"Mutants of Escherichia coli with Abnormal Patterns of Repression of Arginine Biosynthetic Enzymes."

A thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Microbiology at Massey University

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Abstract:

Experimental work was done to attempt to isolate further mutants of the arginine regulatory gene in Escherichia coli W of the same type as a strain known as W2-250. This mutant carries the argR* regulatory gene which confers upon it the diminished ability to repress or derepress its arginine biosynthetic enzymes.

Experimental work involved establishing a suitable method of exaggerating the slow growth rate of this strain on the arginine intermediate N-acetyl-L-ornithine, such that this could be the basis of protecting similar mutants when selecting them from a parent strain (W2-40) in a penicillin selection procedure. This was achieved by growing both strains in diphasic a medium containing 15ug/cm^3 of L-arginine and an excess (30ug/cm^3) of acetylornithine, in which it was found that the argR strain entered a premature stationary phase when the arginine was exhausted but the argR strain continued growth at a slower rate than normal in the acetylornithine. It was also found that a culture of W2-250 which had entered this stationary phase could be diluted 1:1 in fresh minimal medium + acetylornithine and still remain in a stationary condition for up to 24 hours.

Mutants of strain W2-40 produced using ultraviolet light were cycled twice through a system involving this stationary phase in diphasic medium and dilution 1:1 in fresh medium + 500 units of benzyl-penicillin/cm^3 in order to select against the parent-type. Further selection was carried out on minimal agar plates supplemented with acetylornithine and canavanine on which the parent strain will grow, but the argR* type is inhibited until arginine was added to the agar. The colonies which appeared at this stage were screened on variously supplemented solid media to select those most like W2-250.

Those selected on these criteria were then screened using the acetylornithinase assay and the diphasic medium, with W2-250 included for comparison.

A total of nine possible mutants of the desired type were isolated, but problems with the preparation of enzyme samples precluded a definite decision on their identity.

In the course of this work it was also discovered that an arg_8 bradytroph (W2-25/8) could behave similarly to W2-250 in diphasic media. It was also found that there was some transferable "factor" in the medium of cells which had reached the premature stationary phase which could prevent further growth of either W2-250 or W2-25/8 in non-repressive minimal medium. This is discussed in detail.

Acknowledgements:

Sincere thanks are due to Professor Bacon for his invaluable guidance during the experimental work done for this thesis.

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Introduction:

The arginine biosynthetic pathway of Escherichia coli has eight enzyme catalysed steps in which L-glutamic acid is converted to L-arginine (56,65,67,68). There are eight corresponding structural genes, one for each of the eight enzymes (55,68) which are partially scattered and partially scattered at five separate loci on the genome (73). In addition there are genes involved in control (24) coding for permease[a] enzymes (59,73) and for five arginine transfer RNA's (12) and their synthetase (15). There is also an additional gene (arg I) for enzyme four (ornithine transcarbamylase) which has been mapped in E. coli strains B (22) and K-12 (53). (See tables AI & A-II in the appendix.)

The arginine pathway is controlled by a repression (58) mechanism in which the protein product (33,54) of the regulatory gene argR (the apo-repressor) interacts with the corepressor arginine (24), or even its precursors (6,43), and this then interacts with the protein synthesising machinery making the arginine biosynthetic enzymes, causing their cellular levels to decrease (45,58,61). The reverse of this (derepression) occurs in the absence of an external supply of arginine, although the system is sensitive to the internal levels of arginine (48). This form of control occurs in E. coli strains W, K and C (36), and is "negative" insofar as the regulatory gene product switches the structural genes off under appropriate conditions (1,2). However there is some recent evidence for the existence of a second regulatory gene for the arginine pathway in E. coli (8), this being the gene argM or arg 4' which was believed to code for an inducible form of ornithine transcarbamylase identical with that produced by arg 4 (5,19,37,63). It appears that a mutant version of this regulatory gene was responsible for causing the arg 4 gene to induce while it did not prevent repression of all the other structural genes (8). The gene for arginyl-t-RNA synthetase also the control of the arginine regulatory genes (15) as are the synthetases for leucyl, prolyl and methionyl t-RNA's which are under the

[a]Maas, W.K.(1965) Federation Proc. 24 - 1240

control of their respective regulatory genes (4). The arginine genes are seen to increase their cellular levels together at the onset of derepressive conditions (57,58) but in a co-ordinated but non-parallel fashion (61), unlike histidine genes which increase both co-ordinately and in parallel (3).

In strains where the internal supply of arginine from its intermediates is normal, the enzymes will reach a partially repressed "poised" level (48), but in strains where this supply is inadequate for normal growth (restrictive) the enzymes reach fully derepressed levels (45).

This behaviour applies to the similar arginine regulatory patterns of E. coli strains, W, K and C which have the $argR^+$ gene (36) but not to strains with other allelic forms of the $argR^+$. For instance the $argR^+_B$ allele found in E. coli strain B confers upon that strain the property of slight inducibility of all the arginine structural genes in the presence of arginine (24). An $argR^+_B$ allele can be converted by a single point mutation to an $argR^+$ repressible type (36), which suggests that induction and repression are closely related phenomena, a conclusion already arrived at from studies of the "inducible" ornithine-transcarbamylase (63). A single point mutation can also convert the $argR^+$ (repressible) to the $argR^-$, (50) which confers constitutiveness of the arginine structural genes: i.e. the genes are fully derepressed irrespective of arginine. Such $argR^-$ strains are recognised by their resistance to the arginine analogue canavanine (50). A derivative of an E. coli-W $argR^+$ strain is the strain W2-250 which has the unusual regulatory gene $argR^*$ and exhibits diminished repressibility and derepressibility of the arginine structural genes (Bacon: personal communication, 62,68); this feature resembles the control patterns of a tryptophan mutant except that this mutant has a lesion in the tryptophanyl-t-RNA-synthetase gene and not in the "tryp R" gene. Gorini and his coworkers (36) have produced some unusual $argR^+$(K-12)/$argR^+_B$ hybrid alleles which cause hybrid control patterns such as high level inducibility.

The arginine functional group of genes (a regulon[a]) because of their separateness on the genome are a marked contrast to most other known functional groups of genes which fall into single clusters termed operons. This latter term comes from the theory of Jacob & Monod (34) although the concept of operational cluster of contiguous genes came from Hartman (29). Jacob & Monod envisaged a regulatory gene coding for a soluble substance which had the function of switching off a group of contiguous genes when the correct conditions prevail by interacting with a control site at one end of the operon, termed the operator site. Such a model suits the histidine system with its single functional group of genes (3,39) but the arginine regulon is scattered, but is under the control of one regulatory gene, such that one operator or its equivalent must be present for each group of genes at least (61). In fact one operator mutant, the only one so far in the arginine system, has been isolated for the argI gene, and this shows diminished repressibility of the argI gene only (38,53). The single cluster of four arginine genes (21) is actually two regulatory groups (16), one of argE only, and the other of argC, B & H (10), the latter incidentally having its operator sited at the opposite end to that of other known multigene operons in E. coli (3,11,9,64). However it is possible that the arginine genes come together into a spatially close group in the cell, which would facilitate easier control (63,64).

In the original conception of Jacob & Monod's model, it was proposed that the regulatory gene product exerted its effect at the site of the operator-gene DNA, a situation called transcriptional control, which is true for the lactose operon. However there has since accumulated a certain amount of evidence about the arginine pathway which suggests that its regulatory gene product acts at the level of translation of m-RNA into protein. It is envisaged that the gene, the ribosome, the messenger and other factors form the protein-synthesising complex upon which the repressor and arginine act in concert during repression (Vogel-68, See Appendix, also 13, 28,52). This complex for the arginine system has even been seen (47) in the electron-microscope. The nature of the

[a]Maas, W.K. & Clark, A.J. (1964) J.Mol.Biol. 8 - 365

complex appears to vary with the job it is doing: its sensitivity to canavanine is different when it is synthesising acetylornithinase to when it is synthesising acetylornithine transaminase (19), which might give a clue to the pacesetting phenomenon (60) which is exaggerated in the argR$^+$ strain W2-250 indicating the argR$^+$ product may have a greater than normal affinity for the complex.

Studies of N-acetyl-glutamate-semialdehyde-dehydrogenase mutants show that some have diminished repressibility of the whole arginine pathway (8,51), which suggests involvement of this enzyme in the repression complex, whereas the first enzyme of the pathway is involved in feedback inhibition (26). Only some of the revertants of these mutants have regained normal repressibility patterns. The authors were unable to show that this enzyme binds arginine (8) although it has been shown that anthranilate synthetase in the tryptophan pathway will bind to the complex involved in repression which includes tryptophan[a], and histidine enzyme 1 binds histidine (11).

Studies of the possible involvement of arginyl-t-RNA or its synthetase in repression have yielded a negative result for all five t-RNA species (12,43), and the synthetase (31,32,70) unlike the reports of involvement of leucyl-t-RNA (30) histidyl-t-RNA (11) and other t-RNA species (39) in the control of synthesis of their respective amino-acids. The possibility of a small undetectable fraction of arginyl-t-RNA being involved in the repressive complex has not been ignored (31) and there has been one report of changes in the rates of arginyl-t-RNA charging under repressive and derepressive conditions (14).

Some very convincing evidence for translational control of the arginine pathway has come from Vogel and his co-workers (46,68,69) and others (71) as a result of studies using antibiotics to uncouple the various steps such that Vogel's workers managed to uncouple translation and transcription in the arginine system and to show repression of the latter by arginine. However the possibility of even indirect

[a]Somerville & Yanofsky (1965) Studies on the regulation of tryptophan biosynthesis in Escherichia coli, J.Mol.Biol.,11 p.747.

control of transcription would be difficult to ignore completely (19,71).

The actual mechanism of repression is unelucidated at present and probably will not be so until the working of the ribosome are a lot more fully understood, and in the case of arginine at least could be quite complex with so many "possibles" involved. For instance the argCBH messenger RNA is rapidly degraded in the presence of arginine, and may be even read more slowly as well (40), and another report suggests that the repressor substance itself may be more labile in the presence of repressive arginine concentrations (49). There is also some evidence to suggest the synthesis of some important factor during derepression growth, which combines with arginine at the onset of repression causing the levels of the enzymes to drop well below the expected repressed levels before eventually increasing to those levels after a few generations when the "factor" is diluted out (41,42). This suggests that derepression is not just a passive state of "non-control" but is in some way involved in pre-setting the cells for repressive conditions. Wozny et.al (71) have even suggested that translational repression in fact occurs after the initiation of translation which seems to be in keeping with the idea that all genes in a pathway were evolved from a single ancestor gene (Evolving Genes and Proteins. eds V. Bryson and H.J. Vogel (Academic Press: New York) 1965). This could mean that each protein of a given pathway has retained an amino-acid sequence in common with all the others in the pathway, and it is this common sequence which is the site for post-translation-initiation control by some influences involving arginine and the $argR^+$ product at least.

This thesis will deal only with one small part of this very wide field of the control of the arginine biosynthetic pathway in E. coli: this is the mutant strain W2-250 which has the abnormal regulatory gene argR , which means that the arginine structural genes can neither repress or derepress properly under appropriate conditions, (Bacon, personal communication, Vogel 62,68). I will show how I have attempted to isolate further argR mutants from the $argR^+$ strain, W2-40, by utilizing the pacesetting phenomenon in

diphasic medium (60. appendix figure A1) which is exaggerated in the strain W2-250. Then I have screened those mutants further on canavanine supplemented medium after they had grown in the presence of arginine, which differentiates between the $argR^{+}$ strain which is inhibited up to 50% of normal growth rate on such medium (45) and the $argR^{*}$ which is completely inhibited until arginine is added (Bacon: personal communication). Further studies of the nature of the argR were not planned but its growth patterns on diphasic media proved to be both useful and very interesting.

