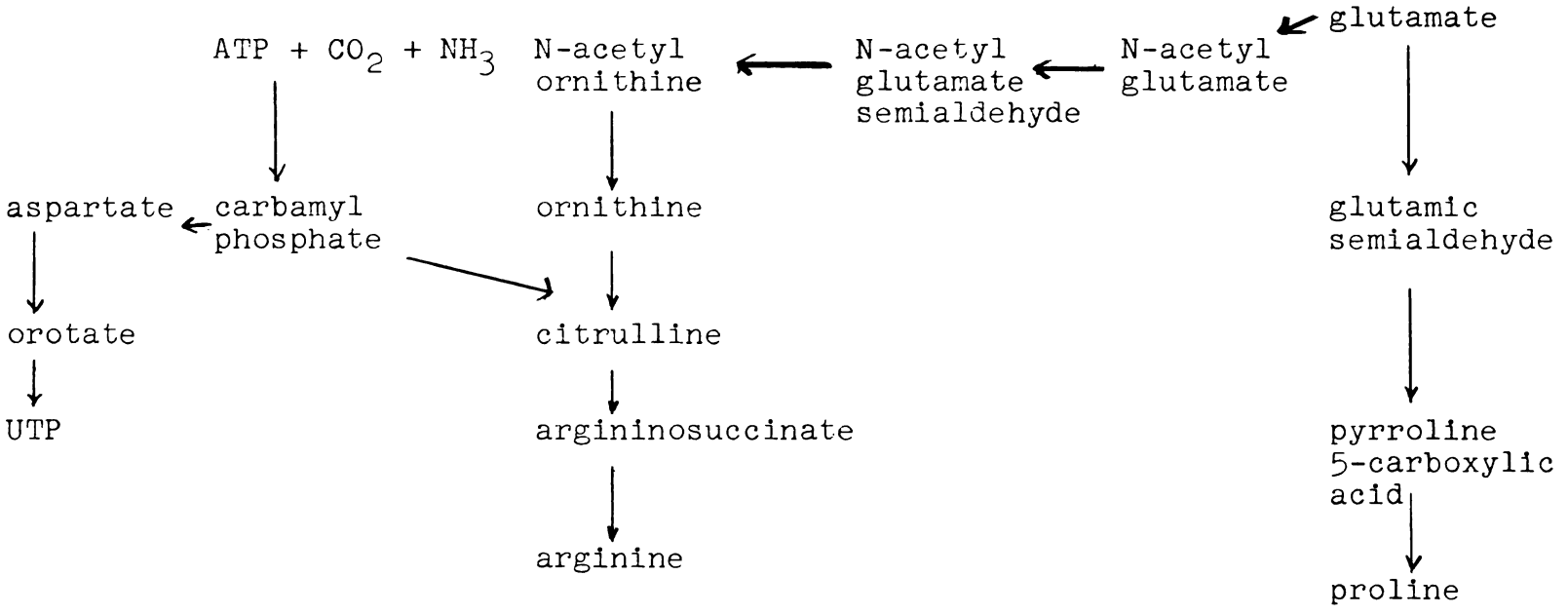


TABLE II.--The proline, arginine, and pyrimidine biosynthetic pathways in E. Coli.



Linearity of Biochemically Related Genes

Auxotrophic mutants have been used extensively for recombination studies with fungi and with bacteria. Such recombinational analysis leads to the representation of the chromosome as a one dimensional diagram each point of which represents a single mutation.

Demerec et al. (1956) have reported that in S. typhimurium genes controlling enzymes in the same biosynthetic pathway are frequently linked on the chromosome. Linkage is of such a nature that the order of genes reflects the order of individual steps in a synthetic pathway. A similar linearity has been found for many enzymes of other Salmonella species and of other bacteria (Demerec and Hartman, 1959).

In the fungi Neurospora and Aspergillus linkage of genes for metabolically related enzymes has been found only rarely (Gross and Fein, 1960; Wagner et al., 1960). In some instance a near random arrangement of genes has been observed for enzymes which are linked in Salmonellae and E. Coli (Pontocorvo, 1958).

In the case of arginine synthesis a linkage of genes controlling metabolically related enzymes has not been observed in Neurospora (Newmeyer, 1957) or in E. coli (Gorini et al., 1961; Maas, 1961). Gene linearity in arginine biosynthesis has not been determined in other bacteria.

S. gallinarum-pullorum has recently been shown to be suited for transduction studies (Snyder, 1961; Vaughn, 1962). Recombinational analysis of the arginine system in this

organism is feasible pending isolation and characterization of various classes of arginine mutants.

Although chromosome maps of genes controlling the formation of enzymes for arginine synthesis do not demonstrate an overall correspondence between linkage and the order of steps in the biosynthetic pathway to arginine, the arrangement of genes is by no means random.

Recombinational studies with E. coli K12 (Maas, 1961) and with E. coli B (Gorini et al., 1961) have made it clear that some of the genes controlling arginine biosynthesis occur in clusters of two or three genes correlated with two or three successive steps in the metabolic pathway. Such a partial linkage of biochemically related genes appears to be peculiar to the arginine system in bacteria.

Cell Regulatory Mechanisms

The enzymes of the arginine pathway have been shown to be repressible on addition of arginine to the culture medium in which E. coli is growing (Vogel, 1961). Vogel (1957) has defined enzyme repression as "a relative decrease, resulting from the exposure of cells to a given substance, in the rate of synthesis of a particular apoenzyme."

Maas (1961) has shown that in E. coli K12 repressibility is controlled by a gene R located some distance on the linkage map from most of the other genes associated with arginine biosynthesis. The action of this gene

represses most, if not all, the enzymes of the arginine pathway (parallel repression).

One model which has been suggested to explain the mechanism of parallel enzyme repression is the operator model (Jacob et al., 1960). Here repression occurs at the level of the gene. According to this model, structural genes governed by the same repressor are linked. An operator gene adjacent to these genes governs repression of their activity. The functional chromosome unit consisting of the operator and a group of continuous structural genes whose activity it controls is called the operon. Another gene, R, not necessarily next to the operator, controls the activity of the operator by production of a repressor substance.

Because of the scattering of arginine loci on the bacterial chromosome (Gorini et al., 1961; Maas, 1961) the operator model may be applied to arginine biosynthesis only if it is assumed that each structural gene or small cluster of genes is governed by a separate operator and that the repressor substance is effective against the operation of all of these (Maas, 1961). Maas (1961) suggests that definite evidence for the operator model in arginine biosynthesis depends upon the isolation of mutants in which a single operator is affected. He further suggests that such mutants in which the rate of snythesis instead of the structure of the enzyme is affected should be discovered among

mutants with a partial block for the ornithine to citrulline conversion isolated as slow-growing revertants from complete auxotrophs.

