COMPETITION BETWEEN STRAINS OF BACTERIA WITH CONTROLLED AND UNCONTROLLED OPERONS

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Abstract

The presence of operon control in bacteria must presumably be due to some selective advantage conferred on organisms possessing it. Four strains of *E. coli* were prepared, two with normal operon control for the lactose operon and two i^- mutants constitutive for the production of the enzymes encoded on the lactose operon. The growth kinetics of the strains in pure culture in minimal media variously supplemented with glucose or lactose were analysed by two methods, the fitting of the parameters of Monod growth curves and the statistical analysis of initial logarithmic growth ratios. Both methods gave rather inconclusive results, although one operon-controlled strain showed a significantly lower initial logarithmic growth rate in lactose than either of the constitutive mutants, which seemed to be corroborated by the appropriate Monod growth curves. In a minimal medium supplemented with both glucose and lactose, the operon-controlled strains showed noticeable diauxy caused by the inhibition of lactose uptake by glucose, while the constitutive mutants showed little or no diauxy. When operon-controlled strains were competed with constitutive mutants in the aforementioned glucose, lactose and glucose + lactose supplemented minimal media, the operon-controlled strain had a significant selective advantage in the glucose medium, whereas the constitutive mutant had a similar selective advantage in the mixed-carbohydrate medium and an apparently greater advantage in the lactose medium. Calculations of the relative differences in growth rate necessary to bring about such rates of selection showed that a relative difference in intrinsic growth rate large enough to explain the observed rate of selection could easily have passed unnoticed, or almost unnoticed, when the strains were grown in pure culture. Computer simulations of the competition experiments using the Monod parameters fitted for the strains in pure culture suggested rather similar conclusions. There is thus no evidence against the proposition that the selective advantages observed are explicable in terms of differences in intrinsic growth rate rather than being interactively generated. It is suggested that the selective advantage of operon-controlled strains in lactose-free media is due to the energy savings made possible by not producing superfluous lactose enzymes, while the selective advantage of constitutive mutants in the presence of lactose may be due to residual repression of the lactose operon in operon-controlled strains. It was impossible to assess from the available data how far the advantage of the constitutive mutant in the mixed medium was due to its lower sensitivity to glucose inhibition of lactose uptake.

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Competition between strains of bacteria with controlled and uncontrolled operons

Introduction

The presence of operon control in prokaryotes has not unnaturally prompted the question as to what selective advantage might arise from possessing it. Presumably such a selective advantage does exist, since constitutive mutant strains, which lack the ability to control some particular operon, seem to be the exception rather than the rule in nature. Considering the fact that operons tend to be demand-responsive, usually either being induced by a substrate of some enzyme coded for in the operon or repressed by a product of such an enzyme, an obvious guess would be that it has the effect of preventing the waste of energy involved in producing superfluous enzyme; and that this allows diversion of energy in other directions, such as production of non-superfluous enzymes, where it will be more effective in maximising the fitness of the strain, either through intrinsic reproductive rate or through resistance to stress. Selection against types using energy or other resources in redundant activities is, of course, a very common phenomenon in evolution in general, being the usual mechanism invoked to account for the 'degeneracy' observed in parasitic organisms as well as for other less extreme cases.

The operon selected for study in this work was the lactose operon of *E. coli*, about which more is probably known, to date, than any other operon. The operon is de-repressed by the substrate, lactose, itself, and includes cistrons for β-galactosidase, which breaks down the lactose into glucose and galactose, lactose permease, which increases the permeability of the cell membrane to lactose, and thiogalactoside trans-acetylase. It was decided to study two related strains of *E. coli* K 12, one possessing the full 'wild type' ability to control the lactose operon, the other being a mutant producing constitutively the enzymes coded for on that operon.

The main aims of the work presented here can be summarised as follows:

i) To test for the presence of a selective advantage arising from the control of an operon, in a situation where the products of the genes in the said operon are redundant, by observing the competition between strains with controlled and uncontrolled operons.

ii) To check the tenability of the hypothesis that this advantage, if it exists, arises from the economy of switching off redundant activity, by investigating the selective advantages (or otherwise) of control of the operon where the products of the operon are not redundant.

iii) To investigate how far (if at all) such a selective advantage is predictable from the population kinetics of the strains when grown in pure culture. This question was considered of interest owing to the fact that not all selective advantages possessed by one bacterial strain with respect to another are predictable from the growth kinetics of the two strains observed in pure culture. Population changes frequently occur in microbial cultures, both in broth and on gelatinous media, as a result of 'ecological' factors such as the accumulation of an inhibitory metabolite to which different types are differentially sensitive, or the production by one type of an inhibitor specific to the other (Braun, 1965). It is to be expected that any sufficiently large fitness difference experienced in mixed culture due to internal economy alone, whether due to differences in intrinsic reproductive rate or in resistance to resource-shortage or pollution stresses arising from overcrowding, would be predictable from observation of the growth kinetics of the two strains in pure culture, qualitatively if not quantitatively.

iv) To look into the effect on fitness differences of the phenomenon of the glucose effect, specific to the particular operon under study. It is known that, when E.coli are placed in mixed media containing both glucose and lactose, the glucose decreases the permeability of the cell membrane to lactose (Magasanik, 1970). In bacteria with a controlled lactose operon, which have not been pre-induced by exposure to lactose, this prevents the synthesis of lactose permease as the lactose never induces the lactose operon in the first place. They are therefore unable to utilise the lactose for growth until all the glucose is consumed, giving rise to biphasic growth kinetics, or diauxy (see Figs. 3.1, 3.2, p.14). Constitutive mutants, and also bacteria of strains with operon control which have been pre-induced, are only partially inhibited in production of β-galactosidase, since they are already producing lactose permease, which partially counteracts the permeability-depressing effects of glucose.

Materials and methods

Bacteria In preparing the strains to be used in the competition experiments, two strains of E coli K12, strains 1510 and 1511, were used as starting material. 1510, with genotype F' met E^- his^- trp^- $lacI^+$ $lacZ^+$ $strA^-$ NalR , was auxotrophic for vitamin B1, methianine, histidine and tryptophan, resistant to streptomycin and nalidixic acid, and able to produce the enzymes encoded on the lactose operon facultatively. 1511, a constitutive mutant derived from 1510 by a single mutation, was of genotype F' $metE^-$ his^- trp^- lacI3 $lacZ^+$ $strA^-$ NalR , as for 1510 except for a single loco I3 mutation rendering it constitutive for synthesis of the enzymes encoded in the genes of the lactose operon. It was decided to use the locus for nalidixic acid resistance or sensitivity as a marker with which to distinguish members of two competing strains in mixed culture. It was suspected that there might be a fitness difference between nalidixic acid resistant and sensitive strains associated with the nalidixic acid resistant/sensitive locus itself. It has, indeed, been shown (Zamenhof, Heldenmuth and Zamenhof, 1966) that azide-resistant mutants are at a selective disadvantage in an azide-free medium, and it is to be expected that relatively rare resistance traits not present in the 'wild-type' E coli K12 must have some selective disadvantage in the absence of the poison to which they are resistant. In order to have a check on whether any fitness difference that appeared was associated primarily with differences in operon control or with differences in nalidixic acid sensitivity, it was decided to carry out two variants of each competition experiment, one in which nalidixic acid resistance was used as a marker for the strain with normal operon control and one in which nalidixic acid resistance was used as a marker for the constitutive mutant. It was therefore necessary to prepare four strains, viz: a nalidixic acid resistant strain with normal operon control, a nalidixic acid sensitive strain with normal operon control, a nalidixic acid resistant constitutive mutant and a nalidixic acid sensitive constitutive mutant. Since the strains 1510 and 1511 were already nalidixic acid resistant, it would not have been feasible to generate the strains required by point mutation, and it was accordingly necessary to generate them by mating the F' strains 1510 and 1511 with the HFr strain K116, which is streptomycin sensitive and has no amino acid auxtrophies. The progeny of both matings was selected for

histidine-synthesising streptomycin-resistant recombinants, and these recombinants were then grid-plated and tested by replica plating for tryptophan prototrophy/auxotrophy and nalidixic acid resistance/sensitivity. The four strains required, henceforth to be denoted 1510 H^+ NalR , 1510 H^+ NalS , 1511 H^+ NalR and 1511 H^+ NalS respectively, were all originated from single colonies chosen from the set of tryptophon-auxotrophic recombinants in order to eliminate selection associated with tryptophon auxotrophy/prototrophy.

Media In competition experiments it is advisable to use media as simple as possible in order to minimise the number of ecological factors affecting selection between the two strains. It was also considered advisable in this case to have a medium with its main energy source sufficiently dilute to be a significant limiting factor on growth rate, so that energy economy would be at a premium with respect to fitness. At the same time, it was necessary to have a great enough concentration of energy source in the medium to allow growth rates to be high enough for differences to show over the period of time (in the order of fourteen days) that was likely to be available for the main competition experiments. Moreover, the concentration of energy source in the medium initially prepared is only an upper bound for the concentration in the medium when bacteria are growing in it, both in batch and in continuous culture. In batch culture on a daily basis, where an innoculum of the previous day's culture is transferred to new medium each day, the concentration is likely to pass through the whole range of values from the initial concentration to zero if the initial concentration is not so high that the energy supply is not exhausted before the end of 24 hours.

It was decided to use a concentration of energy source which might not be significantly limiting in itself, but would probably be reduced to a more limiting level as the organisms consumed the energy source. The initial concentration was roughly equivalent to 18 millimolar for the carbohydrate provided.

The media used were all based on a phosphate-buffered minimal medium.

This was supplemented with the amino acids tryptophan and methionine and the vitamin B1 (thiamine), for which the four strains used were all auxotrophic, at concentrations too high to be limiting, a dose of streptomycin recommended to destroy all streptomycin-sensitive strains (the four strains used were all resistant), and carbohydrate specific to the medium. Three media were prepared, differing only in the specific carbohydrate added, one being supplemented with glucose, another with lactose, and another with a mixture of the two in proportions known, from the literature, to cause diauxy in E. coli with operon control for the lactose operon (Leahy, 1969). The exact compositions of the media (usually prepared in 61.45 cm^3 batches) were as follows:

Ingredients common to all media

Phosphate-buffered minimal medium ($\frac{56}{2}$)	60 cm^3
0.1% vitamin B1 (thiamine) solution	0.05 cm^3
1.0% methionine solution	0.3 cm^3
1.0% tryptophan solution	0.6 cm^3
Streptomycin solution	0.3 cm^3
	61.25 cm^3

Ingredients specific to each medium

1. Glucose medium

20% glucose solution	0.2 cm^3

2. Lactose medium

20% lactose solution	0.2 cm^3

total volume: 61.45 cm^3 per batch

The diauxy medium was prepared by combining the two above media in proportions of 75% lactose medium, 25% glucose medium.