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A. Growth Studies:

1. Bacterial Strains:

These were obtained from the personal stocks of Professor D.F. Bacon of this department, who was partly responsible for their isolation from the parent type, Escherichia coli - strain W (ATCC 9637). They are:

W2-40 ..... pro^- $argR^+$ arg^-_A

W2-250 ..... pro^- $argR^*$ arg^-_A

W2-25/8 ..... pro^- $argR^+$ arg^{low}_H

Other strains will be mentioned in relevant sections. W2-40 was selected because the total supply of arginine to it can be controlled. W2-250 is the original mutant of the type I have attempted to isolate in this exercise, and it was used as a reference for selection procedures. W2-25/8 was selected because of its ability to mimic growth patterns of W2-250.

2. Media:

All stock strains and new isolates were stored in the refrigerator on Difco Brain-Heart-Infusion (1.2%) Agar (1%) slopes in 6 x 1" test-tubes with cottonwool plugs. This BHI agar was also used on petri plates to obtain single-colony isolates from stocks and elsewhere as noted.

The minimal-salts medium (MM) was modified from Davis & Mingioli Medium A (17) and supplemented (SMM) with sterile glucose (0.4%) and proline (30 ug/cc) after autoclaving. To this amino-acids were added as required (30 ug/cc), unless otherwise stated, which were stored as frozen sterile aqueous concentrates (10mg/cm^3).

L-arginine as free base Arg [1] (Sigma: E-grade)
L-ornithine as hydrochloride Orn (Sigma: E-grade)
L-citrulline as free base Cit (Sigma: E-grade)
L-N-acetyl-ornithine as free base AcOrn (Cyclo: I grade)
D-L-proline as free hydroxyproline (Sigma: E-grade)

These were added to SMM agar (1.0%) using the glass spreader method, and directly to liquid media.

1 Abbreviations used in tables and figures.

3. Methods and Results:

a. Preparation of Cells:

Cells obtained from single-colony isolates were inoculated into 10cm^3 of SMM + arginine in 6 x 1" tubes and incubated at 37°C in a water-bath until stationary phase was reached. These cells were harvested and washed in MM using a Sorvall (Model NSE) bench centrifuge (5 min. at 7500 rpm.), and finally diluted to 10^{-5} in MM, 1cc of which was used to inoculate the next step. These had total volumes of 10cm^3 when added to SMM with required amino-acid additives, and are termed "overnight cultures" after 12 hours incubation at 37°C in a water-bath. The cells in these overnight cultures were then harvested and washed as above, and used to inoculate the "growth-tubes" in suitably supplemented SMM to a total volume of 10cc in Klett-Summerson tubes with rubber stoppers (53). These growth-tubes were used in the various growth studies, enzyme studies and in mutant selection studies.

b. Measurement of Cell Growth:

This was done turbidometrically using a Klett-Summerson Colorimeter (Model 800-3) equipped with a red filter (~~=~~.640,-700 mu absorbence), using a sterile-distilled-water filled Klett tube as a zero reference tube, against which all other tubes were corrected when filled with 10cc of SMM + amino-acids, without cells added. Growth-tubes were removed individually for reading, and no more than five were read consecutively at (any one time interval) before the reference tube was used to readjust the meter (if necessary).

It was found that the optical density and the number of cells present were almost directly proportional over the range of values in use (Klett 5 - 120).

Growth was plotted on semilogarithm graph paper, with time on the abscissa (linear) and optical density in Klett-Summerson units on the ordinate (logarithmic).

One doubling time was taken as the time needed to double the optical density (O.D.) of a culture.

c. The Effect of the Overnight Medium Supplement on Monophasic Growth.

Cells of strains W2-40, W2-250 and W2-25/8 were prepared in overnight cultures in which the supplement was varied. Such cultures were used to inoculate growth-tubes containing supplements which were also varied, and the subsequent growth of these cultures was plotted on the graphs in figures 1 & 2.

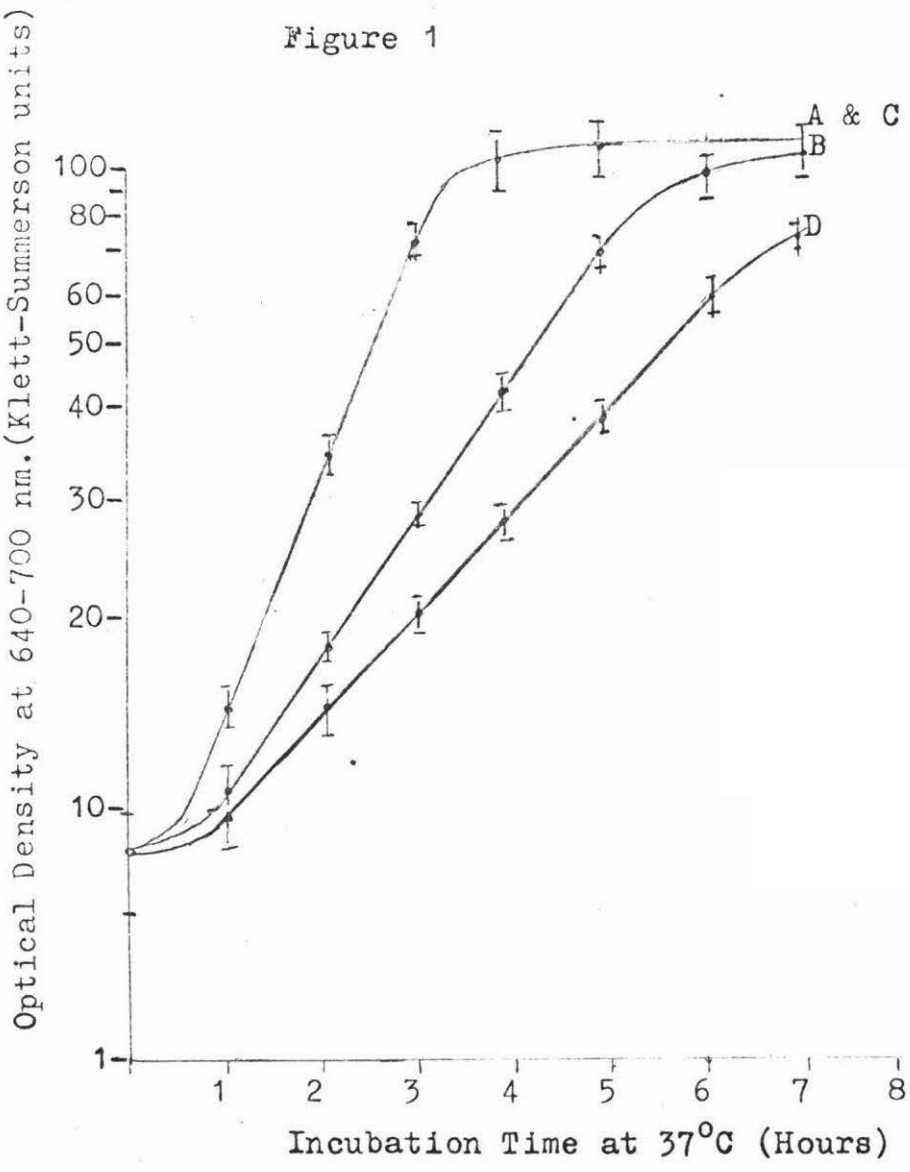
The summary of these results is shown in table I, where these cultures are marked as being monophasic, and in figures 1 & 2.

These results show that the growth of all three strains in SMM + acetylornithine is reduced after overnight growth in arginine in comparison with growth in the same medium after growth in SMM + acetylornithine. This effect is particularly pronounced in the case of W2-250. Citrulline also has a similar effect on the growth of W2-25/8 in unsupplemented SMM, but it is not as great as that of arginine. Although this effect of citrulline on the growth of strains W2-40 and W2-250 in acetylornithine was not investigated, it would probably be similar to that of arginine, as citrulline is known to be able to mimic the repressive effect of arginine, although to a lesser degree of effectiveness, even in strains which cannot convert citrulline to arginine (6).

However this treatment did not achieve the desired effect of completely halting the growth of W2-250 in acetylornithine after overnight culture in arginine, although it did not affect the growth of W2-40 to an unacceptable level.

I decided on this basis to utilize the observation of Vogel (60) of a phenomenon he termed pacesetting, as a possible means of achieving an even greater disparity in the growth rates of the two strains in the same media. (W2-25/8 was included in these studies because of its ability to mimic the growth patterns of W2-250 under certain conditions.)

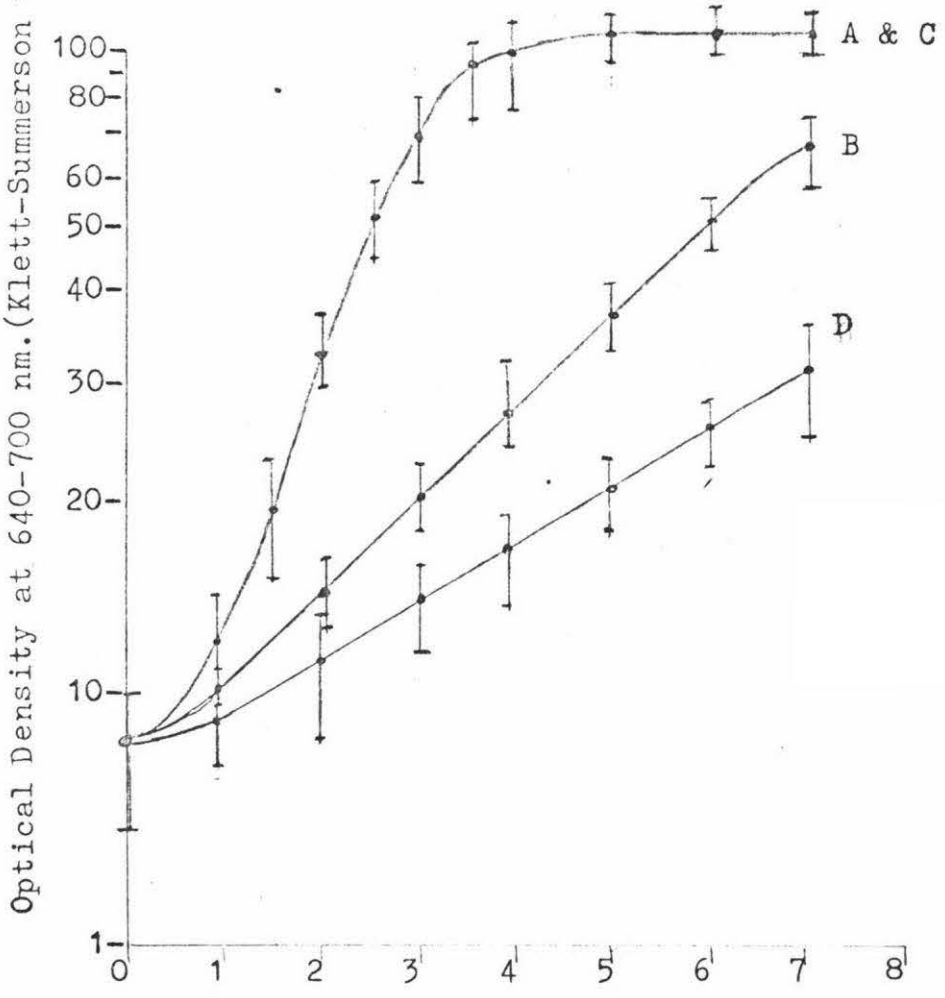
Figure 1



"The Effect of the Overnight Medium on Monophasic Growth of E.coli W2-40."