A second hypothesis to explain the mechanism of parallel enzyme repression, more suited to the arginine system, has been suggested by Gorini et al. (1961). This hypothesis postulates that each enzyme synthesizing unit possesses an identical site of action of the common repressor. According to this second hypothesis the enzymes controlling arginine synthesis should be composed either of two peptide chains, one of which is common to all arginine enzymes, or of a single chain with a region of structure identical to all enzymes responsible for arginine biosynthesis. This common region may be the site of the single repressor substance responsible for parallel repression of the discontinuously arranged genes which govern synthesis of the enzymes in the arginine pathway. If one assumes that arginine enzymes contain a common peptide chain then there should be a single gene which when mutated alters the repressibility of all enzymes responsible for arginine biosynthesis (Gorini et al., 1961). If, alternatively, a portion of the peptide chain is identical in each arginine enzyme, mutations of a structural gene for a given enzyme should alter the repressibility of that particular enzyme alone. Gorini et al. (1961) suggest that such mutuants should be found among back mutants to prototrophy of an arginine repressible strain.

Resolution of the apparent complexities of arginine biosynthesis and its regulation in bacteria is dependent upon further extensive genetic analysis of arginine mutants of various classes, both complete and partial and of back mutants to prototrophy.

Mutagens

Nitrous Acid.--Nitrous acid is one of a class of mutagens which alters resting DNA in such a way that mutations result during subsequent DNA replication (Freese, 1959).

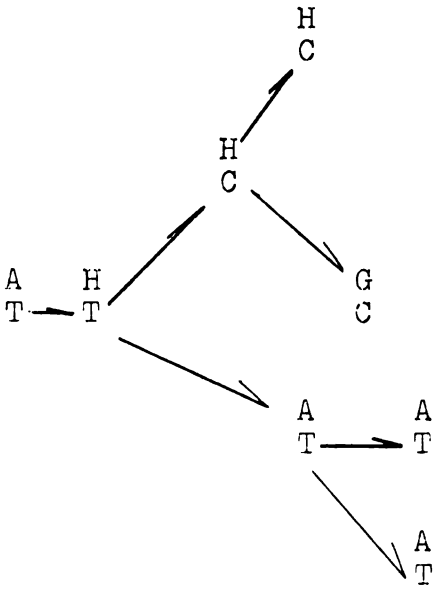
The mutagenesis of nitrous acid was first reported by Mundry and Gierer (1958) for tobacco mosaic virus. Nitrous acid has since been found to be applicable for induction of mutation in plant, animal, and bacterial viruses (Mundry and Gierer, 1958; Boeye, 1959; Tessman, 1959; Freese, 1959; Vielmetter and Wider, 1959), and in bacteria (Kaudewitz, 1959).

The chemical action of nitrous acid on the DNA molecule has been extensively studied by Schuster (1960). This action consists of the replacement of free amino groups of purine and pyrimidine bases by keto groups as a result of oxidative deamination and tautomeric shifts. Direct deamination of DNA bases by nitrous acid results in the formation of new bases with altered pairing properties. These altered pairing properties cause the change of a nucleotide pair so that in subsequent replication a purine is replaced

by a different purine and a pyrimidine is replaced by another pyrimidine.

Nitrous acid alters adenine (A) into hypoxanthine (H) which pairs like guanine (G); cytosine (C) into uracil (U) which pairs like thymine (T); Guanine into xanthine (X) which pairs like guanine (Schuster, 1960).

The following diagram represents a deamination and two subsequent replications which might lead to a mutation (Sanger and Ryan, 1961):



Studies on the reaction velocities of the bases in DNA have shown that adenine and cytosine react slower with nitrous acid than does guanine (Schuster, 1960). As the hydrogen ion concentration of the reaction mixture is increased more adenine and cytosine relative to guanine undergo reaction with nitrous acid. The increased activity of adenine and cytosine is presumably due to the lability

of hydrogen bonds between the amino groups of thymine and guanine at the low pH (Schuster, 1960). Breakage of these hydrogen bonds expcses the amino groups for reaction with nitrous acid.

Deamination of DNA bases by nitrous acid may result in mutation or in inactivatien. Vielmetter and Schuster (1961) have compared deamination rates of guanine, adenine, and cytosine in phage T_2 treated with nitrous acid at various pH values with corresponding rates of mutation and inactivation. These authors have concluded that the deamination of mainly adenine, cytosine, or both, but not guanine is responsible for the induction of mutations. Inactivation may be caused by deamination of adenine, cytosine, or guanine (Vielmetter and Schuster, 1961). Deaminations of cellular proteins may also contribute to inactivation. To increase the rate of mutagenesis with respect to the rate of inactivation cells are treated with nitrous acid at a low pH.

Low pH.--Low pH has itself been shown to be mutagenic for T_4 phages (Freese, 1959). A condition of high hydrogen ion concentration is known to readily remove purines from the DNA molecule (Loring, 1955). Replacement of the removed purine by a different purine or by a pyrimidine in resting DNA or during DNA replication results in the production of point mutations.

2-Aminopurine.--2-Aminopurine is one of a second class of mutagens comprised of close analogues of normal nucleic acid bases. The mutagenic effect of base analogues is probably due to mistakes in complementary base pairing which accompany their incorporation into the DNA molecule (Freese, 1959).

2-aminopurine closely resembles adenine in structure and presumably may be incorporated in place of adenine into the DNA molecule. Because of its structural similarity to adenine, the incorporated analogue would be expected to pair with thymine during a subsequent replication. The original condition rather than mutation would thus be restored. Mutation occurs when a base other than thymine is incorporated opposite 2-aminopurine into the DNA molecule. In each pairing mistake with 2-aminopurine one purine is replaced by another purine.

Both nitrous acid and 2-aminopurine have been reported to be highly mutagenic for S. typhimurium (Kaudewitz, 1959; Hartman et al., 1962).

MATERIALS AND METHODS

Culture Methods

Strain 53 (KCD 35-22-51) of S. gallinarum-pullorum was used throughout this study. The symbol 53W is used to designate wild-type strain 53.

The nutritional requirements of 53W and arginine auxotrophs subsequently isolated were determined using the following chemically defined basal medium and basal medium supplemented with l-arginine, l-ornithine, l-citrulline, l-proline, and argininosuccinic acid, barium salt:

KH_2PO_4	0.2 g	
K_2HPO_4	0.4 g	
NH_4NO_3	1.0 g	
Na_2SO_4	2.0 g	
$MgSO_4 7H_2O$	0.1 g	
$CaCl_2$	Trace	
$KHCO_3$	2.0 g	
Glucose	10.0 g	
Sodium Citrate	0.5 g	
Asparagine	1.5 g	Dissolved in 35 ml 1N KOH
Xanthine	5 mg	
l-Cystine	60 mg	Dissolved in 15 ml 1N HCl
l-Leucine	87 mg	

l-Histidine	39 mg
DL-Valine	78 mg
DL-Serine	59 mg
Decxycytidine HCl	1 mg
Nicotinic acid	5 mg
Thiamine HCl	1 mg
Calcium pantothenate	2 mg
Distilled water	950 ml

(Schoenhard, 1951; Brock, 1958).

Supplements were added in the amount of 43 mg/ml unless otherwise stated. The argininosuccinic acid, barium salt, was dissolved in 1N HCl prior to addition to the basal medium. The basal medium and supplements were sterilized by filtration through Millipore filters.

Wild-type 53 and all mutants were maintained on Brain Heart Infusion (BHI) agar slants.