Culture and sampling methods Cultures were kept in sloping tubes in a 37° C water bath, shaking at speed 65 as indicated on the control dial. The main competition experiments utilised a batch culture method, changing the medium on a daily basis by transferring an innoculum from the previous day's culture each day to a test-tube of fresh medium. Tubes of fresh medium were prepared with 8 cm^3 of the medium desired. At the commencement of the competition experiments, the tubes were innoculated with 0.2 cm^3 of innoculum of each of the two strains, taken from pure overnight cultures in the glucose medium. Subsequently, the change of medium each day was performed by transferring 0.2 cm^3 of innoculum from the previous day's competition tube into a tube of fresh medium. Whenever it was going to be impossible to change the medium on the next day, as happened on Sundays, the culture tube was placed in a refrigerator at 5° C , and the medium changed a day later than was usual.

Samples were taken from the first culture tube immediately after the initial innoculation and seven hours later, and subsequently samples were taken each day from the previous day's culture tube, one hour after the transfer of innoculum to the frsh medium. Since the cells by then were presumably already in stationary phase, this delay was considered not to make a great deal of difference to the proportions of each strain present. For the zero time sampling, samples were diluted in minimal medium by serial dilution to a 10^{-4} dilution, before spreading 0.1 cm^3 on to the plate. Subsequent samples were diluted to a 10^{-5} dilution and spread on to plates in quantities of 0.05 cm^3. Diluted samples were plated on to Luria broth plates initially. To assess the proportion of nalidixic acid resistant individuals, it was initially proposed to grid plate colonies from each Luria broth plate on to a second Luria broth plate supplemented with nalidixic acid. With twelve grid plates to make every day (2 competition mixtures x 3 media, each treatment duplicated), this technique consumed too much time in practice to be considered viable in the long term, and was replaced by replica plating for samplings taken from the third day onwards.

Pure culture population dynamics work In pure culture, cell density was estimated by taking optical density at 660 mμ and subtracting optical density of a sterile blank of the medium in which the culture was growing. Initial innoculum for pure culture work was taken in 0.2 cm^3 volumes from fresh overnight cultures in the medium to be used in the pure culture in the experiment. Pure cultures were grown in tubes containing 8 cm^3 of medium in water baths at temperature and shaking speed as for mixed culture.

Population kinetics of the strains in pure culture

The Monod equations describing the growth of bacteria in batch culture in media where one nutrient is likely to become limiting and the rest are present in excess (see Appendix I) can be shown in a simplified form as

$$\frac{dB}{dt} = Br_{max} \frac{(\hat{B} - B)}{Ky + \hat{B} - B} \qquad (1)$$

where B is bacterial density, $\hat{B}$ is the eventual bacterial density attained when all the 'limiting' nutrient has been exhausted, r_{max} is a maximal logarithmic growth rate reached asymptotically for very high concentrations where concentration of the 'limiting' nutrient ceases to be limiting, y is a yield constant representing the amount of bacterial biomass produced per unit 'limiting' nutrient consumed, and K is a constant. While K is usually interpreted as the Michaelis constant for the permease, or some other enzyme, involved in the uptake of the 'limiting' nutrient, it is obviously unjustifiable to make such precise assumptions here.

Pure cultures of the four strains 1510 H^+ NalR, 1510 H^+ NalS, 1511 H^+ NalR and 1511 H^+ NalS were grown up in each of the three media, and density levels estimated colorimetrically, as described above. The results are shown in Figs. 1, 2 and 3, the curves for growth in pure media (Figs. 1 and 2 , solid lines) being fitted by a computer program working out the best-fit stepwise numerical solution to equation (1) for each set of data derived from pure medium (see Appendix II).

Since equation (1) does not take lag phase into account, the curves were fitted to densities for times from 120 minutes, at which the lag phase can be seen to have ceased. For growth in mixed media, of course, equation (1) is meaningless since two different energy sources are used, both of which are likely to be limiting at different times.

The parameters $\hat{B}$, r_{max}, Ky and B_o (the original bacterial density) describing the fitted curves in Figs. 1 and 2 are set out in Table 1, together with the parameters K and y worked out from the equations

$$y = \frac{\hat{B} - B_o}{c_o} \quad ; \qquad K = \frac{Ky}{y} \qquad (2)$$

where c_o is the initial concentration of the nutrient likely to become limiting, which is arbitrarily considered here in units equal to the initial concentration of carbohydrate in the medium and therefore equal to one for all curves in Figs. 1 and 2 .

The parameters theoretically most likely to be affected by the economies made possible by operon control are r_{max}, the maximal growth rate, and y, the yield of bacteria per unit substrate (and, by implication, B, the eventual bacterial density). It will be noticed that, in glucose, values of r_{max} for both 1510-derived strains are greater than those for both 1511-derived strains, whereas, in lactose, values for r_{max} for both 1511-derived strains are greater than those for both 1510-derived strains. Since, by the Michaelis-Menten theory on which the Monod growth equations are based, r_{max} should be proportional to the amount of the relevant enzyme present (usually interpreted for bacteriological purposes as the permease responsible for uptake of the 'limiting' substrate), it is to be expected that, in glucose-based medium, r_{max} might be greater for the two 1510-derived strains than for the constitutive mutants, since the more energy is diverted into producing β-galactosidase and lactose permease, the less should be available for glucose permease. In the case of lactose, the data for 1510 H^+ NalR look decidedly anomalous, showing what look like biphasic kinetics and an eventual yield far higher than for any other in lactose (see Fig. 2.1), and lead one to suspect accidental contamination of the medium, invalidating

Table 1

Fitted parameters for the curves in Figs. 1 and 2

parameter	1510 H^+NalR	1510 H^+NalS	1511 H^+NalR	1511 H^+NalS
glucose medium				
B_o	0.0297	0.0266	0.0321	0.0220
$\hat{B}$	0.4040	0.3880	0.4010	0.3953
y	0.3744	0.3514	0.3689	0.3733
r_{max}	0.0163	0.0179	0.0158	0.0156
Ky	0.2140	0.2014	0.2147	0.2116
k	0.5716	0.3733	0.5821	0.5668
lactose medium				
B_o	0.0233	0.0413	0.0331	0.0328
$\hat{B}$	0.3450	0.2793	0.3040	0.2835
y	0.3217	0.2375	0.2708	0.2507
r_{max}	0.0139	0.0125	0.0161	0.0153
Ky	0.1830	0.1353	0.1476	0.1349
K	0.5687	0.5685	0.5451	0.5382

that particular result, and the small difference between parameter sizes in general between strains in each medium suggest that there is no certainty of any variation here not attributable to experimental error. A cursory analysis of variance performed on each of the parameters in each of the media showed no significant difference either between nalidixic acid resistant and sensitive strains or between 1510- and 1511-derived strains, except in the case of the parameter r_{max} for strains grown in lactose ($p < 0.01$ for variance ratio derived from differences between 1510- and 1511-derived strains, $0.01 < p < 0.05$ for variance-ratio derived from differences between nalidixic acid resistant and sensitive strains). Since the data for the 1510H^+NalR strain in lactose are very suspect, this significant result is likely to be invalid, and the apparent insignificance of other differences is not surprising in view of the fact that there was