Key: Line A = growth in SMM + Arg, Innoculum Ex SMM + Arg.
Line B = growth in SMM + AcOrn, " Ex SMM + Arg.
Line C = growth in SMM + Arg, " Ex SMM +AcOrn.
Line D = growth in SMM + AcOrn, " Ex SMM +AcOrn.

Figure 2



"The Effect of the Overnight Medium on Monophasic Growth of E.coli-W2-250."

Key: as for figure 1 (page 5)

d. Comparative Growth of Standard Strains in Diphasic Media:

This analysis originally arose out of the observation that cells of W2-250 failed to reach a normal stationary phase optical density (100 to 120 Klett-Summerson units) when grown in an overnight culture medium supplemented with both arginine in sub-maximal concentration (15 ug/cm^3) and acetyl-ornithine (30ug/cm^3).
(This was part of an unsuccessful attempt to exaggerate the growth rate difference between W2-250 and W2-40 in SMM + acetylornithine).

Initially the studies were limited to this system, and the data shown in figure 3 and forming part of table I were the result. This work showed that a doubly supplemented (diphasic) (2, 58, 59, 60) medium containing 15ug/cm^3 arginine and 30ug/cm^3 acetylornithine caused strain W2-250 to cease growth completely at about 55 Klett-Summerson optical density units when the arginine was completely exhausted, whereas strain W2-40 although slower, continued its growth in the acetylornithine. Lower concentrations of arginine in the same system were also tested with the intention of providing the greatest potential for further growth of W2-40 in a penicillin selection procedure, but they did not have the effect of halting the growth of strain W2-250 although its subsequent growth in acetylornithine was greatly slowed. This appears to be a function of the argR regulatory gene which does not permit full derepression of the arginine biosynthetic genes (64), thus exaggerating the effect which is quite minimal for W2-40 which has a normal $argR^+$ regulatory gene. This effect is mimicked by W2-25/8 in 15ug/cm^3 arginine or citrulline + acetylornithine or without a second supplement, but is exaggerated even more in an arginine/citrulline diphasic medium (this is due to the low activity of argininosuccinase in W2-25/8 which can be almost completely repressed by citrulline, causing very slow growth when citrulline is the

only source of arginine (6).

The conclusion of these studies was that cells of W2-40 after mutagenic treatment, should be selected against in a diphasic system (arginine (15ug/cm^3) + acetylornithine) if penicillin is added at a time when any mutant cells which behave like W2-250 become paceset, when the parent type (W2-40) continues growing. To include the possibility of prolonging the growth of the parent type under such conditions to improve enrichment for the new types, it would be necessary to dilute them with fresh medium containing acetylornithine (which would preclude further growth of the mutants). Thus a study of the effects of dilution on cells which had entered a premature stationary phase due to pacesetting was necessary.

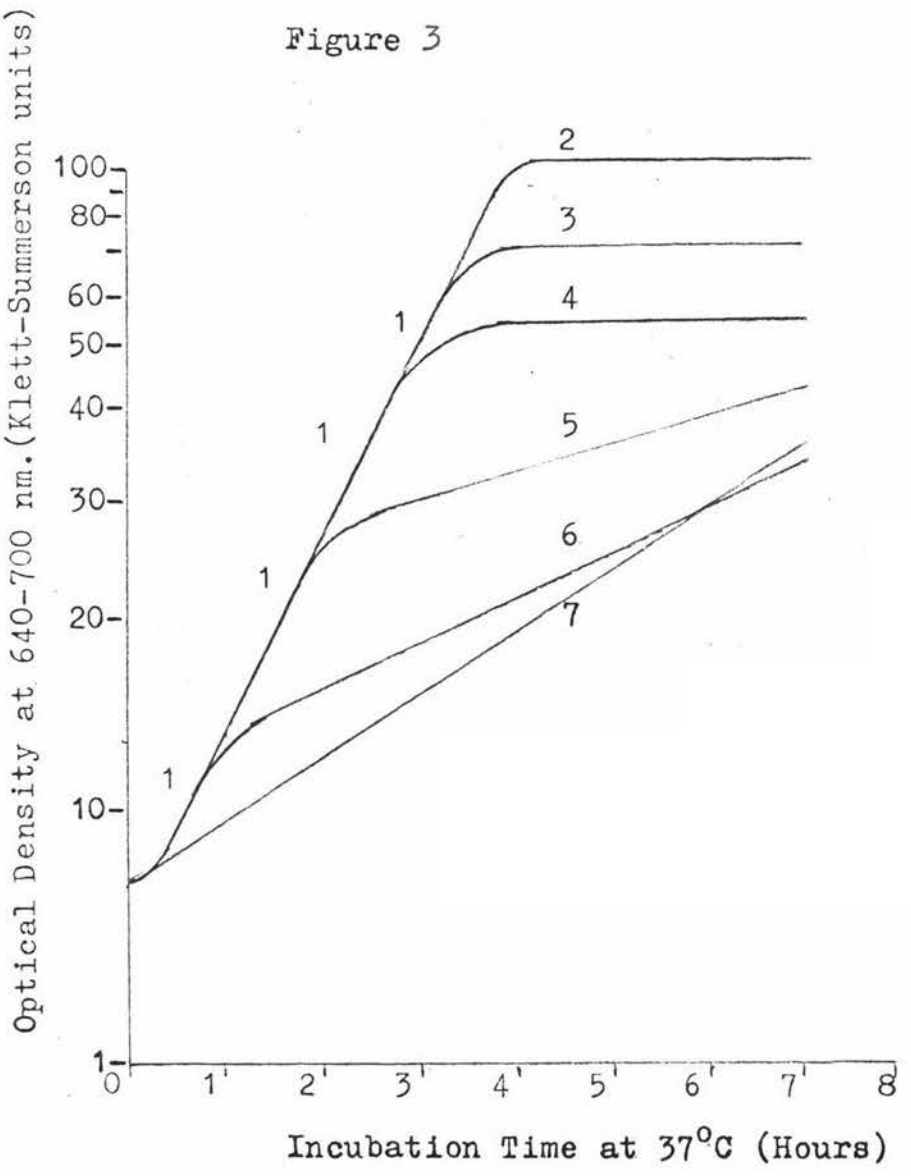
In addition to the studies mentioned above, work was extended to include an investigation of the effect of the supplement in the overnight growth medium on pacesetting and of the effect of different diphasic supplement combinations. The effect of arginine in the overnight medium was already known to carry over into the diphasic stage in the earlier studies with arginine/acetylornithine media where it had the effect of slowing the second phase growth of both W2-250 and W2-40 in acetylornithine when this was compared with an analogous system using acetylornithine only in the overnight medium (see table I).

In all of these investigations the following basic procedure was adhered to:
(1) Inoculate and incubate (12 hours at 37°C) suitably supplemented (30ug/cm^3) overnight culture tubes with cells derived from SMM + arginine liquid culture.
(2) Inoculate Klett-Summerson tubes containing suitably supplemented medium (diphasic, and monophasic controls) using cells from the overnight cultures, incubate and plot growth optically.

The results of these studies are tabulated in table I and show the effect of arginine both in

diphasic medium and in overnight culture is to slow the subsequent growth of a culture in either ornithine or acetylornithine, particularly in the case of W2-250 and W2-25/8. Presumably this feature may select against "leaky" auxotrophs in wild populations of E. coli in favour of prototrophs.

Figure 3



"The Effect of Arginine Concentration on Pacesetting of Strain W2-250."

Key:

1.* growth of cultures 2 to 6 prior to exhaustion of arginine

2.= Control: 30ug/cm^3 arginine only.

3.* 20 ug/cm^3 argimine + 30 ug/cm^3 acetylornithine.

4.= 15 ug/cm^3 arginine + 30 ug/cm^3 acetylornithine.

5.= 10 ug/cm3 arginine + 30 gu/cm3 acetylornithine.

6.= 5 ug/cm^3 arginine + 30 ug/cm^3 acetylornithine.

7.= Control: 30 ug/cm^3 acetylornithine only.

Table I

Growth of E. coli-W arginine Auxotrophs in arginine intermediates.

Strain[2]	Growth Phase: First Phase A1	B	C2	Second Phase A2	C2	D
W2-250	Arg	30	60	Monophasic		
(Arg)	Arg	15	60	AcOrn	S	S
	Arg	10	60	AcOrn	480	420
	Arg	5	60	AcOrn	350	290
	Cit	30	60	Monophasic		
	Cit	15	60	AcOrn	S	S
	Orn	30	60	Monophasic		
	Orn	15	60	AcOrn	270	210
	AcOrn	30	210	Monophasic		
W2-250						
(Orn)	Arg	15	60	AcOrn	S	S
	Orn	15	60	AcOrn	250	190
	AcOrn	30	130	Monophasic		
W2-250						
(AcOrn)	Arg	15	60	AcOrn	S	S
	Arg	10	60	AcOrn	250	190
	Arg	5	60	AcOrn	220	160
	AcOrn	30	120	Monophasic		
W2-40						
(Arg)	Arg	30	60	Monophasic		
	Arg	15	60	AcOrn	210	150
	Arg	10	60	AcOrn	160	100
	Arg	5	60	AcOrn	120	60
	Cit	30	60	Monophasic		
	Orn	30	60	Monophasic		
	AcOrn	30	120	Monophasic		
W2-40						
(AcOrn)	Arg	30	60	Monophasic		
	Arg	15	60	AcOrn	220	160
	Arg	10	60	AcOrn	180	120
	Arg	5	60	AcOrn	100	40
	AcOrn	30	90	Monophasic		

Table I (Continued)

Strain	First Phase			Second Phase		D
	A1	B	C	A2	C2	D
W2-25/8						
(Arg)	Arg	30	60	Monophasic		
	Arg	15	60	Cit	S	S
	Arg	10	60	Cit	480	420
	Arg	5	60	Cit	350	290
	Arg	15	60	Orn	S	S
	Arg	15	60	AcOrn	S	S
	Arg	10	60	AcOrn	600	540
	Arg	5	60	AcOrn	360	300
	Arg	15	60	-	S	S
	Arg	10	60	-	150	90
	Arg	5	60	-	120	60
	Cit	30	150	Monophasic		
	Cit	15	150	Orn	S	S
	Cit	15	150	AcOrn	S	S
	Cit	15	150	-	S	S
	Orn	30	90	Monophasic		
	Orn	15	90	AcOrn	200	110
	AcOrn	30	120	Monophasic		
	SMM	-	100	Monophasic		
W2-25/8						
(Cit)	SMM	-	100	Monophasic		
W2-25/8						
(SMM)	Arg	30	60	Monophasic		
	Arg	15	60	-	S	S
	Arg	10	60	-	150	90
	Arg	5	60	-	120	60
	SMM	-	80	Monophasic		

Key to table I

1 Both monophasic and diphasic data are included here to provide comparable data.

2 The supplement mentioned in brackets immediately below the strain was the one present in the overnight culture medium used to inoculate the growth tubes which provided the adjacent data.

A1 indicates the first or only supplement in the medium.

B indicates the concentration of A1 in ug/cm^3.

C1 is the optical doubling time of the culture in the supplement A1.

A2 indicates the second supplement[a] in the diphasic media.

C2 is the optical doubling time of the culture in A2.

S indicates that the cells entered an optically stationary phase (no apparent growth) due to exaggerated pacesetting.

SMM = supplemented minimal medium with no added arginine or arginine intermediates.

Note: The error in calculating the doubling time of the cultures increases as this increases. This is partly due to errors in the physical estimations from the growth curves on the graphs, and partly due to the comparatively short times over which such slow growing strains were measured during growth.