Induction of Mutation and Inactivation with Nitrous Acid

A 15 hour culture of 53W was sedimented by centrifugation and resuspended in 5 ml 0.5M acetate buffer pH 4.5. One-tenth ml of the suspension was removed to 10 ml 0.038M phosphate buffer pH 7.0 for determination of an original cell count. Immediately one ml of 2M $NaNO_2$ was added to the suspension of 53W in acetate buffer. Samples were withdrawn and plated as shown in Table III. Nitrous acid was inactivated by diluting 1:100 in 0.038 M phosphate

TABLE III.--Plating of cells treated with nitrous acid.

Sample time (sec.)	Sample size	Ml buffer	pH buffer	Decimal dilutions	Amount plated per dilution	Number of plates per dilution
0	0.1 ml	9.9 ml	7.0	10^{0}-10^{-6}	0.1 ml	3
10	0.1	9.9	7.0	10^{0}-10^{-6}	0.1	3
15	0.1	9.9	7.0	10^{0}-10^{-6}	0.1	3
30	0.1	9.9	7.0	10^{0}-10^{-6}	0.1	3
45	0.1	9.9	7.0	10^{0}-10^{-6}	0.1	3
60	0.1	9.9	7.0	10^{0}-10^{-6}	0.1	3
90	0.1	9.9	7.0	10^{0}-10^{-6}	0.1	3
120	0.5	4.5	7.4	10^{0}-10^{-2}	1.0	5
150	0.5	4.5	7.4	10^{0}-10^{-2}	1.0	5
180	0.5	4.5	7.4	10^{0}-10^{-1}	1.0	5
210	0.5	4.5	7.4	10^{0}-10^{-1}	1.0	5
240	0.5	4.5	7.4	10^{0}	1.0	5

buffer pH 7.0 or 1:5 in 0.038M. Aliquots were plated on BHI agar. Plates containing ten to one hundred colonies were replicated for isolation of arginine auxotrophs.

The inactivation rate of a log phase culture of 53W by 0.025M nitrous acid was determined from a graph representing the fraction of survivors plotted against time of contact with nitrous acid in seconds.

Induction of Mutations and Inactivation with Acetic Acid Buffer pH 4.5

To insure that any inactivation and mutagenic effect observed with HNO_2 was due to the nitrous acid alone and not the result of the acetate buffer, the following test was performed.

A 15 hour culture of 53W in BHI broth was sedimented by centrifugation and resuspended in 5 ml 0.5M acetic acid adjusted to pH 4.5 with IN NaOH. Samples were withdrawn and plated as shown in Table IV. Aliquots were plated on BHI agar. All plates containing ten to one hundred colonies were replicated to determine the number of arginine mutants present. The inactivation rate of a log phase culture of 53W by acetic acid buffer pH 4.5 was determined from a plot representing the fraction of survivors versus time of contact with the acid in hours.

Preparation of a Stock Culture Free of Arginine Dependent Mutants

An overnight culture of 53W in BHI broth was sedimented by centrifugation, washed twice, and resuspended in an equal

TABLE IV.--Plating of cells treated with acetic acid.

Sample time (hr.)	Sample size	Ml buffer	Decimal dilutions	Amount plated per dilution	Number of plates per dilution
0	0.1 ml	9.9 ml	10^{-2}-10^{-7}	0.1 ml	3
0.5	0.1	9.9	10^{-2}-10^{-7}	0.1	3
2	0.1	9.9	10^{-2}-10^{-7}	0.1	3
3	0.1	9.9	10^{-2}-10^{-7}	0.1	3
5	0.1	9.9	10^{-2}-10^{-7}	0.1	3
6	0.1	9.9	10^{-2}-10^{-7}	0.1	3
8	0.1	9.9	10^{-2}-10^{-7}	0.1	3

volume of saline. Approximately 10^2 twice-washed cells were transferred to 10 ml basal medium. The culture was incubated with aeration at 37C until maximum turbidity developed. The cells were immediately centrifuged away from the synthetic medium, washed twice, and resuspended in an equal volume of saline. Since arginine dependent mutants are incapable of growth in basal medium not supplemented with arginine, a stock culture free of arginine dependent mutants was obtained.

Isolation of Spontaneous Arginine Mutants

Approximately 10^1 cells of a stock suspension of 53W free of arginine auxotrophs were inoculated into a tube of BHI broth. The culture was incubated with aeration until maximum turbidity developed. The culture was washed twice to remove extracellular metabolites and the final cell count was determined. This suspension was used to inoculate tubes of basal medium plus penicillin according to the penicillin screening techniques of Lederberg and Zinder (1948). Arginine mutants were isolated by the replica-plating technique (Lederberg and Lederberg, 1952).

Induction of Mutation with 2-Aminopurine

Approximately 10^2 cells of a stock culture of 53W free of arginine dependent mutants were inoculated into three tubes containing BHI broth plus 200 mg per ml of 2-amino-purine (Hartman, et al., 1962). The tubes were incubated overnight at 37 C with aeration. Five ml samples from each culture

were washed twice and the cell count was determined. One-tenth ml aliquots of 10^{-3}, 10^{-4}, 10^{-5}, dilutions were inoculated into basal medium plus penicillin according to the penicillin selection technique. Arginine auxotrophs were detected by replica plating.

Isolation of Arginine Auxotrophs

Pencillin screening (selection) technique.--One-tenth ml portions of 10^{-3}, 10^{-4}, 10^{-5} dilutions of twice-washed treated or untreated cultures were transferred to three tubes containing 3 ml basal medium plus 250 units of penicillin per ml. The tubes were incubated at 37 C without aeration for about 24 hours. Penicillin inactivates growing cells by preventing cell wall synthesis. Wild-type cells are able to grow in unsupplemented basal medium and are destroyed by the penicillin (Davis, 1949; Lederberg et al., 1945). Arginine dependent mutants which arise as a result of mutagenic action or which arise spontaneously in a culture are incapable of growth in unsupplemented basal medium and are spared from the action of penicillin. After 24 hours incubation in the presence of penicillin ten 0.1 ml aliquots were removed from each tube and spread on BHI agar plates. Plates were incubated 48 hours. Plates containing ten to one hundred colonies were replicated.

Replica plating technique.--Colonies on a BHI agar plate were imprinted onto a piece of sterile velveteen spread

over the surface of a wooden cylinder by pressing the BHI plate gently against the velveteen. Cells from each colony retained on the fine fibers of the fabric were transferred to a plate containing basal medium and to a plate containing basal medium supplemented with arginine by pressing these plates against the imprinted velveteen. Plates were compared for growth after 24 and 48 hours incubation at 37 C. Colonies which grew on basal medium with arginine but did not grow on unsupplemented basal medium were classified as arginine auxotrophs. All such colonies were transferred from the original BHI plate to BHI agar slants for maintenance prior to characterization.

Characterization of Arginine Auxotrophs by Precursor Utilization

Each arginine auxotroph and 53W was grown overnight in BHI broth. Cultures were washed twice and resuspended in the original volume of saline. One-tenth ml of a 10^{-2} dilution of each saline suspension was inoculated into a series of 16 x 150 mm test tubes containing 2 ml of media as follows: unsupplemented basal medium, basal medium supplemented with proline, with ornithine, with citrulline, with argininosuccinic acid, or with arginine. Tubes were incubated at 37C. The amount of growth in each tube was recorded approximately every twelve hours according tc turbidity estimates (+1, +2, +3, +4) for six to seven days.