Fig. 1 Growth of strains in glucose medium

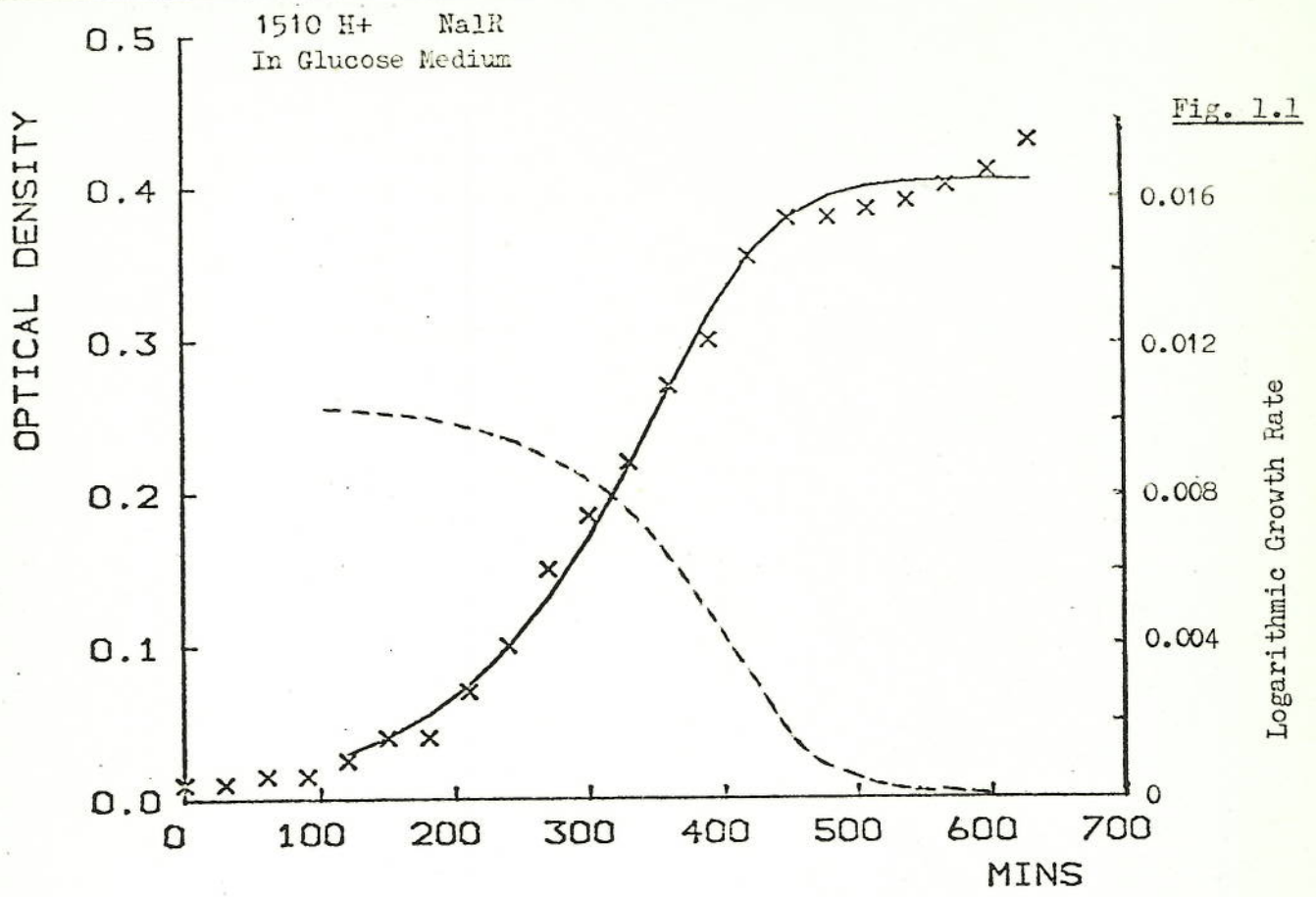


Fig. 1.1

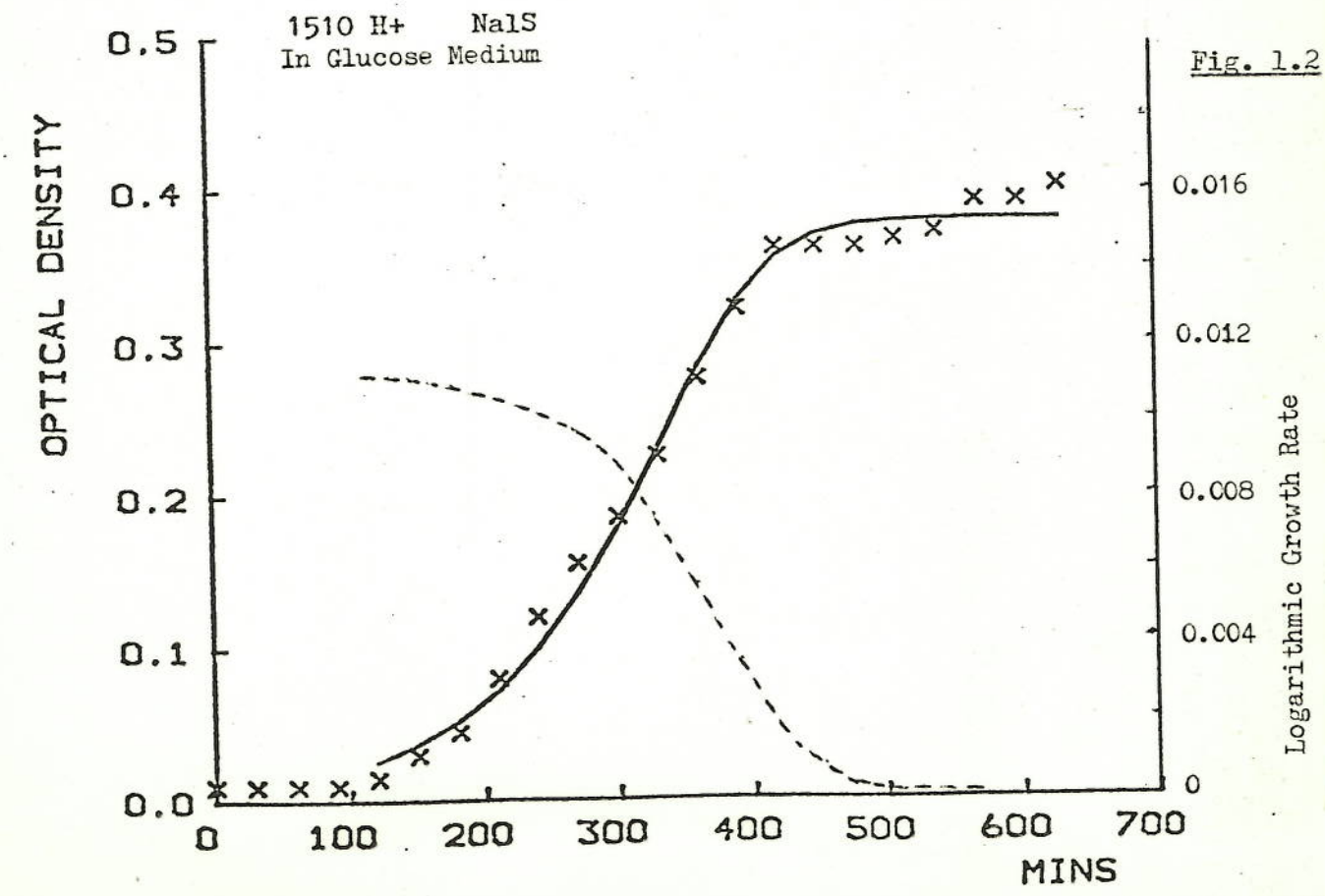


Fig. 1.2

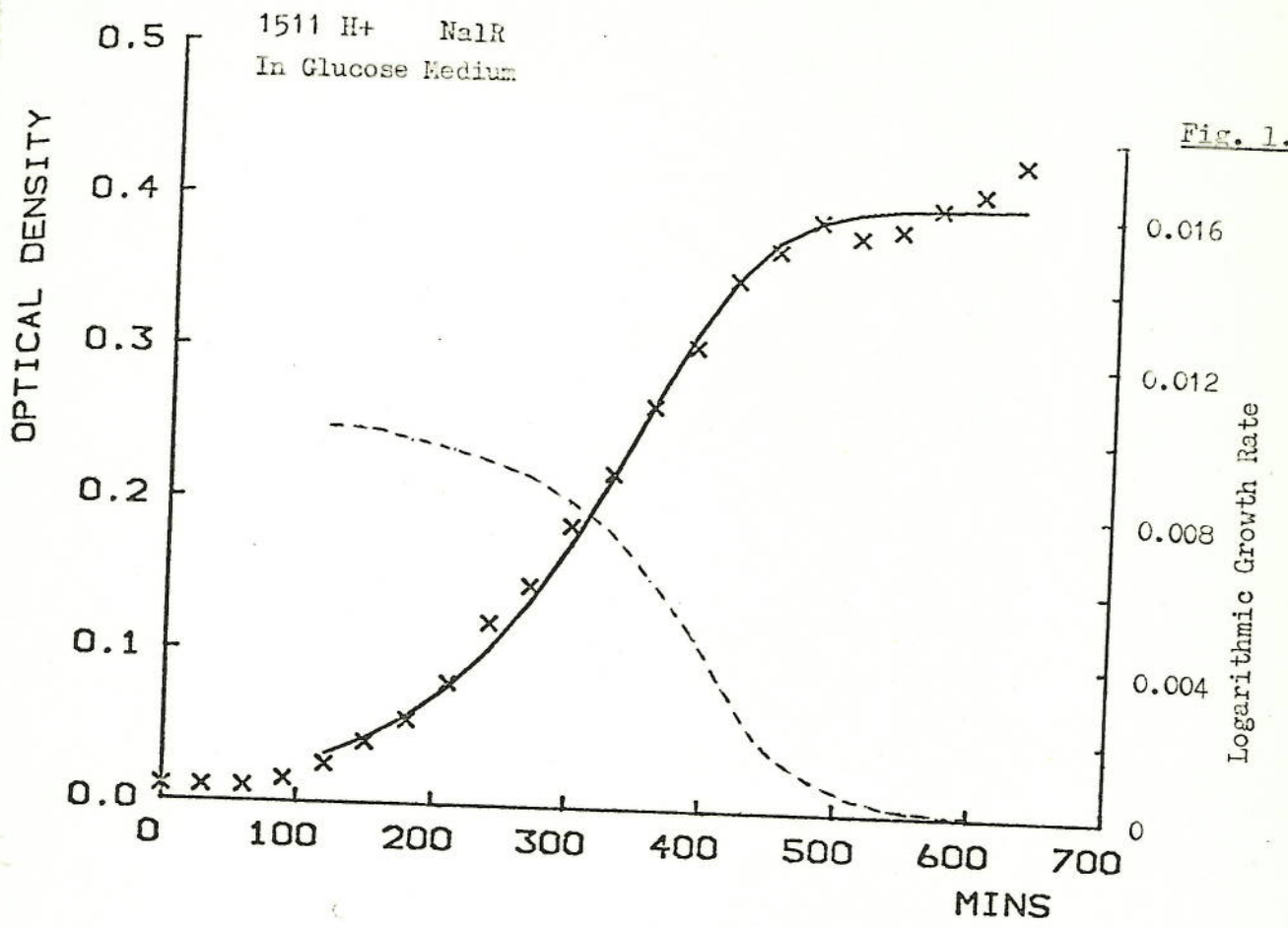

1511 H+ NalR
In Glucose Medium
OPTICAL DENSITY
0.5
0.4
0.3
0.2
0.1
0.0
0
100
200
300
400
500
600
700
MINS
0.016
0.012
0.008
0.004
0
Logarithmic Growth Rate

Fig. 1.3

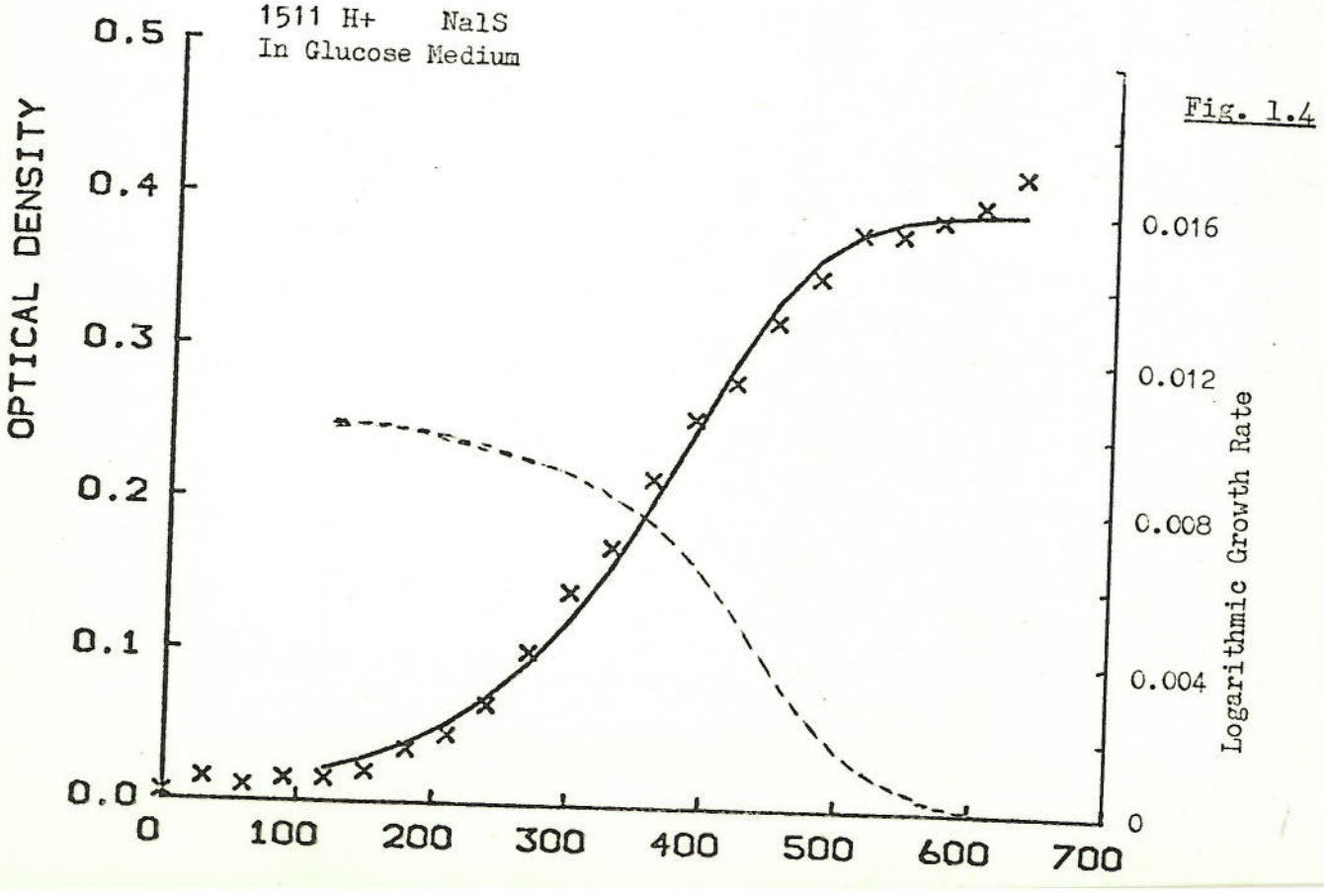

1511 H+ NalS
In Glucose Medium
OPTICAL DENSITY
0.5
0.4
0.3
0.2
0.1
0.0
0
100
200
300
400
500
600
700
0.016
0.012
0.008
0.004
0
Logarithmic Growth Rate