All doubling times quoted in this table represent average values where they represent more than one result for the same situation during different experiments. The deviations are generally less where the doubling times are shorter (see the reasons above)

[a] The arginine intermediates are utilized in the reverse order of their biosynthesis in the arginine pathway

e. The Involvement of the Medium in Pacesetting:

Cells of strains W2-250 and W2-25/8 were prepared in overnight cultures with arginine supplement (30ug/cm^3) and then dealt with separately as follows:

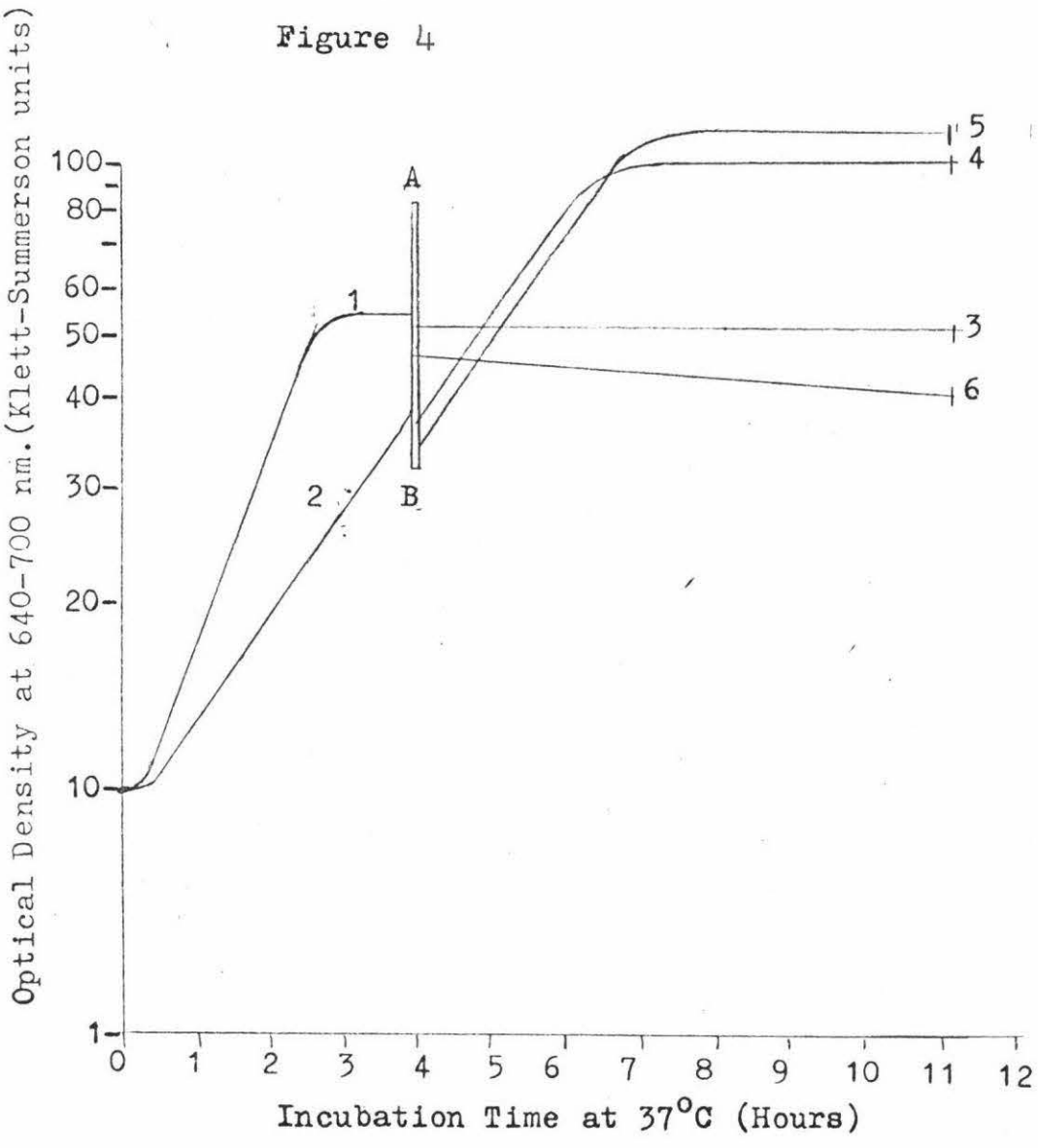
Four parallel Klett tube cultures were inoculated: two contained monophasic[a] medium (acetylornithine 30ug/cm^3) and two contained diphasic[a] medium (arginine 15ug/cm^3, acetylornithine 30ug/cm^3); and these were incubated in parallel at 37°C until the arginine in the diphasic cultures was exhausted and the cells in them had established a paceset stationary phase. All cultures were then harvested by centrifugation, but their media were not discarded.

In one each of the monophasic and diphasic cultures the cells were replaced in their original growth medium to act as controls. The cells from the second diphasic culture were resuspended into fresh SMM + acetylornithine (30ug/cm^3), and the medium from which they originally came was used to resuspend the cells from the second monophasic culture (the medium from which was discarded). Subsequent growth in all tubes was recorded and plotted to give the results shown in figure 4.

Contrary to expectations these results seem to indicate that the effect of pacesetting is in some way transferable in the medium, as evidenced by the inability of non paceset cells which had been growing in the absence of arginine in the medium, to continue growth in medium in which cells had been paceset, despite either the presence of acetylornithine in such medium in the case of strain W2-250, or the ability of strain W2-25/8 to synthesise its own arginine in minimal medium.

([a] Media used for W2-25/8 did not include acetylornithine.)

Figure 4



"The Involvement of the Medium in Pacesetting"

Key: 1 = growth in diphasic medium prior to arginine exhaustion
2 = growth in acetylornithine prior to harvesting.
3 = Control: cells from 1 replaced in original medium.
4 = Control: cells from 2 replaced in original medium.
5 = Pace-st cells from 1 placed in fresh SMM + Acetyl-ornithine.
6 = Non pace-set cells from 2 placed in medium from 1.
Line A=B signifies time of cell transfers.

These results were obtained using strain W2-250, but similar results were obtained using W2-25/8 in an arginine /SMM ONLy diphasic system.

f. The Effect of the Length of Paceset Stationary Phase on Subsequent Growth in Non-Repressive Media.

It was found that cells of either strain W2-250 or strain W2-25/8, remained at the same optical density as long as the medium remained unchanged once these cells had entered a premature stationary phase induced by severe pacesetting in arginine. It was not investigated however whether cell death sets in eventually or not. However in an attempt to isolate "non-paceset" spontaneous derivatives of W2-25/8 with a series of parallel cultures in limiting arginine ($15ug/cm^3$) medium, it was found that the cells remained paceset completely in each culture for 24 hours. These cultures were subcultured into similar conditions and the treatment was repeated: this was repeated again to give a total of six cycles for each culture, and although it did not yield any of the mutants sought, it did demonstrate that pacesetting is repeatable even in sequence, and that it was not "broken" in any of the 24 hour cycles. (This was determined optometrically)

It was decided to study the "life" of the pacesetting effect with respect to time spent in a paceset induced stationary phase.

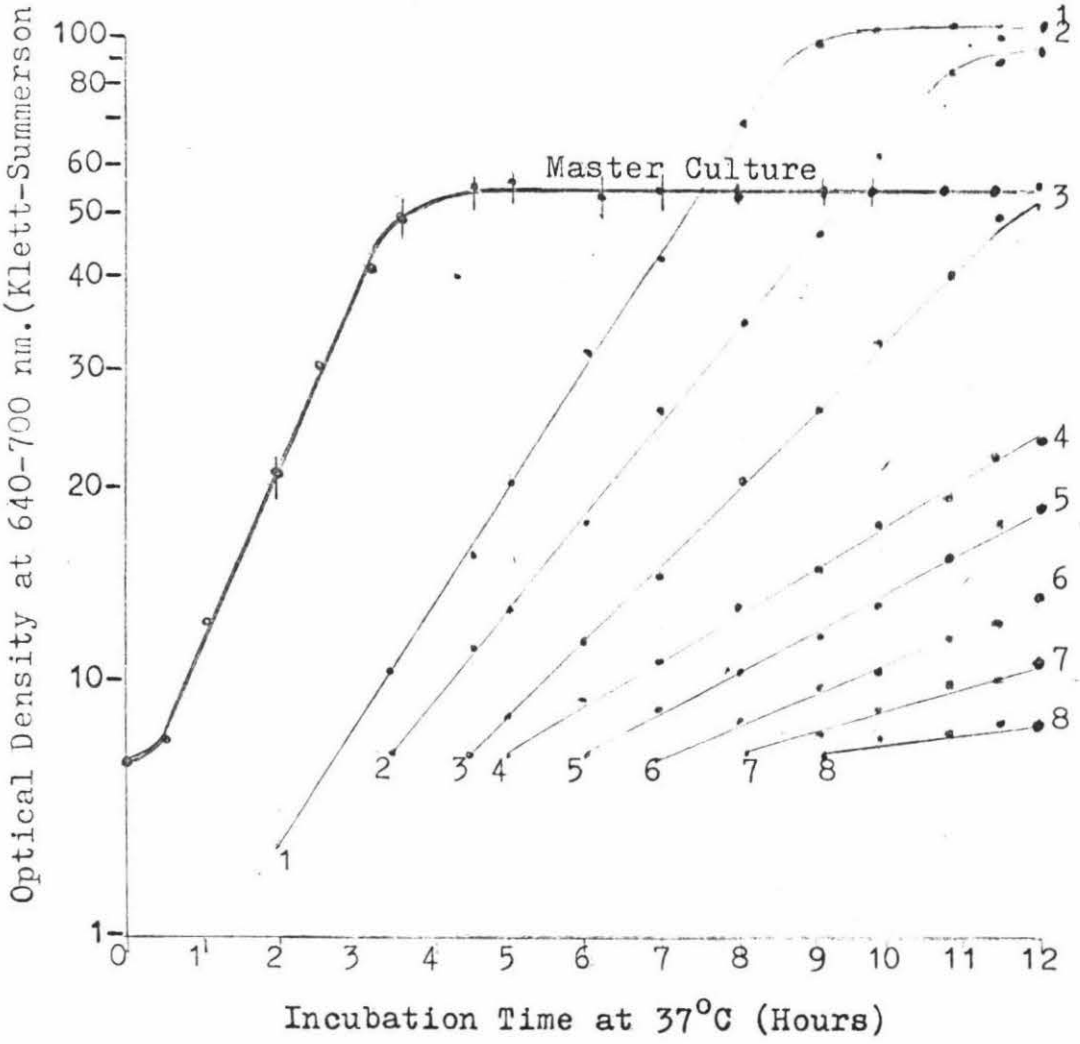
Cells of W2-250, derived from an arginine supplemented overnight medium, were paceset in a diphasic arginine ($15ug/cm^3$)/acetylornithine medium in a bulk culture using a side-arm flask. At intervals during growth and after pacesetting had set in, $1cm^3$ samples were removed from this culture and added to $9cm^3$ of SMM + acetylornithine in Klett tubes to form subcultures which were incubated and their growth followed turbidometrically. (It was known that this complete pacesetting was relieved by one in ten dilution into fresh medium (Section h, Vogel (58)).

Figure 5, and table II, show the results of this work which indicated that the pacesetting effect appears to increase in the paceset stationary phase

with time for at least five hours after the stationary phase had set in.

However cells which had spent different times in a normal stationary phase in SMM + arginine did not appear to show this effect.
(The results shown in figure 5 and table II, are applicable to strain W2-250 only, but the results for W2-25/8 were very similar)

Figure 5



"Effect of Length of Time in Paceset Stationary Phase on Cells of W2-250 or W2-25/8 On Subsequent Growth in Non-Repressive Media".