RESULTS

Inactivation of S. gallinarum-pullorum by Nitrous Acid

Nitrous acid inactivation of bacterial cells was measured as a decrease in the ability of a suspension to form colonies. The inactivation of S. gallinarum-pullorum strain 53 cells in the log phase by 0.025M HNO_2 followed a two-hit curve (Fig. 1). Starting with about 10^8 cells per ml the rate of inactivation was determined over a five log decrease in viable cell count. The inactivation rate was determined to be .126/sec.

A tailing effect was observed after a six log decrease in viable cell count of S. gallinarum-pullorum in 0.025M HNO_2 (Fig. 2, page 25). This tailing effect may represent a selection for cells in the culture which are resistant to the action of nitrous acid.

Induction of Mutations to Arginine Auxotrophy by Nitrous Acid

The spectrum of arginine auxotrophs induced with nitrous acid is given in Table V on page 26.

Nutritional requirements of the arginine auxotrophs isolated were tested in basal medium unsupplemented and in basal media supplemented with proline, with ornithine, with citrulline, with argininosuccinic acid, or with arginine.

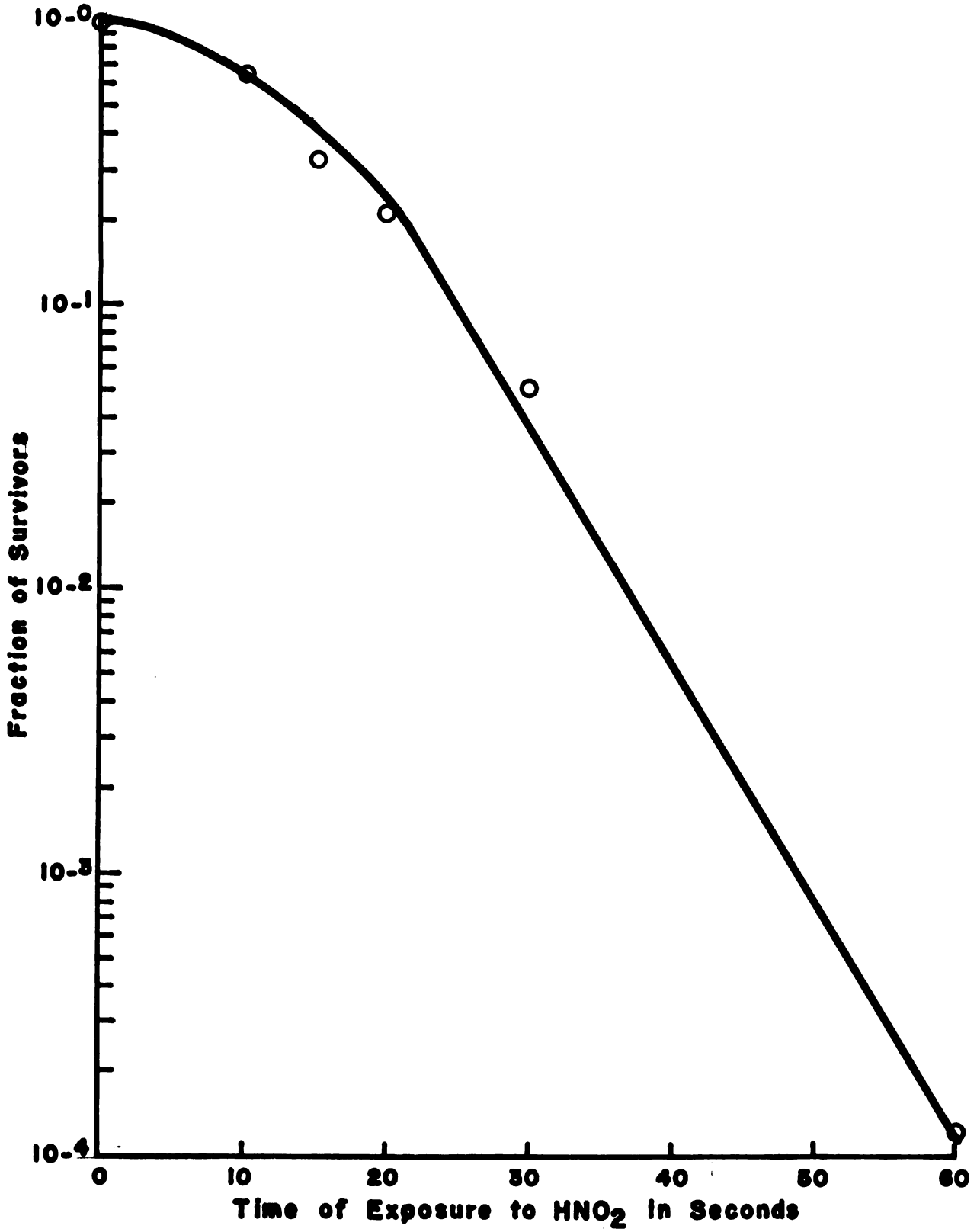


Fig. 1. Inactivation of log phase cells of S. gallinarum-pullorum by 0.025M HNO_2 (pH 4.5) as a function of time.