Fig. 1.4

Fig. 2 Growth of strains in lactose medium

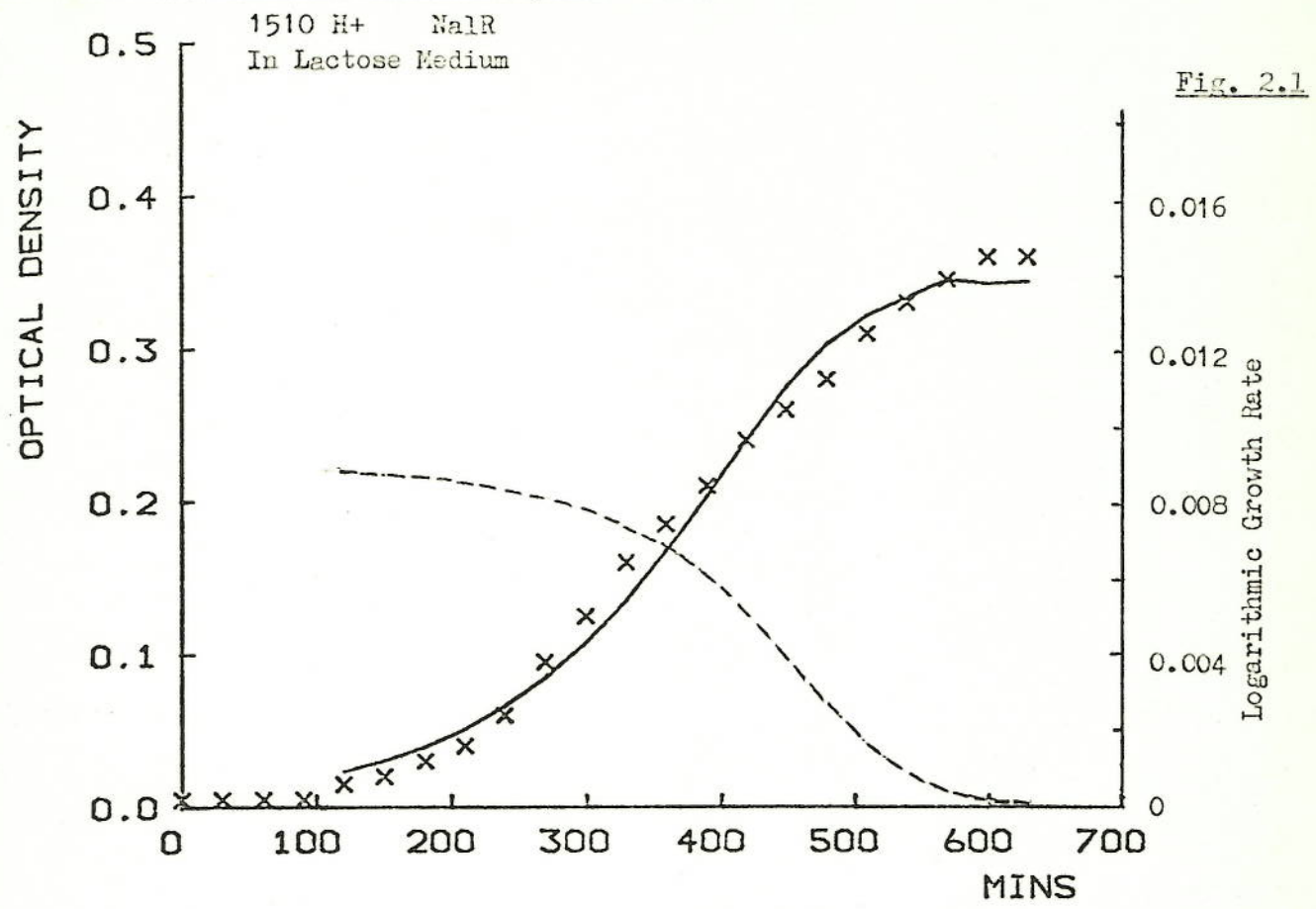


Fig. 2.1

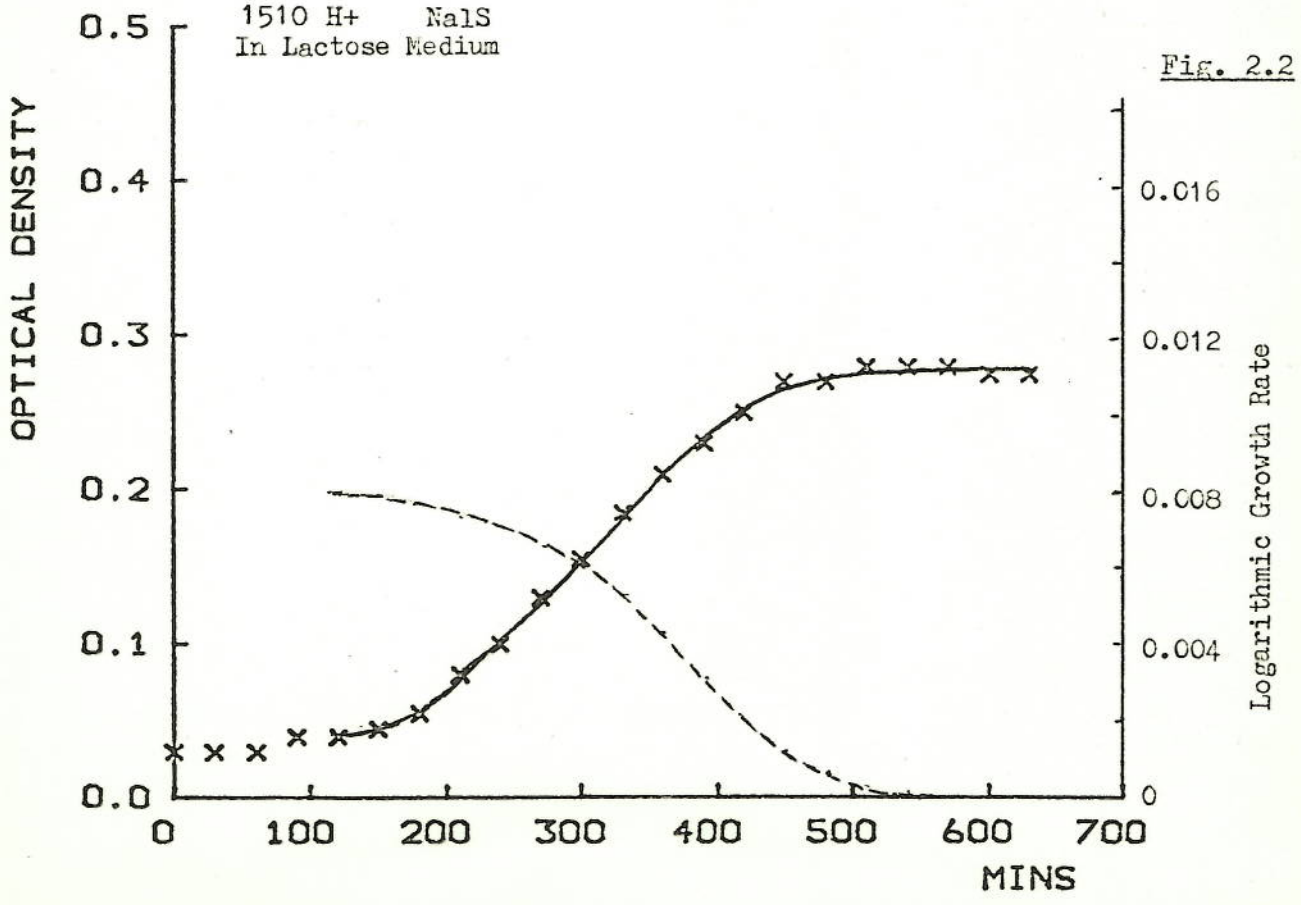


Fig. 2.2

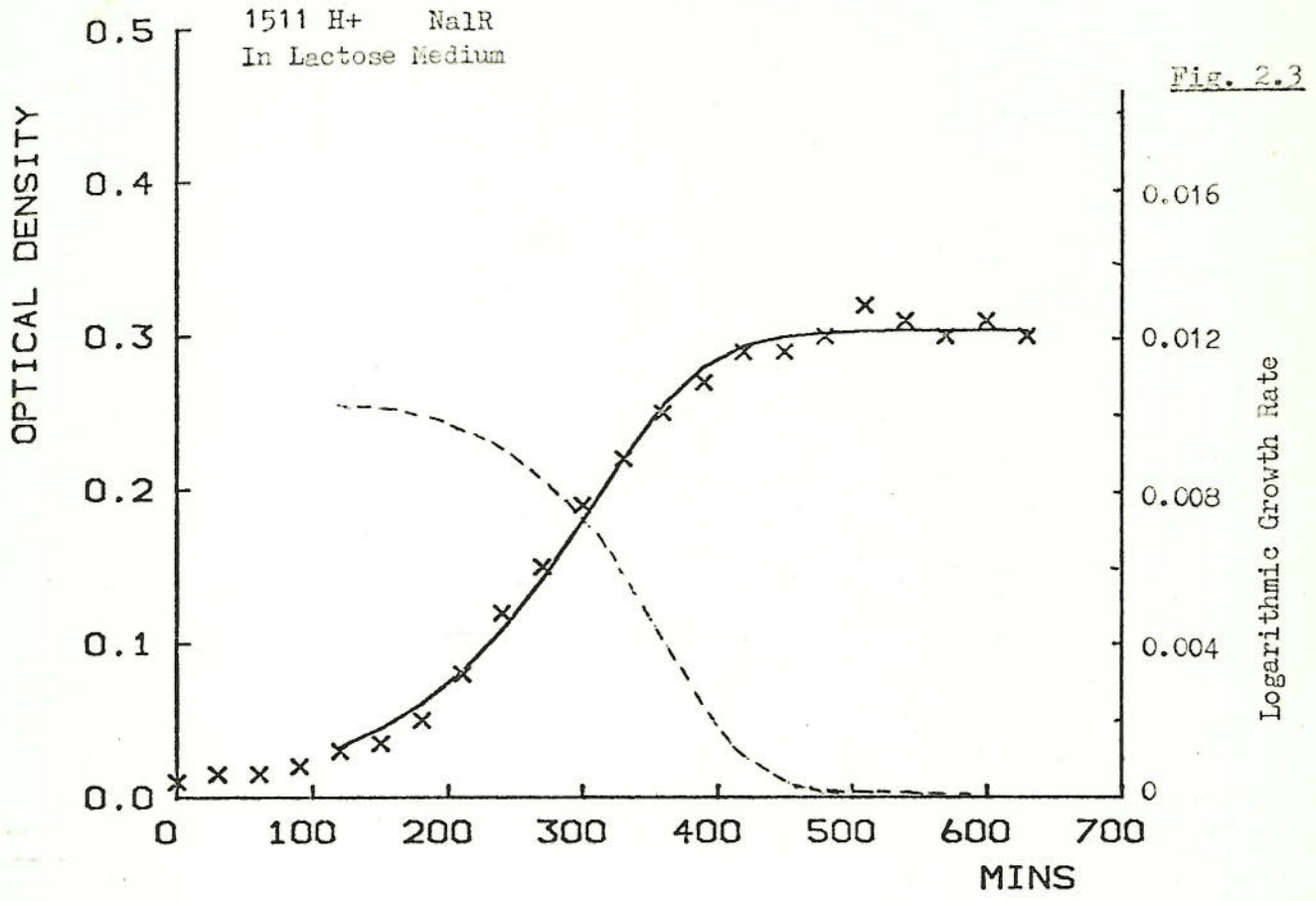

1511 H+ NalR
In Lactose Medium
OPTICAL DENSITY
0.5
0.4
0.3
0.2
0.1
0.0
0.016
0.012