Numbers 1 to 8 represent subcultures of the main culture (heavy line) in chronological order of sampling. See text for further explanation.

Table II

"Effect of the Length of Time in Paceset Stationary Phase on Subsequent Growth in Non-repressive Medium"

A	B	C	D
1	60	-	100
2	60/S	0	120
3	S	30	150
4	S	60	240
5	S	150	300
6	S	210	420
7	S	270	600
8	S	330	c,1000

Key:

A = sample number (in chronological order of removal)
B = doubling time of main culture (minutes)
C = time spent by cells in paceset stationary phase
D = doubling time of cells in subcultures in SMM + AcOrn

S signifies stationary culture-optical density is not changing.

Notes:

1. The inoculum for the main culture was strain W2-250 from an arginine supplemented overnight culture

2. The main culture was grown in SMM + arginine ($15ug/cm^3$) + acetylornithine ($30ug/cm^3$)

3. The subcultures were inoculated with $1cm^3$ samples from the main culture into $9cm^3$ of SMM + acetylornithine ($30ug/cm^3$)

g. The Effect of Acetylornithine on Paceset Stationary Phase Cells:

This experiment was prepared in the same way as in section f., but the main culture was split into six identical 10cm^3 aliquots in Klett-Summerson tubes, and only strain W2-250 was used.

When the cells had entered the premature stationary phase due to pacesetting in the arginine/acetylornithine diphasic medium, enough acetylornithine for a complete growth cycle (30ug/cm^3) was added in sequence to the tubes with 1 hour intervals between the additions. The first tube received its acetylornithine immediately the pace had been "set", and the last five hours later.

This experiment was also done in medium which contained 15ug/cm^3 arginine, but no acetylornithine.

In both experiments the addition of extra acetylornithine at any time during the growth cycle, failed to release the cells from the paceset stationary phase. This indicates that in no way is acetylornithine starvation due to lack of external supply, involved in the pacesetting phenomenon.

h. The Effect of Dilution on Paceset Stationary Phase Cells:

Cells of strain W2-250 from an overnight culture with arginine supplement, were inoculated into a bulk growth culture of diphasic medium (arginine 15ug/cm^3/acetylornithine 30ug/cm^3), and incubated until the arginine had been exhausted and the cells had entered the premature stationary phase. Samples were removed and diluted to various extents into SMM + acetylornithine (30ug/cm^3) in Klett tube growth cultures, and their subsequent growth was measured and plotted on a graph.

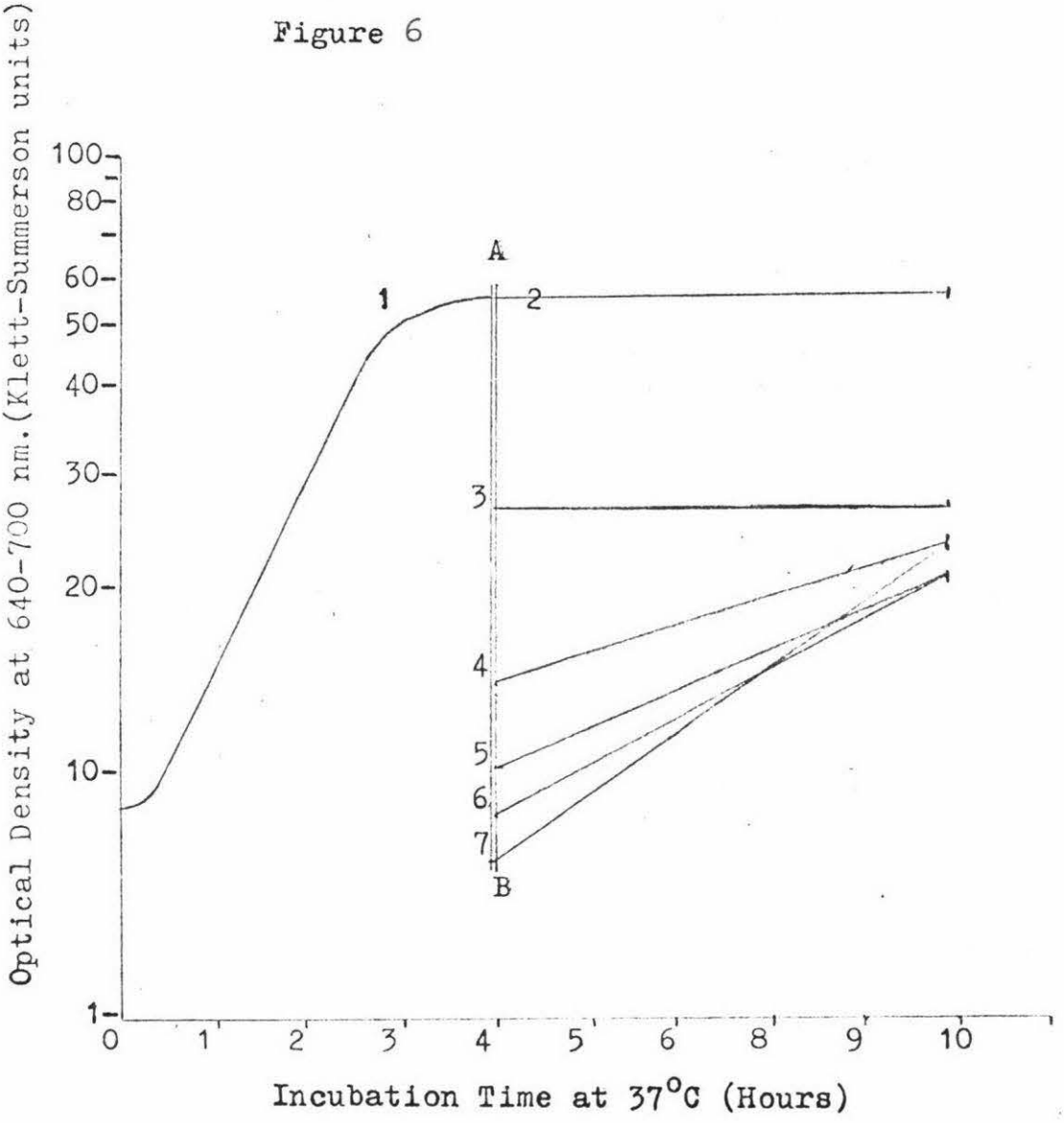
The results of this are shown in table III and in figure 6.

The data indicates that the pacesetting effect can be progressively diluted out, but a dilution of one part inoculum (paceset cells + medium) to one part fresh medium does not relieve the effect to any measurable extent. A dilution of one part inoculum to nine fresh medium appears to almost totally relieve the cells of the pacesetting effect, a feature already observed by Vogel (61). This feature led to the development of the procedure involving penicillin treatment linked to pacesetting and dilution. The dilution step therein was designed to allow for as much growth as possible of the parent type (strain W2-40) which does not paceset into a premature stationary phase, while still ensuring minimal growth of its mutant derivatives which would paceset like W2-250. Thus the selection procedure allowed for only a 1:1 dilution with fresh medium + penicillin.

Table III

Strain W2-250 (ex overnight culture with arginine supplement)	Dilution of subcultures	Doubling time of subcultures
	1:2	Stationary
	1:4	420 minutes
	1:5	360 minutes
	1:8	240 minutes
	1:10	120 minutes
	Undiluted	Stationary

Figure 6



"The Effect of Dilutiom on Paceset Stationary Cells"

Key: Line A=B represents the point of dilution into the sub-cultures 3 - 7

1. = growth of main culture.
2. = undiluted control.
3. = Culture of cells from 1 diluted 1 in 2.
4. = " " " " 1 " 1 in 4.
5. = " " " " 1 " 1 in 5.
6. = " " " " 1 " 1 in 8.
7. = " " " " 1 " 1 in 10.

Cells are diluted into SMM + Acetylornithine(30 ug/cm^3)

1. Development of Penicillin Selection Procedure (23):

This procedure used the pacesetting differential between the two strains, W2-40 and W2-250.

Parallel cultures of W2-40 and W2-250 were prepared in overnight cultures with arginine supplement. These were used to inoculate each of three growth tubes containing 10cm^3 of arginine (15ug/cm^3)/ acetylornithine diphasic medium to give two parallel sets (one set per strain), which were incubated until the arginine was exhausted. The first tube of each set was allowed to continue as before at this juncture, but the second and third tubes of each set were reduced to a volume of 5cm^3 and then diluted back to the original volume with fresh SMM + acetylornithine (30ug/cm^3). The third tube of each set also had penicillin added to give a final concentration of 500 units in 10cm^3.

0.1cm^3 samples were removed from these third tubes at hourly intervals starting with the addition of the penicillin, and were plated at suitable dilutions on to BHI agar (after washing in MM) in order to determine the comparative numerical survival of the two strains. Tube three of each set was also used for an optical comparison of the two strains, with tube 1 & 2 of each set as controls. (See figures 7A & 7B)

Results of Penicillin treatment:

Table IV

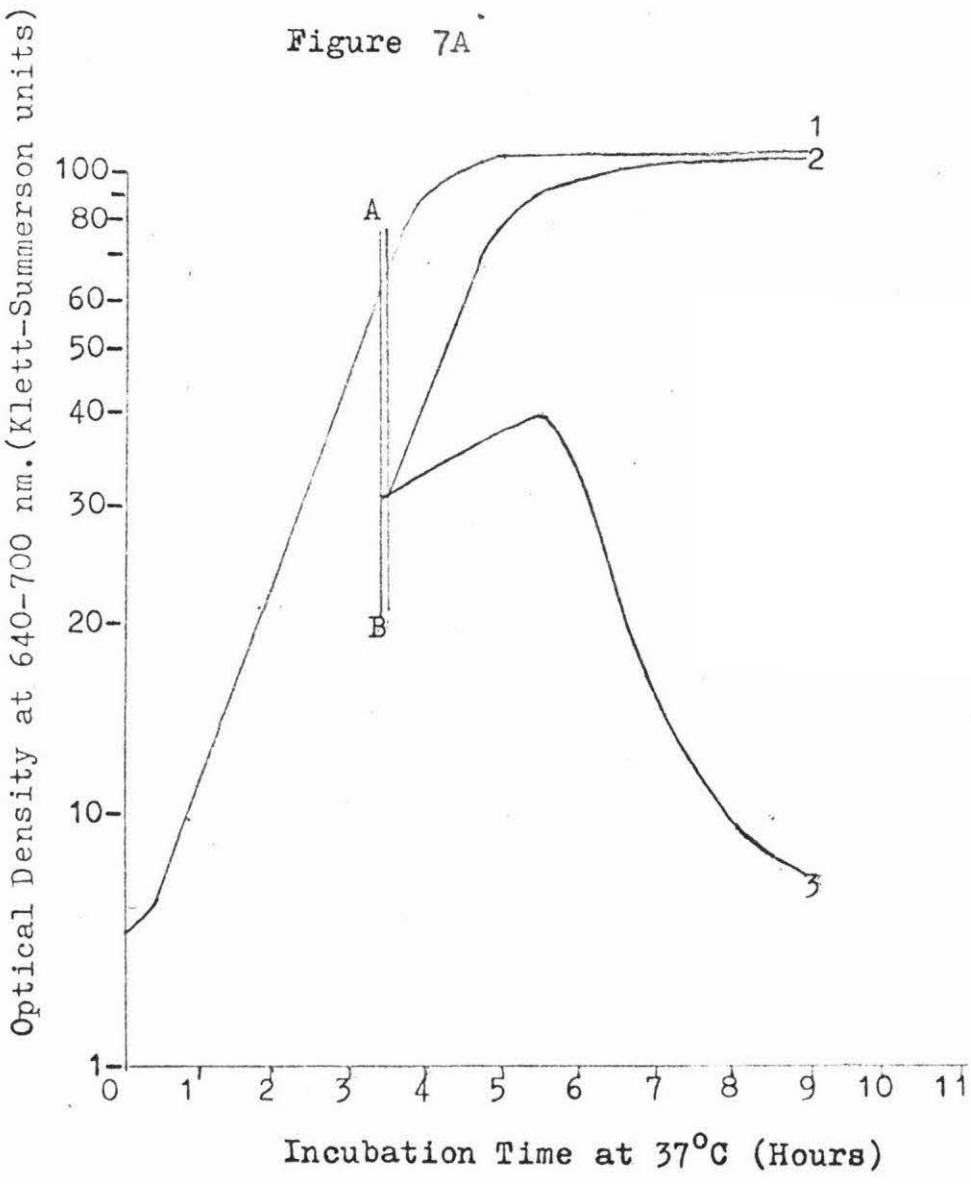
Relative Survival

Time in Penicillin	Strain W2-40	W2-250
0 hours	1.00	1.00
1 hour	0.15	0.30
2 hours	0.03	0.09
3 hours	0.006	0.016
4 hours	0.003	0.015

1. Sucrose is omitted as it inhibits growth of these strains.
2. Based upon plate counts; relative value of 1.00 is approximately equivalent to 2×10^8 cells/cm^3.