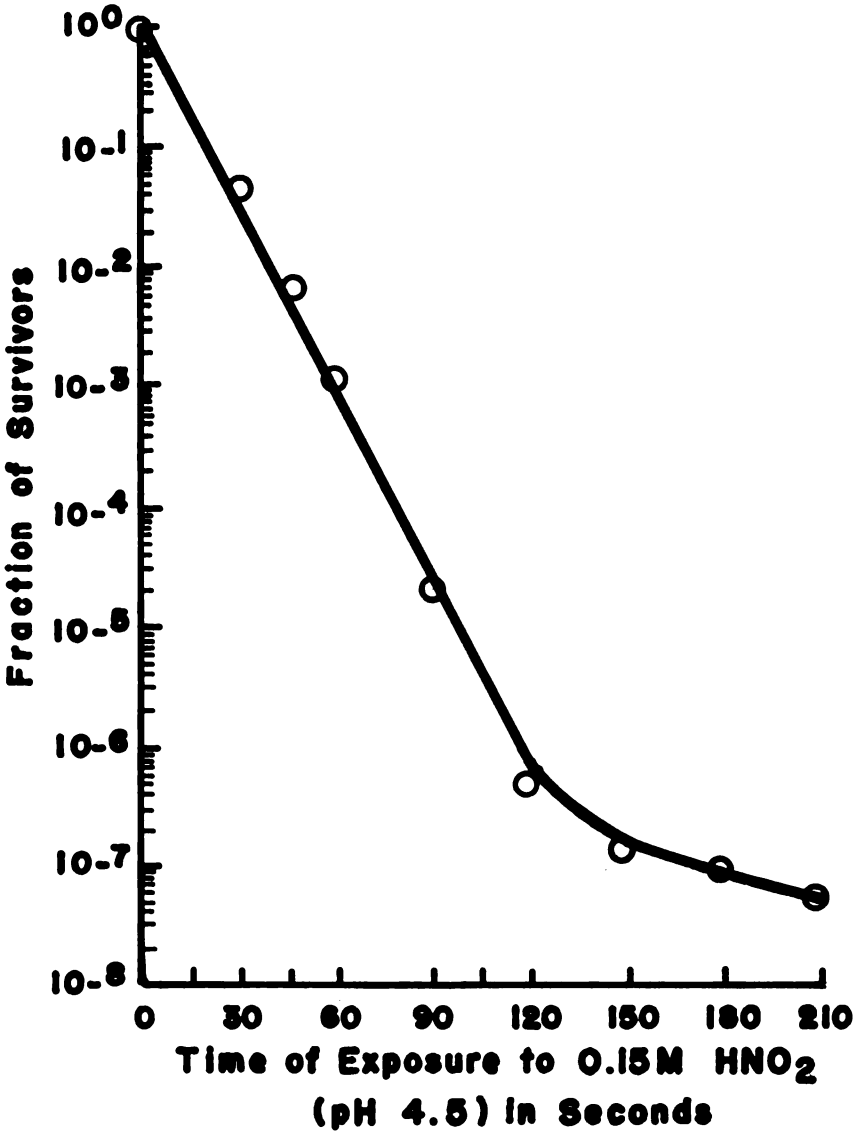


Fig. 2. Tailing effect of nitrous acid inactivation of S. gallinarum-pullorum.

TABLE V.--Distribution of nutritional requirements of arginine auxotrophs of S. gallinarum-pullorum strain 53 induced with nitrous acid.

Nutritional requirements	Metabolic block	Number of arginine auxotrophs isolated	Number of bradytrophs isolated
Ornithine	glutamate → ornithine	4	4
Citrulline	ornithine → citrulline	24	7
Argininosuccinic Acid	citrulline → argininosuccinate	2	1
Arginine	argininosuccinate → arginine	1	0
Proline	glutamate → proline	3	0

Growth was recorded every twelve hours for six to seven days. Wild type strain 53 gave a +4 reaction in all testing media after 36 hours. Mutants which demonstrated a growth differential of 48 hours or more among the testing media after an initial incubation period of 36 hours were classified as arginine auxotrophs. The genetic block of a mutant was determined according to the supplement earliest in the arginine cycle which would support its growth. Examples of growth patterns of three representative nitrous acid-induced arginine auxotrophs and of 53W are presented in Table VI.

Proline was included in tests for the nutritional requirements of arginine auxotrophs to separate proline mutants whose metabolic block can be overcome in the presence of arginine from mutants deficient in arginine biosynthesis. The primary requirement of mutants able to grow on proline appears to be proline rather than arginine (Vogel and Kopac, 1959) while those unable to grow on proline exhibit genetic blocks in the arginine cycle.

The exact nutritional requirements of mutants with metabolic blocks priorto ornithine were not determined since N-acetylated intermediates to ornithine synthesis are not commercially available.

Mutants which demonstrated a 36 to 48 hour growth differential among the various media but which did not show growth in unsupplemented basal medium until the fifth or sixth day of incubation and mutants which consistantly

TABLE VI.--Patterns of nutritional requirements of 53W and three representative nitrous acid-induced arginine auxotrophs of S. gallinarum-pullorum.

	Supplement						
	Arginine	Arginino-succinate	Citrulline	Ornithine	Proline	Unsupplemented	Hours of incubation
W53	+3	-	+3	-	-	-	12
	+4	+4	+4	+4	-	-	24
	+4	+4	+4	+4	+4	+4	36
			Genetic Block:	o → c			
No_{2-5}	+4	-	-	-	-	-	36
	+4	-	+4	-	-	-	48
	+4	-	+4	-	-	-	60
	+4	+3	+4	-	-	-	72
	+4	+4	+4	-	-	-	84
	+4	+4	+4	-	-	-	96
	+4	+4	+4	-	-	-	108
	+4	+4	+4	-	-	-	117
			Genetic Block:	glutamate → o			
N_{2-26}	+4	-	+1	+1	-	-	36
	+4	-	+4	+2	-	-	48
	+4	+3	+4	+4	-	-	60
	+4	+4	+4	+4	-	-	72
	+4	+4	+4	+4	-	-	84
	+4	+4	+4	+4	-	-	96

TABLE VI.--Continued

	Supplement						
	Arginine	Arginino-succinate	Citrulline	Ornithine	Proline	Unsupplemented	Hours of incubation
	Genetic block: proline mutant						
S-2-16	+4	-	+4	-	±	-	36
	+4	-	+4	+4	+1	-	48
	+4	+4	+4	+4	+4	-	72
	+4	+4	+4	+4	+4	-	84
	+4	+4	+4	+4	+4	-	96
	+4	+4	+4	+4	+4	-	108

showed reduced growth in media supplemented with metabolites prior to the proposed genetic block were classified as partial mutants, bradytrophs. The growth patterns of two partial mutants are presented in Table VII.

Mutation Rate to Arginine Auxotrophy with Nitrous Acid

Nitrous acid is an effective mutagen for S. gallinarum-pullorum 53W. With an inactivation of about 10^{-6}, 2.2 to 3.0 per cent of the surviving cells were arginine auxotrophs. With inactivations greater than 10^{-6} the percentage of survivors which are arginine auxotrophs decreases considerably. This decrease in the number of recoverable mutants may represent a selection for cells in the culture resistant to the action of nitrous acid, as did the tailing effect (Fig. 2). Figure 3, page 32, represents the relationship between the percentage of induced arginine mutants and exposure of log phase cells to nitrous acid.

Inactivation of S. gallinarum-pullorum by Acetic Acid Buffer pH 4.5

The inactivation of 53W cells in the log phase by 0.5M acetic acid buffer pH 4.5 is demonstrated by Figure 4. The rates of inactivation of 53W in nitrous acid and in acetic acid buffer are compared in Figure 5, page 34.

The inactivation rate of 53W in acetic acid buffer was determined over six logarithmic cycles on the ordinate: K = 1.74/hr. or 0.00048/sec.

TABLE VII.--Patterns of nutritional requirements of two bradytrophs isolated from *S. gallinarum-pullorum*.

	Supplements					Unsupplemented	Hours of incubation
	Arginine	Arginino-succinate	Citrulline	Ornithine	Proline		
			Genetic block: C → as				
AP_1	+4	-	-	-	-	-	36
	+4	-	-	-	-	-	48
	+4	-	-	-	-	-	60
	+4	+4	+1	-	-	-	72
	+4	+4	+4	-	-	-	84
	+4	+4	+4	-	-	-	96
	+4	+4	+4	+4	-	-	108
	+4	+4	+4	+4	+4	-	120
			Genetic block: o → c				
N_{24}	+4	-	-	-	-	-	36
	+4	-	+2	-	-	-	48
	+4	-	+2	-	-	-	60
	+4	-	+4	+2	-	-	72
	+4	+2	+4	+2	-	-	84
	+3	+2	+4	+3	-	-	96
	+4	+4	+4	+3	+2	+2	108
	+4	+4	+4	+3	+2	+2	120
	+4	+4	+4	+3	+2	+2	144