0.008
0.004
0
Logarithmic Growth Rate
0
100
200
300
400
500
600
700
MINS

Fig. 2.3

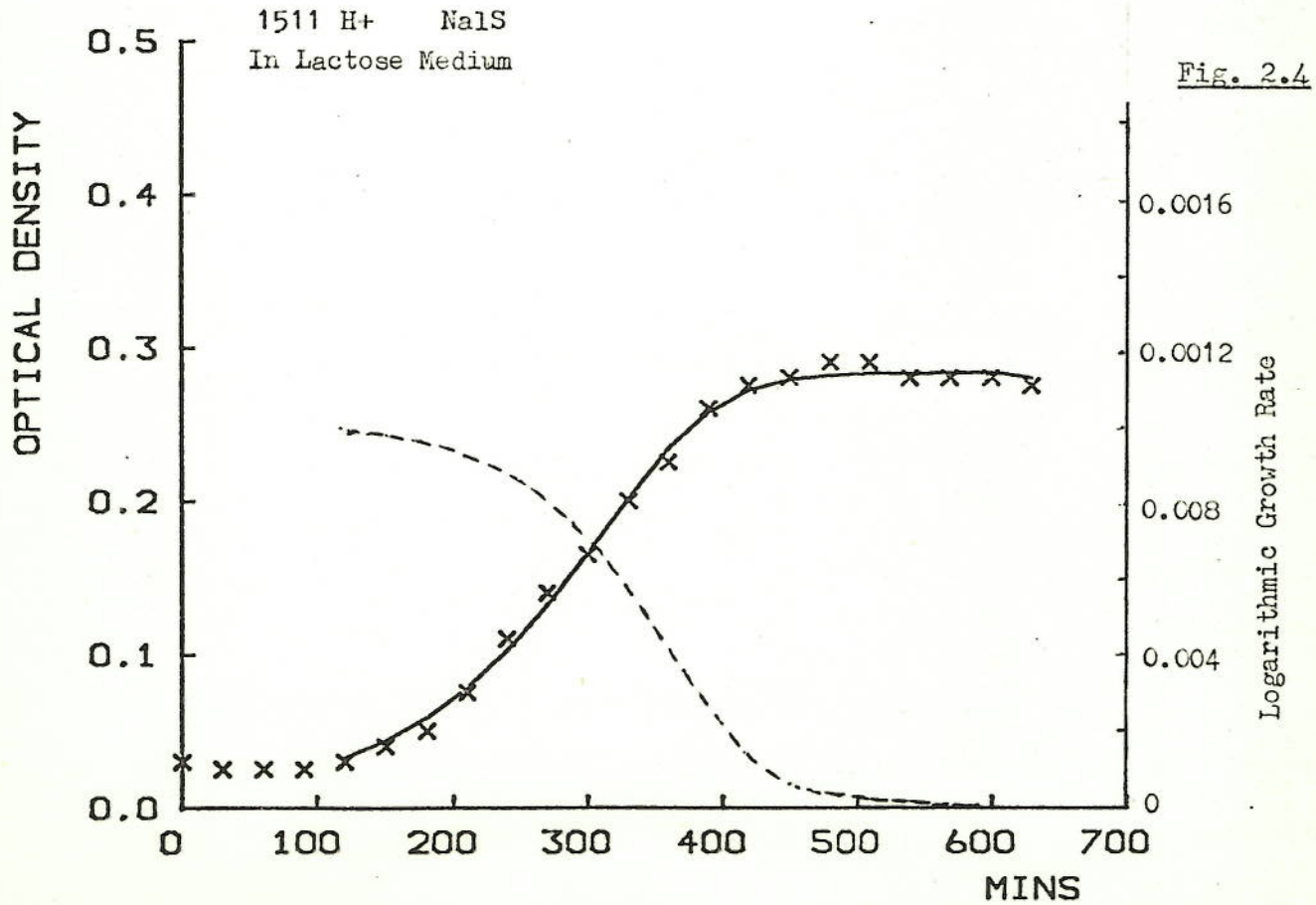

1511 H+ NalS
In Lactose Medium
OPTICAL DENSITY
0.5
0.4
0.3
0.2
0.1
0.0
0.0016
0.0012
0.008
0.004
0
Logarithmic Growth Rate
0
100
200
300
400
500
600
700
MINS