These results favoured a treatment time of 3-4 hours in penicillin under the conditions as prescribed above.

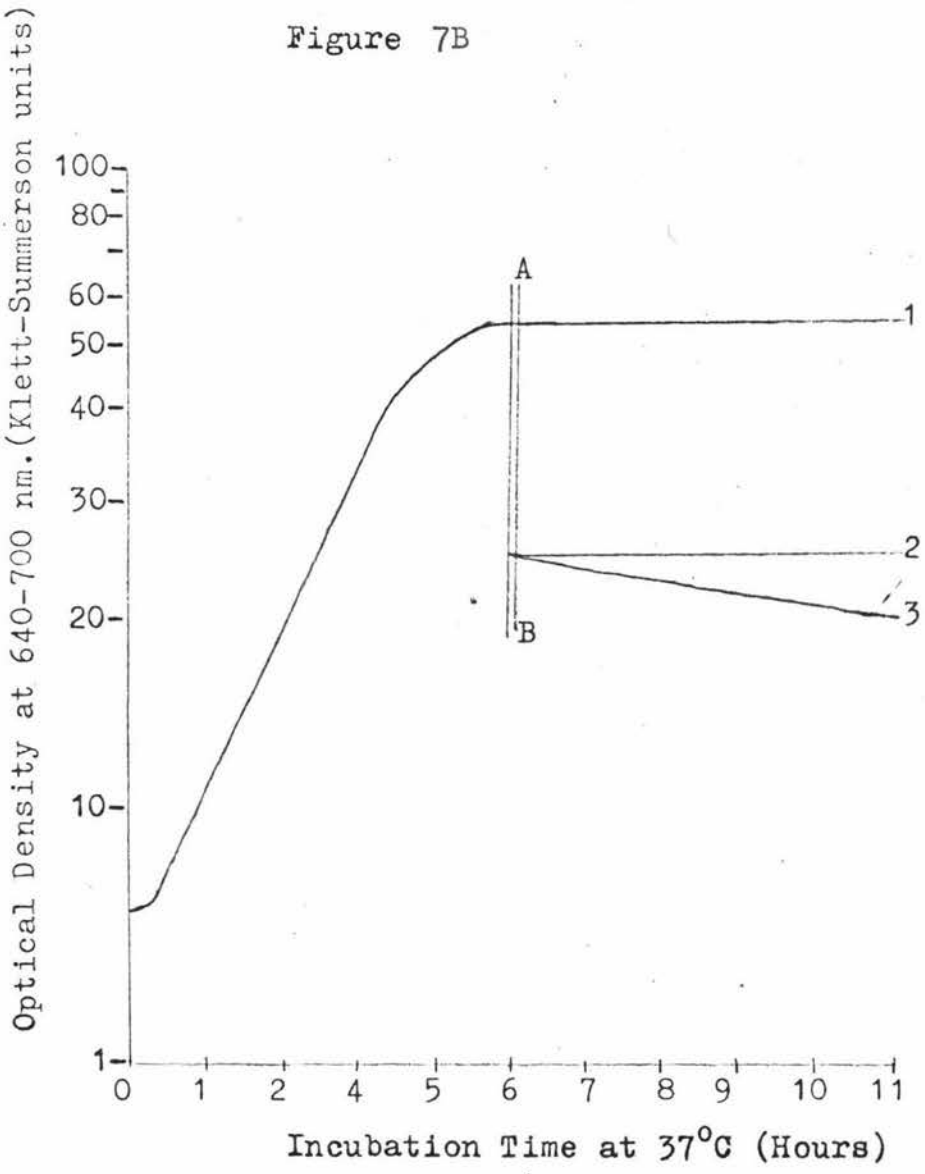
Figure 7A



"Survival of Paceset Stationary Phase Cells when Diluted into Penicillin Medium: A-strain W2-40"

Key see figure 7 -B.

Figure 7B



"Survival of Paceset Stationary Phase Cells when Diluted into Penicillin Medium: B- W2-250"

Key:

1. = undiluted control.
2. = culture diluted 1;2 into SMM + AcOrm.
3. = culture diluted 1:2 into SMM + AcOrn + penicillin.

Line A=B shows point of dilution for 1 & 3.

j. Ultraviolet Mutagenesis:

This was performed using a Chromatolux 2L(cat.no. 633,000) ultraviolet lamp using only one of the two emitters; this was set 60cm above the sample for exposure, an incidental light was reduced to prevent excessive photoreactivation of damaged loci[a].

Cells of the wild-type, E. coli -W, were prepared in the usual manner in MM + 0.4% glucose, and when Klett 30-40 was reached, they were diluted, $1cm^3$ into $9cm^3$ of MM, washed and resuspended into the same volume of MM. $7.5cm^3$ of this is then placed in the base of a standard petri-dish, and exposed to the uV source with gentle swirling of the contents by hand. $0.1cm^3$ samples were removed at intervals and plated at suitable dilutions onto BHI agar (1%). After incubation these were counted and the percentage survival calculated relative to the time immediately prior to exposure. The results of this indicated that 45 seconds exposure under the given conditions was necessary to achieve 0.1% survival as required.

[a]Rupert, C.S. (1961). "Repair of ultraviolet damage in cellular DNA". J. Cell. comp. Physiol., 58 - (Suppl.1), - 57.

k. Canavanine Selection (50) of Mutants on Solid Media:

Cells from exponentially growing cultures of W2-40 and W2-250 in SMM + arginine were harvested and washed in minimal medium, and then plated at suitable dilutions on SMM agar + acetylornithine (20ug/cm^3) and canavanine at a range of concentrations. Incubation at 37°C followed for 48 hours and the plates were inspected to determine which concentration of canavanine gave the most inhibition of W2-250 and the least of W2-40. Then the agar of the plates was lifted carefully (intact), and 50ug of arginine was added in solution to the bottom of the plate and the agar replaced. Incubation for a further 24 hours at 37°C followed and the recovery of W2-250 was noted.

It was found that 15ug/cm^3 of canavanine gave the desired differentiation between the two strains, although the distinction was never sharp. (Any one plate had large and small colonies, and the concentration of canavanine which completely inhibited W2-250 also inhibited W2-40 quite significantly. However W2-250 showed full recovery to the added arginine.)

B. Isolation of Paceset Mutants of E. coli W2-40:

1. Method:

An overnight culture of W2-40, with arginine supplement was used to inoculate a culture for ultraviolet mutagenesis (exponentially growing cells in SMM + arginine). Treated samples were diluted into SMM + arginine and incubated at 37°C overnight. These were used to inoculate growth tubes containing arginine (15ug/cm^3) and acetylornithine (30ug/cm^3), (total vol. 5cm^3) and allowed to grow until the optical density reached about 55 Klett-Summerson units. Then the cultures were diluted with an equal volume of SMM + acetylornithine (30ug/cm^3) + penicillin (final concentration of 500 units/cm^3) and incubated for a further three hours, and then chilled, harvested and washed with MM. The whole procedure was then repeated on these same cultures excluding the irradiation step.

Diluted samples of the cultures were plated onto SMMagar + acetylornithine + canavanine and incubated for 48 hours at 37°C. Colonies which had appeared were "marked" and 0.1cm^3 of arginine (stock solution) was added to each plate by lifting the agar with a sterile spatula and injecting it underneath. The plates were incubated for a further 24 hours and inspected for newly apparent colonies, which were sampled and transferred to stocks on SMM + arginine agar slants. In all six separate cultures of W2-40 were subjected to irradiation procedures, and six samples of each were subjected to selection by pacesetting + penicillin, giving a potential of 36 new clones. In fact more clones than this were selected off the canavanine plates, as some colonies appeared smaller than others.

All clones were then replica plated on selective media in order to eliminate types which could not utilise acetylornithine or were "slow growers" on acetylornithine like W2-25/8 because of low enzyme activities (enzymes 6, 7 & 8). The selective plates were SMM supplemented with:

acetylornithine
acetylornithine + canavanine
ornithine
citrulline.

The master plate was supplemented with arginine. Only those clones which behaved like W2-250 on these media were selected for further study: i.e. they grew well on arginine, citrulline or ornithine; slowly on acetylornithine; and not at all on acetylornithine + canavanine, or on SMM only.

2. Enumeration of New Isolates:

All new isolates have the pre-title "W2-40" having been derived from this same strain. The next symbol, in the code indicates which ultraviolet treatment the mutant originate in, and the following symbol which of the six samples from that treatment the mutant was selected from using pacesetting/penicillin and canavanine procedures. The final symbol, where present, indicates which clone the mutant was, if more than one clone was selected off any one dilution set. Thus the new strain W2-40/24-B was produced in the second ("2") mutagenesis treatment, was selected for in the fourth ("4") sample therefrom, and was the second B clone isolated from the dilution sets for that sample on selective agar.

C. Screening of New Isolates by Enzyme Assay and Growth Studies:

1. Bacterial Strains:

In addition to those standard strains previously mentioned, the following which were obtained from the personal stocks of Professor Bacon, were also used in the enzyme assays:

E. coli -W2 ..... pro^-
E. coli -W2-R2 ..... pro^- $argR^-$
E. coli -W-160-37D2 .. arg_5^-

2. Materials:

a. Protein Assay:

Alkali Reagent: 2% Na_2CO_3 in 0.1 M NaOH.
Copper Reagent: 0.5% $CuSO_4$ in 1.3% NaK-tartrate ($4H_2O$).
Phenol Reagent: one volume of Folin-Ciocalteau Reagent to two volumes of distilled water.
Standard protein: 1.0mg/cm^3 Bovine Serum Albumin (aqueous) stored frozen in 1cm^3 aliquots until needed.

b. Acetylornithinase Assay:

0.1M potassium phosphate buffer (pH 7.0) which is 1.0M with respect to glutathione.
L-N^{α}-acetylornithine (3 x 10^{-2}M) aq frozen in storage.
L-ornithine (1 x 10^{-3}aq) " " "
Cobaltous chloride (1.0 mM)
Ninhydrin Reagent: 2 volumes of 1% ninhydrin in Methyl-Cello solve + 1 volume of 0.4 M aqueous citric acid (mixed immediately before use).
Alkali: 0.7 M aqueous NaOH.

c. Potassium phosphate-glutathione diluent:

0.1M K-phosphate buffer (pH 7.0) which is 0.1mM with respect to glutathione.

d. Glutathione was stored as frozen stock solutions to be added to buffers as required, but these were freshly prepared every third day:

Diluent: 10^{-1}M (30.1mg/cm^3)
Enzyme Assay: 4 x 10^{-3}M (1.23mg/cm^3)

3. Methods and Results:

a. Preparation of Cells:

All strains were prepared as required in arginine ($30ug/cm^3$) supplemented overnight media (10^{-5} dilution of stationary phase cells used to inoculate overnight cultures) - 12 hours at $37^{\circ}C$. This was used as inoculum for the growth phase in Klett-Summerson tubes containing $10cm^3$ of suitably supplemented SMM. These were incubated until the optical density of the cultures had reached 50-55 Klett-Summerson units; then chilled and harvested at $0-5^{\circ}C$ in a Sorvall RC2-B refrigerated centrifuge (5 minutes at 12,350 g.[a]), and resuspended in $1cm^3$ phosphate-glutathione diluent per culture.