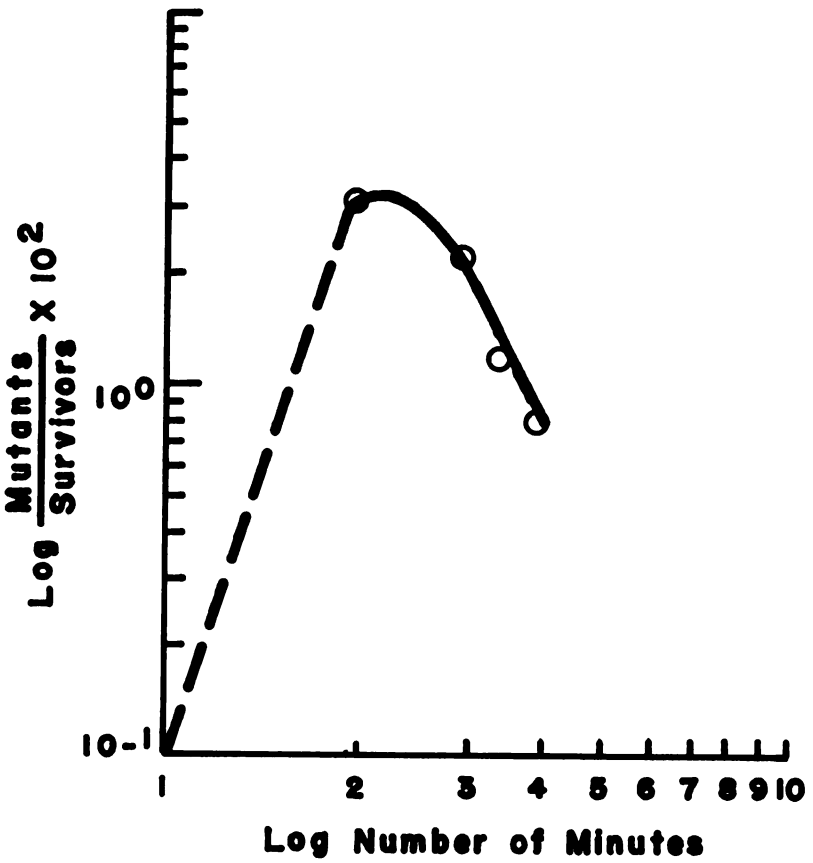


Fig. 3. Relationship between the percentage of induced arginine mutants and the exposure of log phase cells to 0.025M HNO_2.

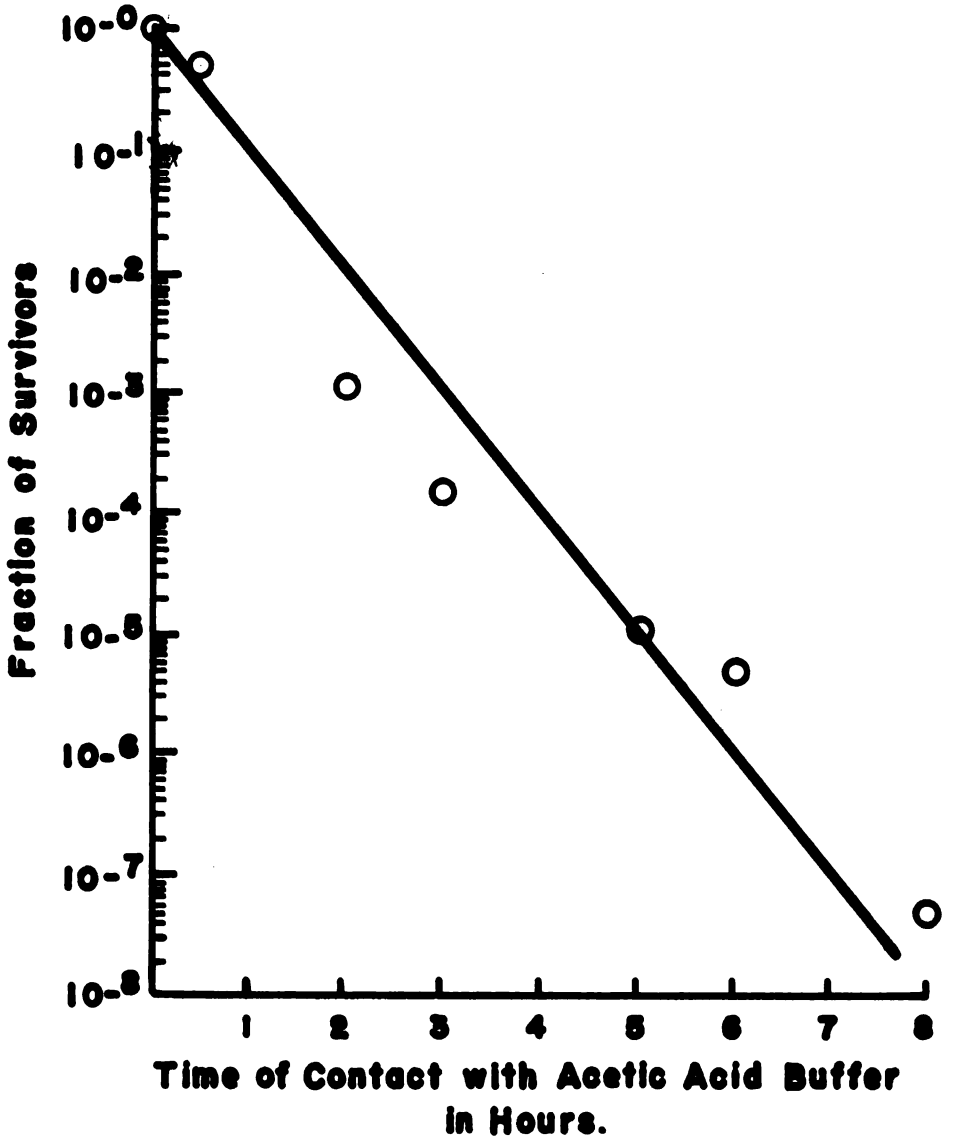


Fig. 4. Inactivation of log phase cells of S. gallinarum-pullorum by acetic acid buffer as a function of time.