Fig. 2.4

Fig. 3 Growth of strains in glucose/lactose medium

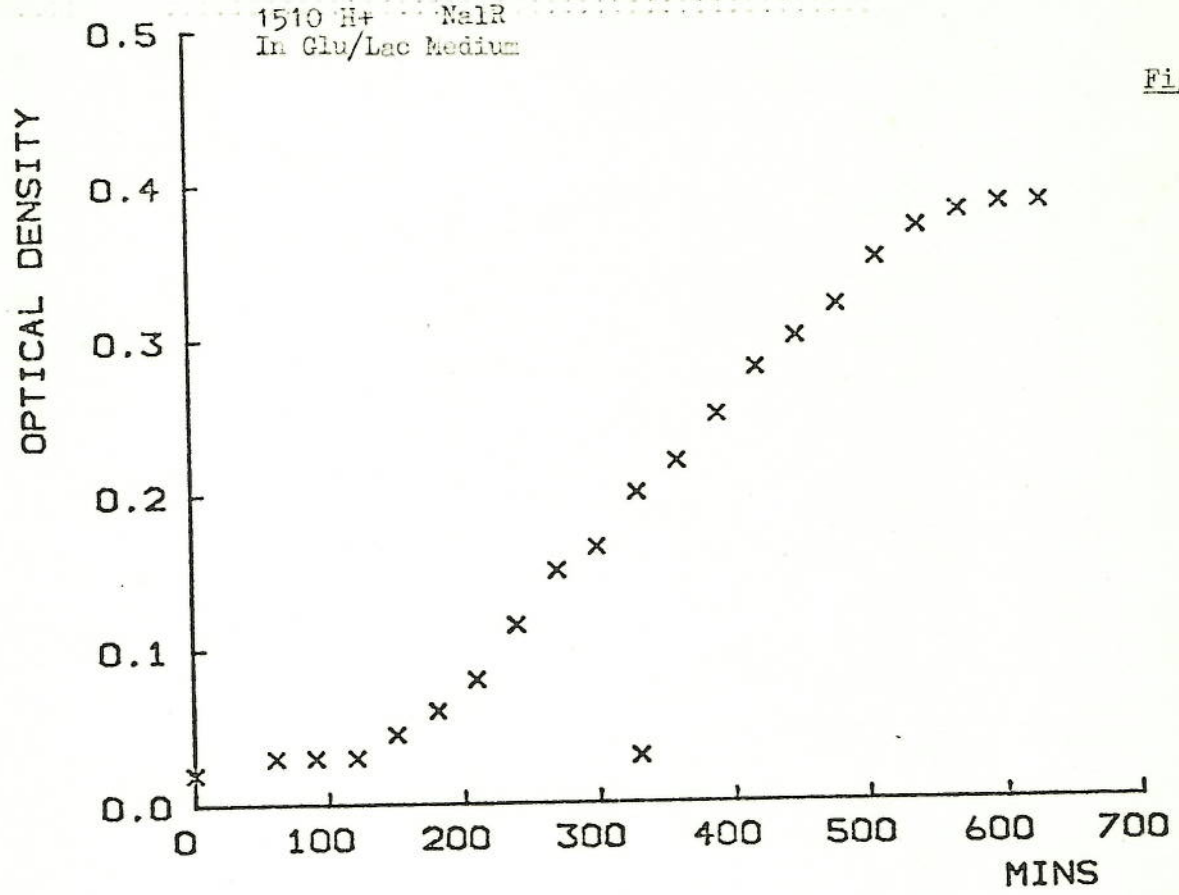


Fig. 3.1

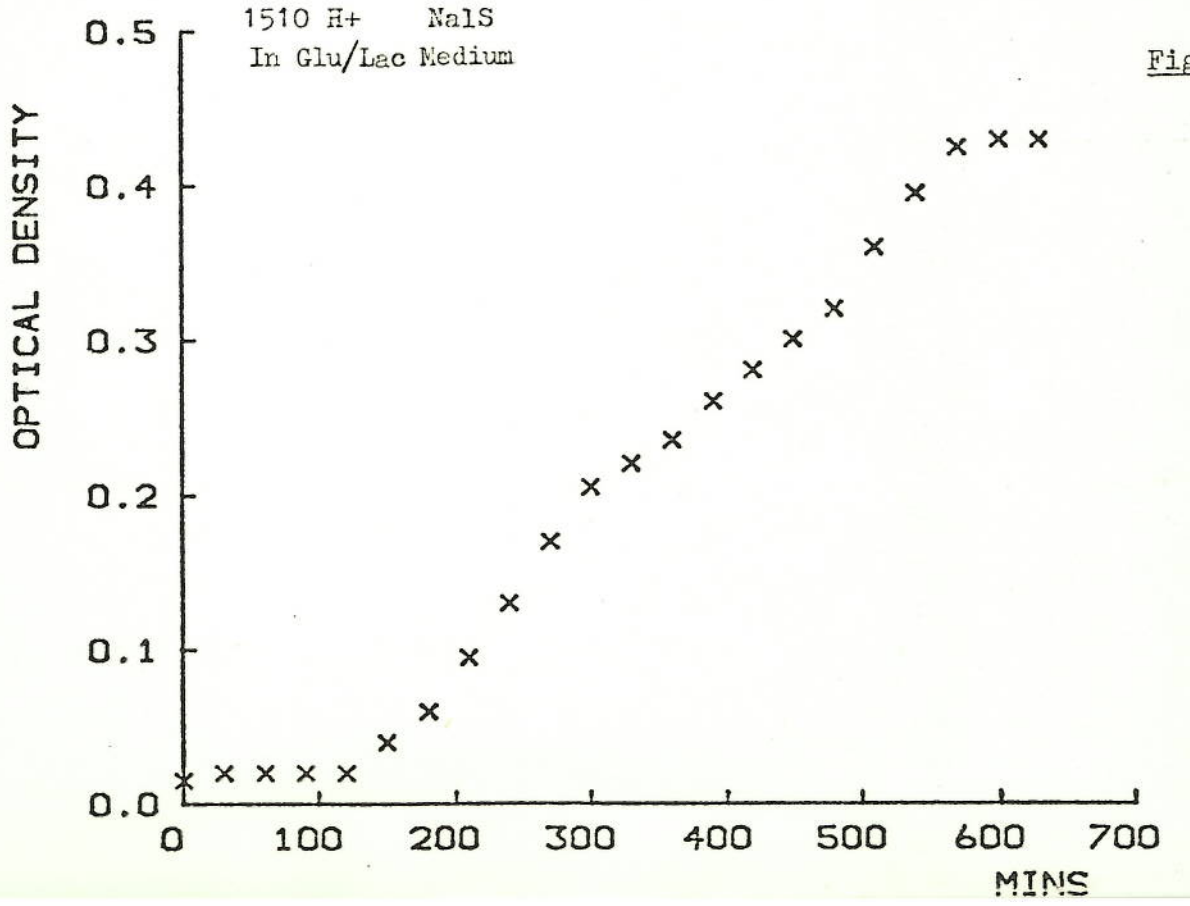


Fig. 3.2

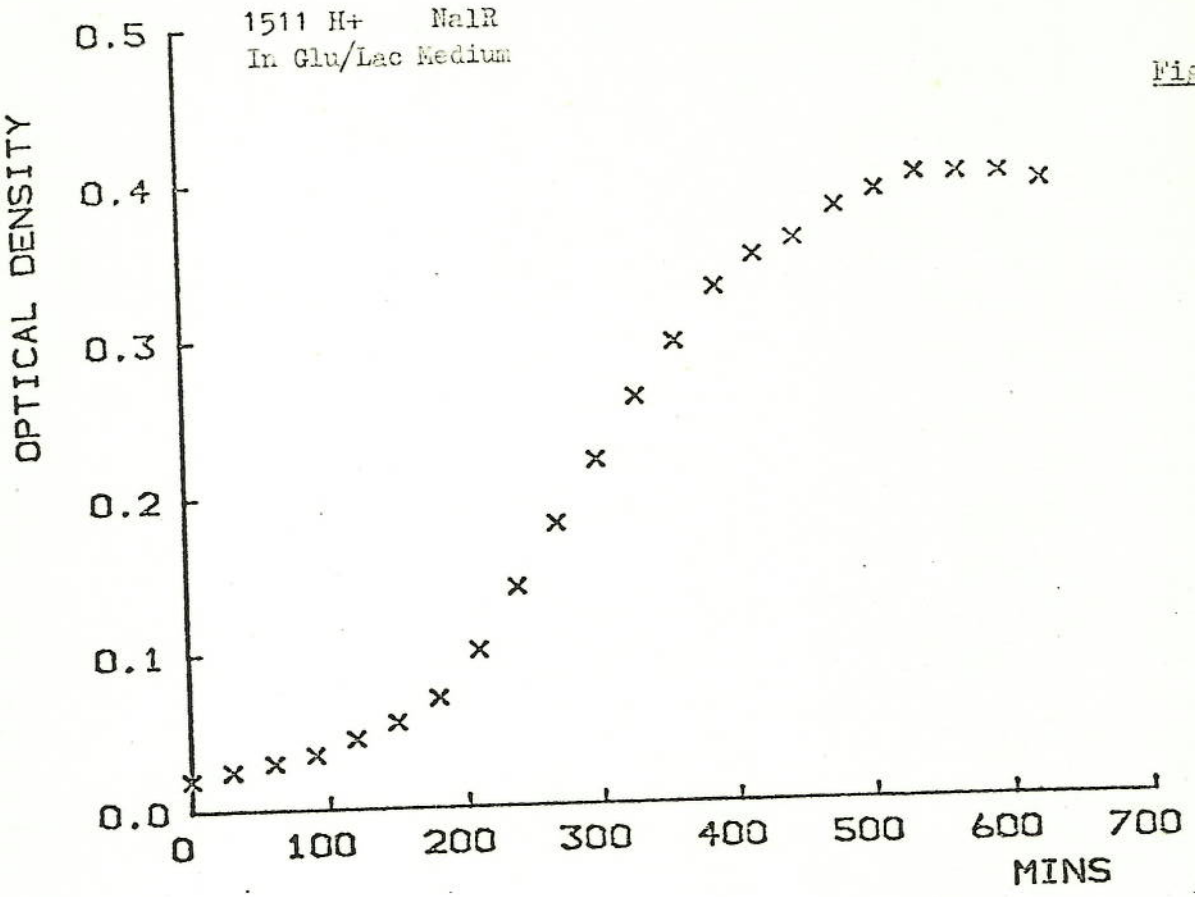

1511 H+ NalR
In Glu/Lac Medium
OPTICAL DENSITY
0.5
0.4
0.3
0.2
0.1
0.0
0
100
200
300
400
500
600
700
MINS

Fig. 3.3

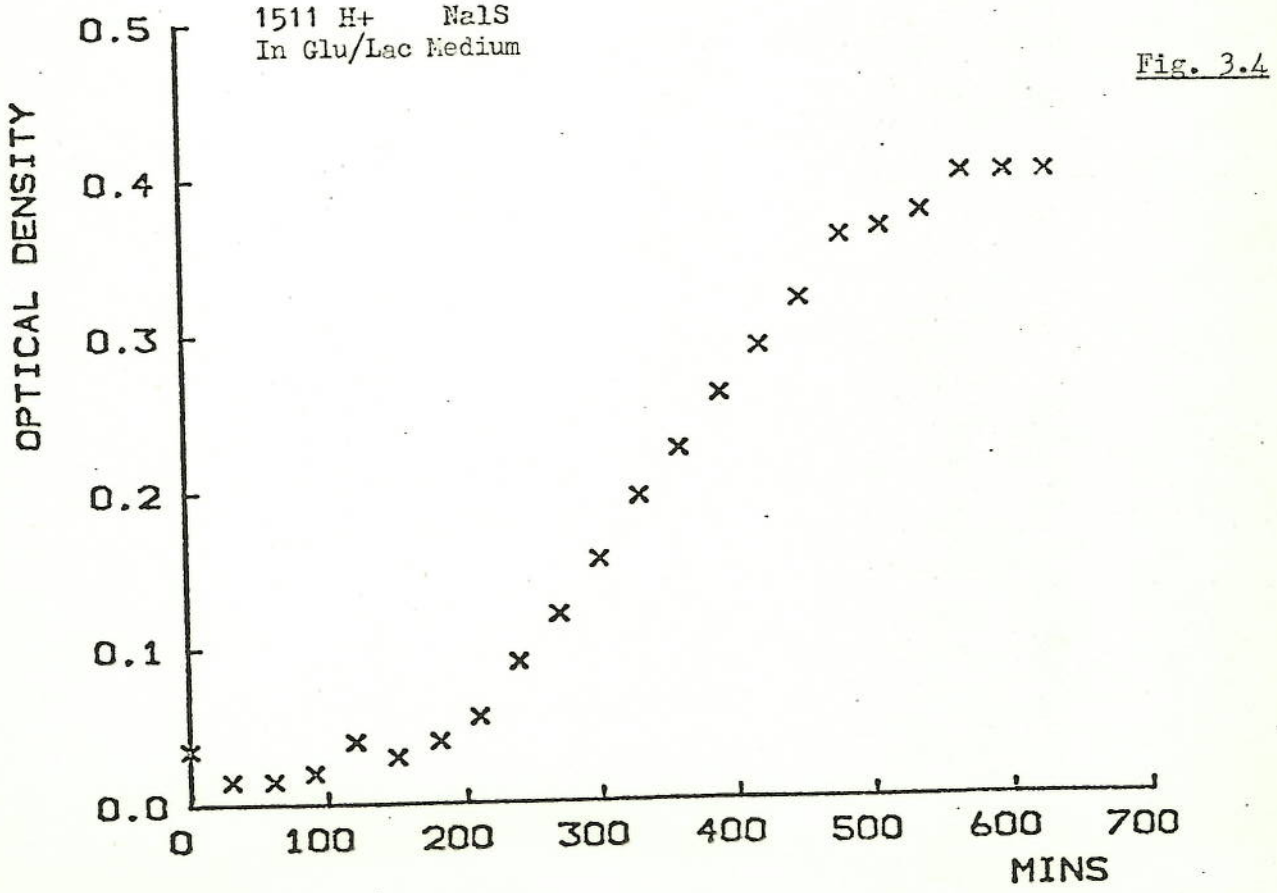

1511 H+ NalS
In Glu/Lac Medium
OPTICAL DENSITY
0.5
0.4
0.3
0.2
0.1
0.0
0
100
200
300
400
500
600
700
MINS

Fig. 3.4

only one replicate of each individual treatment. Had it been possible to repeat this set of experiments several times, a significant difference in one or more of the parameters might have been conclusively detected. (A greater knowledge of the statistical techniques used to estimate the confidence limits of individual fitted parameters from the matrix of second derivatives of the residual sum of squares with respect to these parameters might also have been useful.)

It is still interesting, however, in view of the insensitivity of the statistical methods available with such small samples, that, in glucose, both the maximal relative growth rates of the 1510-derived strains (with operon control) are greater than both the maximal relative growth rates of the 1511-derived strains (constitutive mutants); and that, in lactose, even ignoring the suspect result for 1510 H^+NalR, the remaining operon-controlled strain, 1510 H^+NalS, has a much lower fitted maximal relative growth rate than either of the two constitutive mutant strains.