These were either sonicated, then assayed immediately or frozen. Fresh glutathione was added to all frozen cell sonicates before they were assayed. (The decrease in activity of cellular acetylornithinase is negligible in frozen samples (Professor Bacon, personal communication)). (Standard strains were included for comparison in preparations for screening batches of new strains.)

b. Sonication Procedure:

Harvested cells in phosphate buffer/glutathione diluent were transferred to chilled 2 x 1 inch sonication tubes fitted with special caps, and then subjected to sonication using an MSE 100W Ultrasonic Disintegrator with chilling at all stages using ice.

Initially only the standard strains were sonicated, with variations on the time of treatment and the type of probe needed to achieve optimum enzyme activity, as this did not appear to be detailed in any of the literature or in the sonicator manual.

The results indicated that the largest probe (25925-Tit-318A) gave the most efficient release of the enzyme, and that a single 15 second exposure at maximum

[a]SM-24 rotor

amplitude (8.0-8.5mu) appeared to be the optimum time. (See Baumberg (7)), although the latter did not appear to be very critical.

It is apparent from the results of the acetylornithinase assays (next section) that some part of the system was not functioning properly and I feel that it was largely due to the sonication procedure. This is probably because I failed to find a satisfactory standard procedure for the sonication step, and this was no doubt due to the trial and error search for it, the time for which was somewhat limited, and was aided only slightly by the literature.

c. Protein Assay:

This was done using the method of Lowry et. al (44).

Protein standards were prepared with Bovine Serum Albumin over the range of 0-250ug/cm^3, and gave linear proportionality.

0.1cm^3 of cell sonicate was assayed for protein, and was expected to approximate 100ug/cm^3 in each sample, two BSA standards being included with each set of sonicates (100ug/cm^3 and 150ug/cm^3).

Samples were measured with the Klett-Summerson colorimeter using the red filter.

d. Acetylornithinase Assay:

Acetylornithinase: α-N-acetyl-L-ornithine amido-hydrolase: EC 3.5.1.16.) activity in cell sonicates was assayed by the method of Vogel & Bonner (66) under the following <u>standard conditions</u>; and always included reference strains: the assay was performed in 5 x ½ inch test tubes containing: 0.1cm^3 of 0.1M potassium phosphate buffer at pH 7.0 (50 umoles); 0.1cm^3 of N$^{\alpha}$-acetylornithine (3 umoles); 0.1cm^3 of $CoCl_2$ (0.1 umoles) 0.1cm^3 of glutathione (0.1 umoles), to which was added 0.1cm^3 of enzyme (as cell sonicate) after prewarming to give a total volume (in aqueous solution) of 0.5cm^3. Standard time was 10 minutes at 37°C in a water-bath with aluminium foil caps on the

tubes. The reaction was halted by the addition of the ninhydrin reagent.

The contents were finally transferred to Klett-Summerson tubes and read against a reagent blank at 350-490 mu (No. 42 filter).

Each assay included:

O - reference blanks (no enzyme, no substrate)

C - enzyme blanks (acetylornithine added after incubation)

A - substrate blanks (no enzyme)

B - ornithine standard (0.1 umole = 10 units/cm^3) (enzyme, no substrate)

D is the reaction tube

Calculation of enzyme activity:

(OD of B) - (OD of A) = a

(OD of D) - (OD of C) = b

$\frac{b \times 10}{a}$ = c = units of activity/cm^3 in tube D

Value "c" is then corrected to units/mg of protein using protein assay data.

A unit of acetylornithinase activity is defined as the amount of enzyme which will catalyse the formation of one micromole of L-ornithine under standard conditions.

The results of assays of standard strains are shown in table V.

Table V

"Acetylornithinase Activities of Standard Strains"

Strain	Repressed Supplement A	Activity A	Range	Derepressed Supplement B	Activity B	Range	Ratio $\frac{B}{A}$
W2	Arg	5.5	(1.4-8.4)	-	17.4	(17.3-17.5)	3.16
W2-40	Arg	5.2	(0.7-13.0)	AcOrn	12.2	(3.0-26.0)	2.39
	Orn	10.4	(6.3-12.5)				1.17
W2-250	Arg	6.0	(1.4-12.4)	AcOrn	8.1	(2.1-13.5)	1.39
	Orn	8.0	(one sample)				c. 1.00
W2-25/8	Arg	4.2	(1.6-9.8)	-	28.2	(20-47)	6.57
	Orn				38.3	(one sample)	c. 1.00
W2-R2	Arg	28.5	(one sample only)		32.5	(one sample only)	c. 1.00 ($argR^-$)
W-160-37-D2	Arg	0.00					arg_5^-

It is apparent from these results that the activities of these various strains with respect to acetylornithinase are substantially less[a] than other literature on this aspect of this topic (see Appendix - Table A3).

In attempts to iron out this problem various parameters of the assay were tested in addition to the sonication studies already mentioned (section b). Preparation of any of the additives for the assay tubes in a fresh batch each day did not improve the results, nor did extension of the incubation time, or the use of different buffers (NaK phosphate instead of Kphosphate) or different reducing agents. Attempts to improve cell disruption in order to release more enzyme included the combination of EDTA or toluene treatment with sonication, or separately and although toluene treatments (10 minutes incubation at 37°C) yielded no improvement, the EDTA actually caused a decrease in activity, probably because it bound the cobalt ions necessary for activity of the enzyme (66).

It was decided to take into account not only the actual repressed and derepressed acetylornithinase activities of the various strains for screening purposes, but also the ratio of derepressed activity:repressed activity, and whether or not the new strains showed the paceset premature stationary phase in arginine ($15ug/cm^3$)/ acetylornithine ($30ug/cm^3$) diphasic medium as did W2-250.

e. Pacesetting of new mutants:

This was done in the manner described for the standard strains, in section, using diphasic medium which causes premature stationary phase in W2-250.

[a]The activities varied from day to day with any given strain but I was unable to isolate any valid reason for this.

4. New Mutant Strains of Interest:

Of the new isolates only a few exhibited the ability to establish a premature stationary phase due to pacesetting in the same way as W2-250 or W2-25/8. These were W2-40/12B, W2-40/12C, W2-40/13B, W2-40/15B, W2-40/45C, W2-40/21B, W2-40/51B, W2-40/51D, W2-40/52C, W2-40/54C, W2-40/62B, W2-40/62C and W2-40/63A.

Of these strains which could be paceset, the following produced comparatively high repressed levels and comparatively low derepressed levels: W2-40/12B, W2-40/12C, W2-40/13B, W2-40/15B, W2-40/45C, W2-40/52D, W2-40/54A, W2-40/54C, W2-40/62B and W2-40/62C. (A total of 9 out of 66 clones selected from 36 penicillin selection runs). The other pacesettable mutants did not have these comparable enzyme activities, typically having low repressed levels.

There were also some other mutants, which although they were not paceset like W2-250 (even W2-250 failed to paceset on three occasions for no apparent reason), showed enzyme activities which were comparable with those of W2-250 and the strains mentioned above. These were strains W2-40/11A, W2-40/11B, W2-40/15A, W2-40/41A, W2-40/45B, W2-40/46, W2-40/56C, W2-40/61B, W2-40/61D, W2-40/63C, W2-40/63D, W2-40/64C, W2-40/66A, W2-40/66B, W2-40/65A and W2-40/65B. Some of these are probably sister clones of the most likely strains as mentioned in the second paragraph above (e.g. W2-40/15A).

II. Conclusions:

It is evident from the results of the final section of the experimental work that I would be unjustified if I claimed that I had succeeded in isolating further arginine regulatory gene mutants of the type argR as found in *E. coli* W2-250. The reasons for this are discussed in the relevant sections, and I must reiterate my belief that the fault lies in the sonication procedures. This view is supported by the fact that the various blank tubes prepared for the enzyme assays gave optical density readings of the expected magnitude (Bacon; personal communication, Vogel & Bonner (66)). Further to this the growth rates of the various standard strains were as expected in the media in use (Bacon, personal communication).

In spite of this I do feel that I have probably succeeded in isolating some new mutants which show growth characteristics in diphasic media like those of strain W2-250, and appear to have abnormal acetyl-ornithinase levels in repressive and non-repressive media, all of which suggests the possibility that they may be argR mutants. Even with a successful assay however, it would be necessary to assay the activity of some of the other arginine biosynthetic enzymes under similar conditions, and probably also to map the sites of the mutations responsible in the new strains; such that it could be definitely proved that any, or all of these new strains are of the same type as W2-250. Time did not permit me to progress this far, as delays were caused by the difficulties with the sonication procedure and by the fact that none of the strains selected for growth studies would tolerate the presence of sucrose (38) as an osmotic support in the penicillin selection procedures as originally proposed by Gorini (23).

The studies on pacesetting provided an interesting basis for further analysis. This was the apparent ability of some "pacesetting factor" to be transferred

from a culture medium in which cells had entered a premature stationary phase caused by pacesetting, into a culture where the cells were genetically derepressed, thus causing such cells to be paceset in to a stationary phase also, despite the absence of arginine in their medium. The first studies of this were with strain W2-250 which has acetylornithine supplement in derepressed cultures, suggested that the acetylornithine permease enzyme seemed to be subject to repressive effects from the first phase growth in arginine, a feature attributed to a "memory effect" by Vogel (59) who also noted that this could be relieved by dilution into fresh medium. But the apparent involvement of the acetylornithine permease cannot be applied to the strain W2-25/8 which can synthesize its own arginine supply in minimal medium (at a slow rate), and yet shows marked pacesetting effects, especially when citrulline is present in the second phase. All of this points to another idea from Vogel (60) in which he suggests that some component of the enzyme forming system decreases as the length of time in arginine increases, thus implying a decrease in the number of functional enzyme forming sites per cell. This seems hard to reconcile with the rapid recovery of completely stationary paceset cells when diluted into fresh medium; how can they suddenly develop enough enzyme forming sites in functional condition to suddenly begin almost normal growth in acetylornithine when diluted 1:10 from such an "abnormal" stationary phase? Vogel (60) states that the cell density does not appear to be a factor in second phase growth rates, so that this possibility can be discounted. However neither of these models explain my observation of the transferability phenomenon, and I am tempted to invoke some "external" influence; either the lack of something essential in the medium (which cannot be provided by the simple addition of more acetylornithine) or the presence of some inhibitory factor which increases in the presence of arginine. In the latter case one would be faced with the interesting possibility of

intercellular "communication". The former case seems more likely and the increasing effect of pacesetting might possibly be easily abolished by some simple addition such as adjustment of the pH or extra Mg^{++}. This factor was certainly not acetylornithine, as a culture of W2-250 which had entered a premature stationary phase, caused either by pacesetting or a lack of arginine (see section 3g) could not be relieved from this stationary phase by the addition of acetylornithine ($30ug/cm^3$ extra) as long as the cells remained in the same medium at the same density. Nevertheless the effect of the overnight medium supplement, particularly when it is repressive like arginine or citrulline, cannot be ignored. This suggests that some intracellular effect is present, because an inoculum derived from such a repressive medium, and despite washing in unsupplemented minimal medium, will show the effects of such an origin in its subsequent growth in acetylornithine or minimal medium, and even in diphasic media involving first phase growth in arginine. (Results compared with non-repressive overnight medium for inoculum) (see section A3).