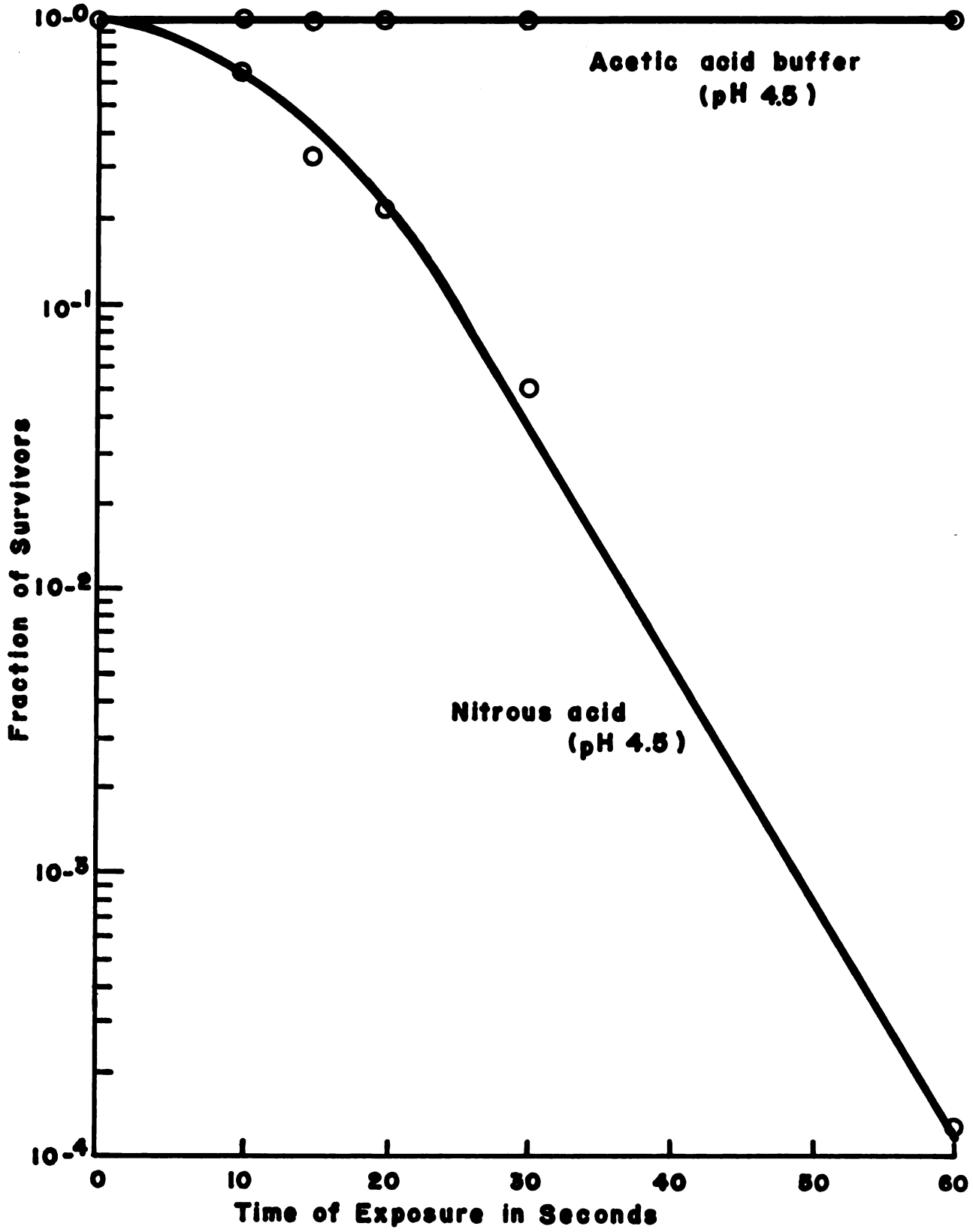


Fig. 5. Comparison of the inactivation rates of S. gallinarum-pullorum in nitrous acid and in acetic acid buffer.

Induction of Mutation with Acetic Acid Buffer pH 4.5

In three separate inactivation trials all plates containing ten to one hundred colonies were replicated for detection of arginine auxotrophy. No arginine mutants were isolated.

Spontaneous Arginine Auxotrophy of S. gallinarum-pullorum 53W and Spontaneous Mutation Rate to Arginine Auxotrophy

The spectrum of spontaneous arginine auxotrophs isolated from 53W is given in Table VIII, page 36.

The spontaneous mutation rate to arginine auxotrophs in S. gallinarum-pullorum was determined according to the formula $\mu = \ln 2 \frac{M}{N}$ where μ is the mutation rate, M is the number of arginine auxotrophs per ml, and N is the number of cells produced from a small inoculum, and was found to be 4.8×10^{-4} mutations/cell.

Induction of Mutation with 2-Aminopurine and Mutation Rate to Arginine Auxotrophs with 2-Aminopurine

The distribution of 2-aminopurine induced arginine mutants isolated from S. gallinarum-pullorum is given in Table IX.

The mutation rate to arginine auxotrophy in 53W with 2-aminopurine was determined by the same technique as was the spontaneous rate and was found to be 2.6×10^{-3}.

TABLE VIII.--Distribution of nutritional requirements of spontaneous arginine auxotrophs of S. gallinarum-pullorum 53W.

Nutritional requirements	Metabolic block	Number of arginine auxotrophs isolated	Number of bradytrophs isolated
Ornithine	glutamate ⟶ ornithine	12	1
Citrulline	ornithine ⟶ citrulline	13	4
Argininosuccinic Acid	citrulline ⟶ argininosuccinate	1	3
Arginine	argininosuccinate ⟶ arginine	0	0
Proline	glutamate ⟶ proline	3	0

TABLE IX.--Distribution of nutritional requirements of 2-aminopurine-induced arginine auxotrophs of S. gallinarum-pullorum 53W.

Nutritional requirements	Metabolic block	Number of arginine auxotrophs isolated	Number of bradytrophs isolated
Ornithine	glutamate → ornithine	21	1
Citrulline	ornithine → citrulline	13	5
Argininosuccinic Acid	citruline → arginiosuccinate	0	1
Arginine	argininosuccinate → arginine	0	0
Proline	glutamate → proline	1	0

DISCUSSION

Attempts by investigators to isolate spontaneous or induced arginine auxotrophs from a variety of Salmonellae have in the past met with little success. In this study one hundred and eighteen arginine dependent mutants of spontaneous, nitrous acid-and 2-aminopurine-induced origin were isolated from S. gallinarum-pullorum 53W. Mutants blocked at all steps in the arginine pathway tested for were found. The techniques employed in this study were similar to those used by previous investigators (Clarke, 1962; Kaudewitz, 1959; Plough et al., 1950) with one exception. Two mutagens, nitrous acid and 2-aminopurine, not previously applied to S. gallinarum-pullorum for induction of mutation to arginine auxotrophs were employed. Both were found to be mutagenic for arginine loci in S. gallinarum-pullorum.

Certain differences in the efficiency of mutagenesis of 2-aminopurine and nitrous acid were observed. Nitrous acid appears to be most effective for the production of a genetic block for the ornithine to citrulline conversion Sixty-seven per cent of the nitrous acid-induced arginine auxotrophs demonstrated a nutritional requirement for citrulline. Eighteen per cent were found to contain a genetic block prior to ornithine.

2-Aminopurine appears to be most effective for the production of genetic blocks prior to ornithine in 53W. Fifty-two per cent of the 2-aminopurine-induced auxotrophs isolated demonstrated a metabolic deficiency in ornithine synthesis. Forty-three per cent were blocked at the ornithine to citrulline conversion. From Tables IV and V it may be seen that the spectrum of arginine auxotrophs isolated following induction with 2-aminopurine most closely resembles the distribution of arginine dependent mutants of spontaneous origin. The difference observed in the efficiency of the two mutagens for induction of specific gene mutations may reflect some difference in the isolation procedures employed or a difference in the mode of action of the mutagens at the molecular level.

The number of bradytrophs isolated from 2-aminopurine-induced cells is nearly identical to the number isolated as spontaneous mutants from the original stock culture of 53W. Induction of mutation with nitrous acid resulted in the production of a ten per cent increase in the number of bradytrophs over that found among spontaneous or 2-aminopurine-induced arginine auxotrophs.