The broken lines on the graphs in Figs. 1 and 2 represent the fitted relative, or logarithmic, growth rate,

$$r = \frac{d\ln B}{dt} = \frac{1}{B}\frac{dB}{dt} = r_{max}\frac{(\hat{B} - B)}{Ky + \hat{B} - B} \qquad (3)$$

calculated from the fitted parameters of equation (1). This is plotted with respect to time in order to indicate more clearly how the relative growth rate itself, rather than absolute density, is changing. If the model being used is correct, as it usually is for cultures in 'clean' media prepared from minimal medium plus known organic nutriments, then the carbohydrate concentrations in the media are only slightly limiting at first, become increasingly limiting fairly soon, and remain limiting for a significant proportion of the time taken to reach stationary phase. A more concentrated supply of carbohydrate would have produced a curve for logarithmic growth rate which stayed almost constant ($r \simeq r_{max}$) for a longer proportion of the time taken to reach stationary phase, and would have plummetted down faster when the time came (hence the convention, among workers using very energy-rich media, to use a semilog plot and draw a regression line whose slope is equal to the logarithmic growth rate). Conversely, a less concentrated supply of medium would have produced a curve which descended a lot less steeply. The curve

for logarithmic growth rate is intended to show more clearly, and more graphically, the differences in relative growth rate between media and strains, especially between strain 1510 H^+NalS and the two 1511-derived constitutive mutants in lactose.

It will also be noticed that all the values of y, the yield constant, for strains grown in glucose are higher than any value for y obtained in lactose, although this is not necessarily very important as regards competition between two strains in the same medium.

As regards the growth kinetics of the strains in mixed cultures, the operon-controlled 1510-derived strains showed varying degrees of diauxy (although the diauxy of the strain 1510 H^+NalR becomes a lot more apparent when an attempt is made to fit a non-diauxic Monod growth curve to it), while no obvious diauxy (but perhaps some very slight diauxy) is shown in the two 1511-derived constitutive mutants. This is to be expected, since constitutive mutants are able to partially counteract the glucose effect causing diauxy by production of lactose permease, which is encoded in the lactose operon. The operon-controlled strain, although it had probably become induced at some stage in the overnight culture from which the innoculum was taken, had possibly become repressed again by the time the innoculum was taken owing to exhaustion of lactose from the medium. The relative insensitivity to the glucose effect shown by the constitutive mutant might be expected to be an asset in competition with the operon-controlled strains in a mixed medium.

It can be seen that the data points from strains in glucose tend to show very visible discrepancies from the fitted curves towards the end of the experiment. Considering the fact that optical density is printed on a logarithmic scale on the colorimeter gauges, and is thus subject to greater absolute error at higher optical densities, this discrepancy seems small enough to be explained by subconscious experimenter bias due to a slight feeling of impatience with the glucose cultures for taking so long to equilibrate compared to the lactose cultures. It is conceivable that a weighting procedure ought to have been used in the curve fitting to take account of the

logarithmic scale on the colorimeter gauge, perhaps by minimising relative, rather than absolute, deviations between the experimental results and the theoretical curve. Meanwhile it is fortunate that the maximal reproductive rate r_{max}, which, it will be argued, is probably the most important parameter as regards outcome of the competition experiments, is not likely to be a very sensitive parameter with respect to the parts of the curve closest to the stationary phase.

Curve-fitting in general tends to be rather error-prone for many reasons, and, in general, the more parameters have to be fitted, the more error-prone it is likely to be. (A suspected error source in fitting a Monod curve to the data here is the fact that oxygen supply probably tended to become co-limiting with carbohydrate owing to suboptimal shaking speed. This was brought about by an initial error made when setting up the competition experiments, which, when discovered some days later, was not corrected since it would destroy the long-term uniformity of the culture conditions. In order that the growth in pure culture should have the potential of reflecting something about what happened in competition culture, it was decided to grow the pure cultures at the sub-optimal speed.) Accordingly, it was decided, for the cases of the cultures in single-carbohydrate media, to estimate an initial logarithmic growth rate for each culture by the standard practice of finding the regression of the natural logarithm of bacterial concentration with respect to time over the period of most active growth. The seven points from t = 120 to t = 300 were chosen for this purpose on the basis of appearance on a log plot. The initial logarithmic growth rates thus fitted are given in Table 2, complete with their 95% confidence limits. Each pair of growth rates from different strains in the same medium, or from the same strain in different media, was t-tested, and the results are also given in Table 2.

In glucose, no significant differences in initial growth rate were observed between any two strains, although the value of 't' for the pair 1511 H^+ NalR/1510 H^+ NalS is very nearly up to significance level for $p = 0.05$. In lactose, the strain 1510 H^+ NalS grew significantly more slowly than either of the 1511-derived strains. The culture of 1510 H^+ NalR in lactose, whose data have already been mentioned as suspect, seemed to grow significantly faster than either 1510 H^+ NalS or 1511 H^+ Nal S in lactose.

Table 2 : Results of Statistical Analysis of the Initial Logarithmic Growth Rates[2] of the Strains in Pure Culture

1. Strains Grown in Glucose Medium

Strains	Growth Rate	95% Conf. Limits	Pairwise 't' tests between regressions: 1510H+ MalR	1510H+ MalS	1511H+ MalR
1510H+MalR	0.0114	+/-0.0015			
1510H+MalS	0.0141	+/-0.0023	1.9067		
1511H+MalR	0.0111	+/-0.0012	0.2540	2.2105	
1511H+MalS	0.0125	+/-0.0008	1.3654	1.2130	1.9861

2. Strains Grown in Lactose Medium

Strains	Growth Rate	95% Conf. Limits	Pairwise 't' tests between regressions: 1510H+ MalR[1]	1510H+ MalS	1511H+ MalR
1510H+MalR	0.0121	+/-0.0006			
1510H+MalS	0.0081	+/-0.0008	7.7258****		
1511H+MalR	0.0111	+/-0.0012	1.4314	4.0583***	
1511H+MalS	0.0100	+/-0.0009	3.6283***	3.0833**	1.3870

3. 't' tests for differences between growth rates in Glucose and in Lactose for each strain

	1510H+ MalR	1510H+ MalS	1511H+ MalR	1511H+ MalS
't'	0.8782	4.7991****	0.0541	4.1075***

(For 't' tests the No. of degrees of freedom is 10 throughout)

** = p less than 0.02

*** = p less than 0.01

**** = p less than 0.001

Footnote 1 The culture of 1510H+MalR in Lactose showed anomalous growth kinetics and contamination with Glucose was suspected (see text)

Footnote 2 The initial logarithmic growth rate is reckoned as the regression coefficient of Log_e Optical Density at 660 mu with respect to time in minutes, from 120mins. to 300mins. after innoculation.

Apart from that, there were no significant differences between any other two strains in the same medium, although both of the NalS strains grew significantly faster in glucose than in lactose. However, there is no way of telling for certain that this is not due to random environmental variation between tubes rather than variation between strains or media, since only one culture was grown per strain per medium. Since it was not possible to have unlimited use of the colorimeter, it was not possible to quantify variation in growth rate among cultures of the same strain in the same medium. Had this been done, it would have been possible to be more conclusive about inter-strain differences in growth rate of such small magnitudes as observed.

Competition experiments

In the main competition experiments, two factors were varied, the carbohydrate in the medium and the pair of strains competing. The three media were the glucose, lactose and mixed glucose/lactose diauxy media described above. The two pairs of strains put together in the competition experiment were 1510 H^+NalR/1511 H^+NalS and 1510 H^+NalS/1511 H^+NalR, the two pairs differing in whether the operon-controlled strain or the constitutive mutant was marked by nalidixic acid resistance.

The measure of frequency of one of two competing strains most likely to be roughly linear with respect to time (see Appendix I) is the natural logarithm of the ratio between the two strain densities,

$$\ln R = \ln \frac{B_1}{B_2} \qquad (4)$$

B_1 is here taken as the density of the 1511-derived constitutive mutant, and B_2 is taken as the density of the 1510-derived operon-controlled strain. This quantity was plotted for each treatment (see Fig. 4, pp.26-8). Time is taken here as days of active growth, i.e. not including the time spent refrigerated at 5° C over weekends, owing to the impossibility of access to the laboratory to change the medium on Sundays, but including the time presumably spent in 'stationary phase' in the water baths.

The results were analysed by t-test for significance of regression, and then subjected to pairwise t-tests to check for the presence of any

significant differences between the logarithmic rates of change of ratio (see Table 3). The regression slopes for cultures in glucose were all negative, i.e. the ratio of constitutive mutants to operon-controlled cells decreased with time. Since all regressions were apparently significant ($p < 0.001$) except one (1511 H^+NalR / 1510 H^+NalS, replicate 2, no significant regression), a selective advantage of operon control where the product of the operon is not required is indicated, at least for the lactose operon. The single insignificant regression out of the four derived from glucose medium does not differ significantly from the strongly significant ($p < 0.001$) regression derived from its replicate, and so it is unlikely that selection is failing to take place for operon control in glucose where the operon-controlled strain is nalidixic acid sensitive and the constitutive mutant is nalidixic acid resistant, although it was found that the regression for the first of the two replicates of the combination 1511 H^+NalS / 1510 H^+NalR in glucose differed significantly ($p < 0.02$) from those for both of the two replicates for the competition 1511 H^+NalR /1510 H^+NalS in glucose. The difficulty of repeating a two-week (actual time) competition experiment in a five-week project is to be regretted in this connection, as is the difficulty of obtaining less error-prone results.

Selection for the constitutive mutant in a lactose medium seemed to be, if anything, more intense than selection for the operon-controlled strains in a glucose medium, although pairwise t tests on the modulus of the regression coefficient, rather than the regression itself, showed a significant difference only in some cases; i.e. in only some cases was the rate of selection for the constitutive mutant in lactose significantly greater than the rate of selection for the operon-controlled strain in glucose.

In the mixed glucose/lactose diauxy medium, the regression of lnR with time was significantly positive ($p < 0.001$) for both replicates of both treatments. However, all the regression coefficients for competitions in the mixed medium were smaller than any regression coefficient for a competition in the lactose medium, and some regressions from the mixed medium were significantly less than some from the lactose medium, although this was not true of all such pairs of experiments (see Table 3).