Table IA

Arginine Biosynthetic Pathway:-

Basic Steps (68)

Step	Reaction catalysed	Gene	Map Position	Ref.
1.	L-glutamate → N-acetyl-L-glutamate	A	54"	(73)
2.	N-acetyl-L glutamate → N-acetyl-L-glutamyl phosphate	B	77"	(73)
3.	N-acetyl-L-glutamyl phosphate → N-acetyl-L-glutamic-γ-semialdehyde	C	77"	(73)
4.	N-acetyl-L-glutamic-γ-semialdehyde → N^{α}-acetylornithine	D,T	64"	(73)
5.	N-acetyl-ornithine → ornithine	E	77"	(73)
6.	ornithine → citrulline	F	5"	(73)
7.	citrulline → L-arginino-succinate	G	61"	(73)
8.	arginino-succinate → arginine	H	77"	(73)
	Other Associated Genes:			
	Repression control of the arginine biosynthetic pathway via a repressor protein product	R	62"	(73)
	An arginine inducible enzyme 4, or second Rgene	M,4'	73"	(53)
	An arginine repressible ornithine transcarbamylase reported in K-12 (21) & B (19)	I	84"	(21,19)
	arginyl-t-RNA-synthetase	S	35"	(73)
	arginine permease	P	57"	(73)

Table IIA (68)

Enzymes of Arginine Biosynthesis in Escherichia coli.

Step	Enzyme	
	Common Name	Systematic Name
1.	N-acetylglutamate synthetase	Acetyl-CoA:L-glutamate N-acetyl transferase (EC 2.3.1.1.)
2.	N-acetyl-γ-glutamo-kinase	ATP:N-acetyl-L-glutamate-5-phosphotransferase
3.	N-acetylglutamic-γ-semialdehyde dehydrogenase	N-acetyl-L-glutamate γ semialdehyde:NADP oxidoreductase (phosphorylating)
4.	Acetylornithine - δ - transaminase	α-N-acetyl-L-ornithine:2-oxoglutarate aminotransferase (EC 2.6.1.11)
5.	Acetylornithinase	α-N-acetyl-L-ornithine amidohydrolase (EC 3.5.1.16)
6.	Ornithine transcarbamylase	Carbamoyl phosphate:L-ornithine carbamoyltransferase (EC 2.1.3.3)
7.	Argininosuccinate synthetase	L-citrulline:L-aspartate ligase (AMP) (EC 6.3.4.5)
8.	Argininosuccinate lyase	L-argininosuccinate arginine-lyase (EC 4.3.2.1)

Table A-III

"Some Acetylornithinase Activities"

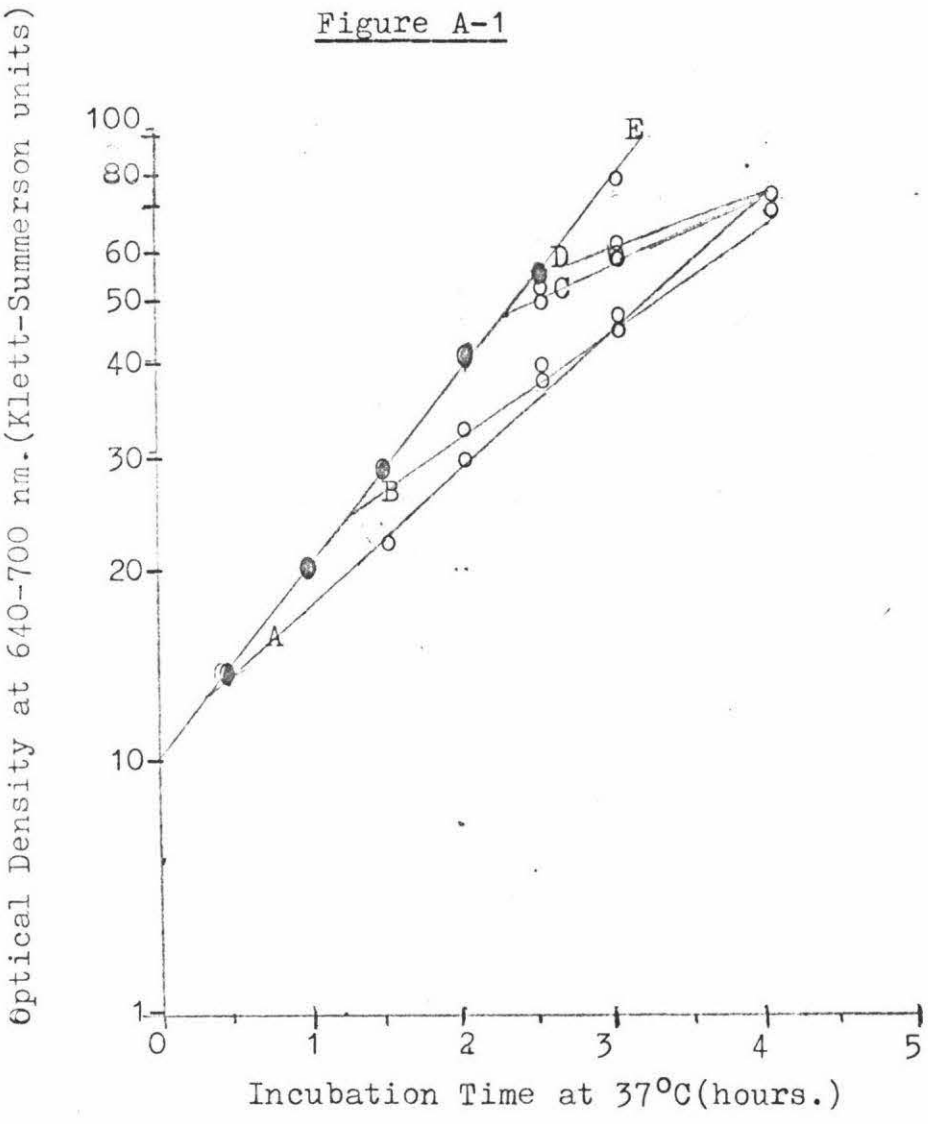
Strain	Acetyl ornithinase activity Repressed	Poised	Derepressed	Units	Reference
39A-23R3	11	-	119	units/mg	(59)
K-12	0.08	0.20	1.00	relative	(61)
W-wild	10	37	-	units/mg	(5)
B-wild	3.34	4.35	-	units/mg	(36)
39A-23R1	8.3	-	1.0	relative	(58)
K-12	0.8	21.0	-	units/mg (total dry weight)	(25)
Wc2 ($argR^-$)	120		125	as above	(25)
39A-23R3	15	39	100	relative	(9)

The data here are for comparison with those in section C, table V page 36, with particular respect to the values shown by the W strains: 39A-23R1 and 39A-23R3, W wild type and Wc2. I expected my strains to produce the following acetylornithinase activities: in units/mg of protein:

Strain	SMM + AcOrn	SMM + Arg
W2-40	100	10-15
W2-25/8	120-130	10-15
W2-250	70-80	20-30

Data: personal communication with Professor Bacon.

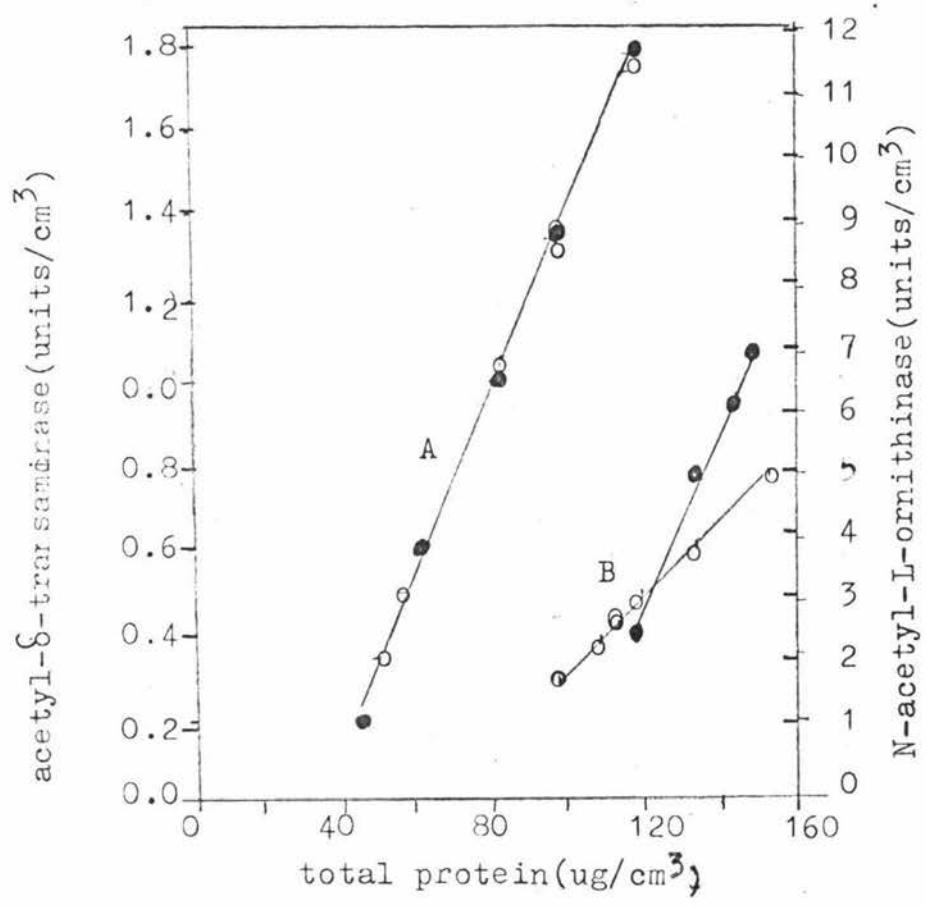
Figure A-1



"Diphasic growth of strain W-39A-23 on mixed supplements of N^{α}-acetyl-L-ornithine(50 ug/cm^3) and L-arginine hydro-chloride
A; 2 ug/cm^3 B: 4 ug/cm^3 C: 8 ug/cm^3 .
D: strain W-39A-23R3 in acetylornithine(50 ug/cm) and arginine at 15 ug/cm^3
E: both strains in an excess of arginine (50ug/cm^3):solid symbols represent points common to all curves.

Adapted from Vogel:(1960 figure 1) ref:59
:(1961 figure 3) ref:61

Figure A - I.



Differential rates of formation of acetylornithinase and acetylornithine-δ- transaminase :adapted from Vogel(60,61)

A - early onset of derepression.

B - late onset of derepression.

o - acetylornithine transaminase.

o - acetylornithinase.

This figure shows the slower rate of formation of acetylornithinase compared with that of the transaminase when a culture enters the second phase of growth in a diphasic medium after a longer phase in arginine than the cells represented by line A in the figure.This appears to be the basis of the pacesetting phenomenon, at the physiological level,and cuold be explained by the proposed difference in the repressive complexes of the two enzymes(ref.19)

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