A high number of arginine mutants with growth patterns characteristic of bradytrophs may result from the induction of gene unstabilization with nitrous acid. Gene unstabilization refers to a condition in which a gene mutates to an altered gene with a higher spontaneous mutation rate

(Zamenhof, 1961). One type of gene unstabilization is reflected in the mutation of a stable enzyme producer (prototroph) to an unstable enzyme producer which mutates to an enzyme non-producer with a high spontaneous mutation rate. Gene unstabilization may be induced in the genetic region controlling production of the enzyme responsible for the ornithine to citrulline conversion (ornithine transcarbamylase). The resultant unstable mutant would be capable of synthesizing ornithine transcarbamylase (OTC) at a rate limited by the rate of spontaneous mutation of the unstabilized gene to an enzyme non-producer. At any given time only a portion of the cells of such a mutant would be capable of snythesizing OTC. A delayed utilization of ornithine in an ornithine supplemented medium would result. The high spontaneous mutation rate of an unstable gene to an enzyme non-producer would result in a decrease in the number of OTC producing cells with an increase in incubation time.

Since growth of 53W in unsupplemented medium lags considerably behind the level of growth in ornithine supplemented medium, it is possible that the constantly decreasing number of OTC producing cells may not reach a concentration high enough to produce visible turbidity for 48 to 72 hours following the appearance of turbidity in ornithine supplemented medium. The growth pattern of mutants resulting from gene unstabilization would not differ significantly from the expected growth pattern of a bradytroph in the same testing media.

The isolation of a large number of stable auxotrophs from unstable nitrous acid-induced mutants may serve to distinguish auxotrophs arising as a result of nitrous acid-induced gene unstabilization from bradytrophs.

Few auxotrophs exhibiting a genetic block after citrulline in the arginine pathway were isolated. The rarity of these mutants suggests a low mutation rate, spontaneous or induced, of genes controlling enzymes for later conversions in the arginine cycle. Two recently described modifications of the penicillin selection technique may be useful in the future for the isolation of rare arginine auxotrophs. Gorini and Kaufman (1960) have isolated arginine auxotrophs of E. coli blocked after citrulline by inoculating starved, treated cells into a penicillin basal medium containing 2,000 units of antibiotic per ml for time intervals as short as 90 minutes. The main advantage of this method lies in keeping the exposure to penicillin so brief as to eliminate cross-feeding by prototrophic cells that lyse during penicillin treatment. A decrease in the degree of cross-feeding allows screening of population densities as high as 5×10^8 cells per ml, greatly increasing the possibility of recovering mutants which arise at low frequencies.

Lubin (1962) has been able to isolate rare mutants deficient in potassium transport in E. coli following enrichment of the auxotrophic population by recycling.

Recycling refers to a process of alternating the growth of cells between an enriched medium and a basal medium supplemented with penicillin. By use of this technique progressively more mutants are selected for, resulting in an increase in the population of rare auxotrophs to a detectable concentration.

The modified penicillin selection techniques may be readily incorporated into the procedures described for the isolation of arginine auxotrophs of spontaneous and 2-aminopurine-induced origin. Incorporation of these techniques into the nitrous acid procedure may be accomplished following a modification of the described isolation method. Nitrous acid must be inactivated in a highly buffered basal medium supplemented with arginine. Incubation of HNO_2 treated cells in this medium allows the mutants induced to become phenotypically expressed. The cells may then be washed and starved and treated as described in the modified penicillin techniques, of Lubin (1962) and of Gorini and Kaufman (1960).

Each mutagen employed in this study presents both advantages and disadvantages to its use for the production of arginine auxotrophs in S. gallinarum-pullorum 53W. Nitrous acid treatment has the advantages over 2-aminopurine induction of rapidity of action and of a considerably higher induced mutation rate to arginine auxotrophy. Approximately one-third of the mutants isolated following nitrous acid induction, however, are bradytrophs. Induction of mutation

with 2-aminopurine although a more lengthy procedure, results in the isolation of 10 per cent fewer partially blocked auxotrophs.

Indications that nitrous acid primarily induced a genetic block in the synthesis of OTC while 2-aminopurine induces mostly metabolic blocks prior to ornithine may dictate that the mutagen selected for use be determined by the position of the genetic block being sought.

Preliminary attempts to characterize arginine auxotrophs by intermediate accumulations have confirmed the genetic blocks predicted from precursor utilization studies for two mutants tested. Two mutants deficient in ornithine synthesis were shown by the technique of two dimensional chromatography performed on supernatants of the cultures grown in a limiting supply of arginine to accumulate glutamic acid or glutamic acid derivatives.

The relatively high spontaneous mutation rate to arginine auxotrophy in S. gallinarum-pullorum (4.8×10^{-4}) is surprising on the basis of previous reports concerning the rarity of arginine mutants in Salmonellae.

Stokes and Bayne (1958) examined six strains of Salmonella pullorum and twelve strains of Salmonella gallinarum for arginine dependence. All strains were found to be prototrophic with respect to arginine. Growth of all strains was, however, stimulated by the presence of arginine in the test medium. A similar stimulation of growth by

arginine has been observed in S. gallinarum-pullorum 53W. In testing the nutritional requirement of 53W a delay of 12 to 24 hours in the appearance of turbidity in ornithine supplemented medium and in unsupplemented basal medium was consistently observed (see Table 2). Even in tests using 53W cells maintained in the logarithmic phase in unsupplemented basal medium to insure that the level of production of enzymes in the arginine cycle was at its maximum, a twelve hour lag in the appearance of growth in these media was observed. The delay of growth in ornithine supplemented and in unsupplemented media suggests that 53W exhibits a partial genetic block in the production of the enzyme necessary for the conversion of ornithine into citrulline. The existance of a partial genetic block in the arginine pathway may account for the stimulation of growth observed when 53W is supplied with arginine.

The isolation of mutants more effectively blocked in a particular metabolic conversion than a partially blocked parental type has been described by Fincham (1962). Similarly, the arginine auxotrophs and bradytrophs isolated in this study may represent either varying degrees of a more complete genetic block between orinthine and citrulline or a newly induced block at some other step in the arginine cycle.

SUMMARY

In this study a series of arginine auxotrophs and bradytrophs of spontaneous, nitrous acid-and 2-aminopurine-induced origin in S. gallinarum-pullorum 53W were isolated and characterized by precursor utilization. Mutants blocked at all steps tested in the arginine pathway were found. Nitrous acid and 2-aminopurine were shown to be mutagenic for arginine loci in 53W, increasing the rate of mutation to arginine auxotrophy above the spontaneous rate by a factor of 10^1 and 10^2, respectively. Nitrous acid was found to be most efficient for the induction of a genetic block for the ornithine to citrulline conversion and for the induction of bradytrophs. The spectrum of arginine auxotrophs isolated following 2-aminopurine treatment was found to be most similar to the distribution of arginine auxotrophs of spontaneous origin. Mutants deficient in ornithine synthesis were more efficiently induced with 2-aminopurine than with nitrous acid. Evidence is presented which suggests that 53W contains a partial block for the ornithine to citrulline conversion and that the arginine auxotrophs and bradytrophs isolated represent either varying degrees of a more effective genetic block between ornithine and citrulline or a newly induced block at some other step in the arginine pathway.

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