Table 3 Statistical analysis of regression of log ratio with time for competition experiments

	strains competing		significance of regression	pairwise comparisons between regression coefficients										
				1	2	3	4	5	6	7	8	9	10	11
1	1511H$^+$NalS/1510H$^+$NalR in glucose Rep.1	't'	45.98											
		d.fr	8 ****											
2	1511H$^+$NalS/1510H$^+$NalR in glucose Rep.2	't'	5.68	1.38										
		d.fr	7 ****	15										
3	1511H$^+$NalS/1510H$^+$NalR in lactose Rep.1	't'	31.16	8.34	5.55									
		d.fr	7 ****	15****	14****									
4	1511H$^+$NalS/1510H$^+$NalR in lactose Rep.2	't'	17.62	7.16	4.61	0.96								
		d.fr	9 ****	17****	16****	16								
5	1511H$^+$NalS/1510H$^+$NalR in glu/lac Rep.1	't'	7.75	6.90	3.41	3.48	2.29							
		d.fr	9 ****	17****	16***	16***	18*							
6	1511H$^+$NalS/1510H$^+$NalR in glu/lac Rep.2	't'	8.89	6.66	3.66	2.70	1.60	0.68						
		d.fr	9 ****	17****	16***	16**	18	18						
7	1511H$^+$NalR/1510H$^+$NalS in glucose Rep.1	't'	9.72	2.82	0.59	6.39	5.20	4.18	4.27					
		d.fr	8 ****	16***	15	15****	17****	17****	17****					
8	1511H$^+$NalR/1510H$^+$NalS in glucose Rep.2	't'	1.09	4.20	1.57	5.33	4.14	2.67	2.98	1.44				
		d.fr	9	17****	16	16****	18****	18**	18***	17				
9	1511H$^+$NalR/1510H$^+$NalS in lactose Rep.1	't'	9.03	5.54	3.84	0.83	0.03	1.73	1.24	3.98	3.17			
		d.fr	8 ****	16****	15***	15	17	17	17	16***	17***			
10	1511H$^+$NalR/1510H$^+$NalS in lactose Rep.2	't'	37.03	8.72	5.95	0.52	1.46	4.01	3.22	6.83	5.80	1.25		
		d.fr	9 ****	17****	16****	16	18	18****	18***	17****	18****	17		
11	1511H$^+$NalR/1510H$^+$NalS in glu/lac Rep.1	't'	9.37	7.06	3.55	3.29	2.11	0.24	0.47	4.37	2.88	1.59	3.83	
		d.fr	10. ****	18****	17***	17***	19*	19	19	18****	19**	18	19***	
12	1511H$^+$NalR/1510H$^+$NalS in glu/lac Rep.2	't'	18.70	8.00	3.99	3.05	1.82	0.78	0.44	5.18	3.58	1.35	3.60	0.52
		d.fr	10 ****	18****	17****	17***	19	19	19	18****	19***	18	19***	20

p less than 0.05 *
p less than 0.02 **
p less than 0.01 ***
p less than 0.001 ****

Throughout the experiment, no two replicates of the same treatment differed significantly in the size of the regression, and, apart from the examples previously noted from the glucose medium, no two competitions in the same medium gave significantly differing regressions, suggesting that the effect of the nalidixic acid resistance/sensitivity polymorphism on the competitions was small, if it existed at all.

A tendency will be noticed, especially in the case of the competition experiments in lactose, for the residual scatter around the regression line to increase with time. This, of course, is due to the fact that the experimental error is tied to density of bacteria and proportion of nalidixic acid resistant individuals, and tends to be distorted with transformation to natural log of ratio.

It was considered desirable to have some idea of the order of magnitude of the relative differences in growth rate required to produce selection at the rate observed. (It would seem rather unreasonable to ask for anything more precise than that.) In order to do this, it was decided to use an equation relating relative difference in growth rate to change in ratio over one cycle in batch culture:

$$\frac{R_{t+1}^{(1-\frac{1}{s})}}{1 - R_{t+1}} - \frac{B_o}{\hat{B}} \frac{R_t^{(1-\frac{1}{s})}}{1 - R_t} = 0 \qquad (21)$$

where B_o is bacterial density immediately on inoculation, $\hat{B}$ is bacterial density at stationary phase, R_t is the ratio of density of one strain to density of the other at the start of cycle t, R_{t+1} is the ratio at the start of cycle t+1 (i.e. at the end of cycle t), and s is the relative difference in growth rate:

$$s = 1 - \frac{r_2}{r_1} \qquad (22)$$

where r_1 and r_2 are the logarithmic growth rates of the constitutive mutant and the controlled strain respectively. Equation (22) assumes that the ratio (and therefore the relative difference s) of the two growth rates remains constant (although the growth rates themselves may change through the cycle), and that the ratio between B_o and $\hat{B}$ remains constant through successive cycles (see Appendix III). The

equation was only used for data from single-carbohydrate media, since the former assumption should not apply in diauxy medium.

Reliable values for ratios at the beginning and end of any one daily cycle were not available owing to the size of sampling error. Instead, it was decided to use the long-term regression of ln R on time to estimate the change in value in ln R over a 'typical' daily cycle. The ratio of initial to final bacterial density for such a cycle was reckoned as equal to the ratio of volume of initial inoculum to culture volume, i.e. neglecting possible differences in density at stationary phase owing to the difference between yields per unit carbohydrate of the two strains, which would seem to be relatively slight. Equation (22), therefore, was solved for s using the values

$$R_t = 1 \; ; \quad R_{t+1} = e^{24b} \; ; \quad \frac{B_o}{\bar{B}} = \frac{0.2}{8.2} \qquad (23)$$

where b is the regression coefficient.

The optimum estimates of s thus arrived at are shown in Table 4. The estimated maximum and minimum values there were derived as for the optimum, but inserting the upper and lower 95% confidence limits, respectively, of the regression as the value for b in (23). Relative differences between the growth rates observed in pure culture are also included in the table, not because they were significant (most were not), but to show that a difference in intrinsic growth rate large enough to account for the selection observed in competition culture could have passed undetected in the pure cultures simply owing to its low order of magnitude. In this connection, it may be interesting that the only relative difference in pure culture included in Table 4 which is both significant and derived from two non-suspect cultures, namely the value for the pair 1511 H^+NalR/1510 H^+NalS in lactose, is well within the (admittedly very broad) confidence limits for the relative difference estimated from both replicates of the competition culture.

What Table 4 shows, therefore, is that, although genuine differences in intrinsic growth rate were mostly not detected in pure culture, it does not in any way follow that such differences were not there. Thus the proposition that the selection is brought about by intrinsic difference in growth rate rather than by some more complex interactive mechanism has not been refuted.

Table 4 : Relative Differences in Growth Rate in Competition Cultures Calculated from the Regression of Log_e Ratio 1511/1510 with respect to Time

Medium	Strains	Rep.	Regression Slope	95% Confidence Limits	Relative differences in Growth Rate			
					Est. from Pure Culture	Est. from Competition Culture		
						Max.	Optimum	Min.
Glucose	1511H+NalS/1510H+NalR	1	-0.0090	+/-0.0026	0.0925	-0.0424	-0.0603	-0.0785
"	1511H+NalS/1510H+NalR	2	-0.0054	+/-0.0044	0.0925	-0.0062	-0.0356	-0.0659
"	1511H+NalR/1510H+NalS	1	-0.0039	+/-0.0024	-0.2609	-0.0094	-0.0254	-0.0418
"	1511H+NalR/1510H+NalS	2	-0.0013	+/-0.0025	-0.2609	0.0075	-0.0086	-0.0249
Lactose	1511H+NalS/1510H+NalR	1	0.0117	+/-0.0041	-0.2096	0.0986	0.0737	0.0484
"	1511H+NalS/1510H+NalR	2	0.0089	+/-0.0041	-0.2096	0.0816	0.0562	0.0303
"	1511H+NalR/1510H+NalS	1	0.0088	+/-0.0057	0.2725	0.0904	0.0555	0.0196
"	1511H+NalR/1510H+NalS	2	0.0133	+/-0.0043	0.2725	0.1090	0.0834	0.0572

Fig. 4 Results of competition experiments

Fig. 4.1

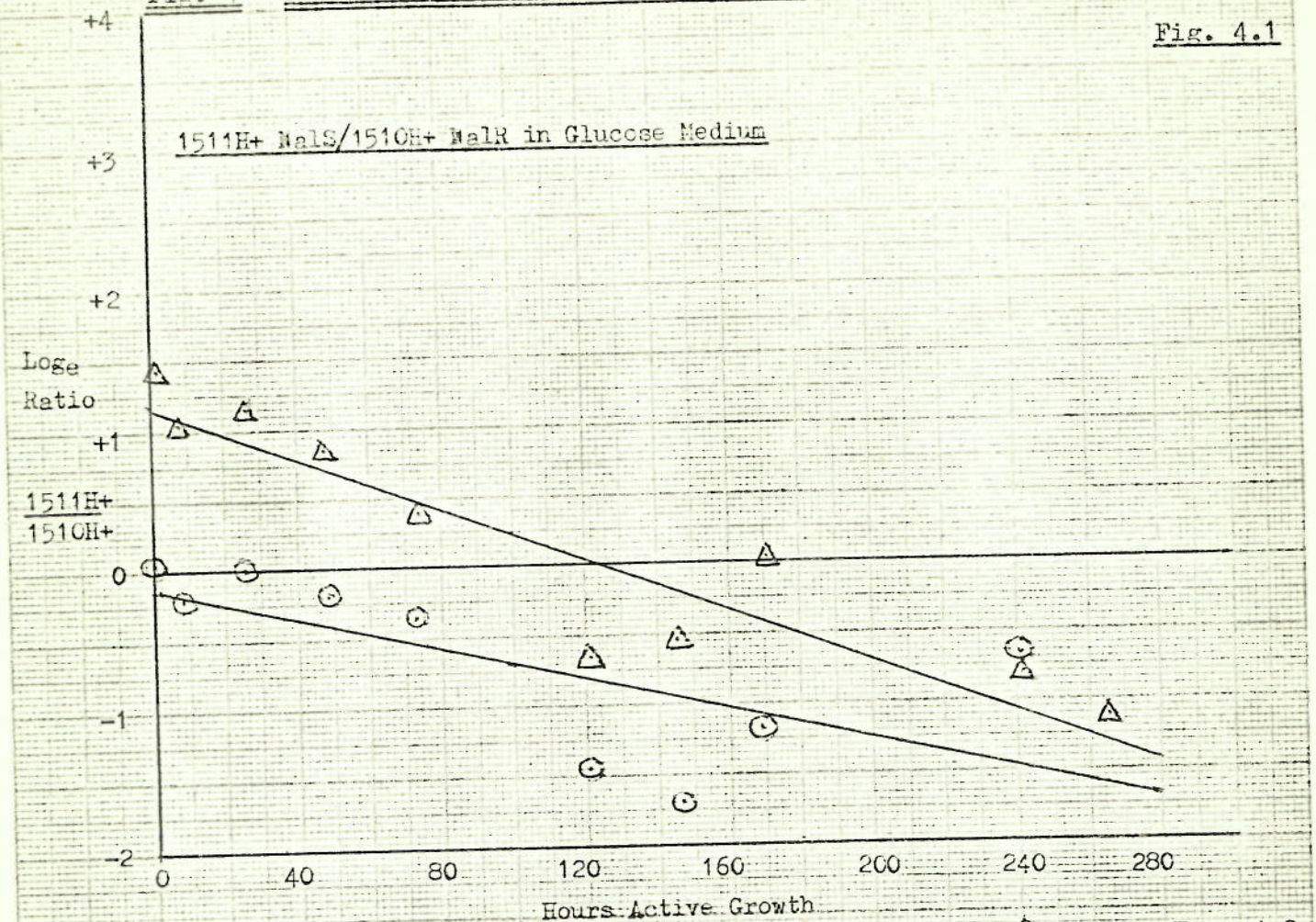


Fig. 4.2

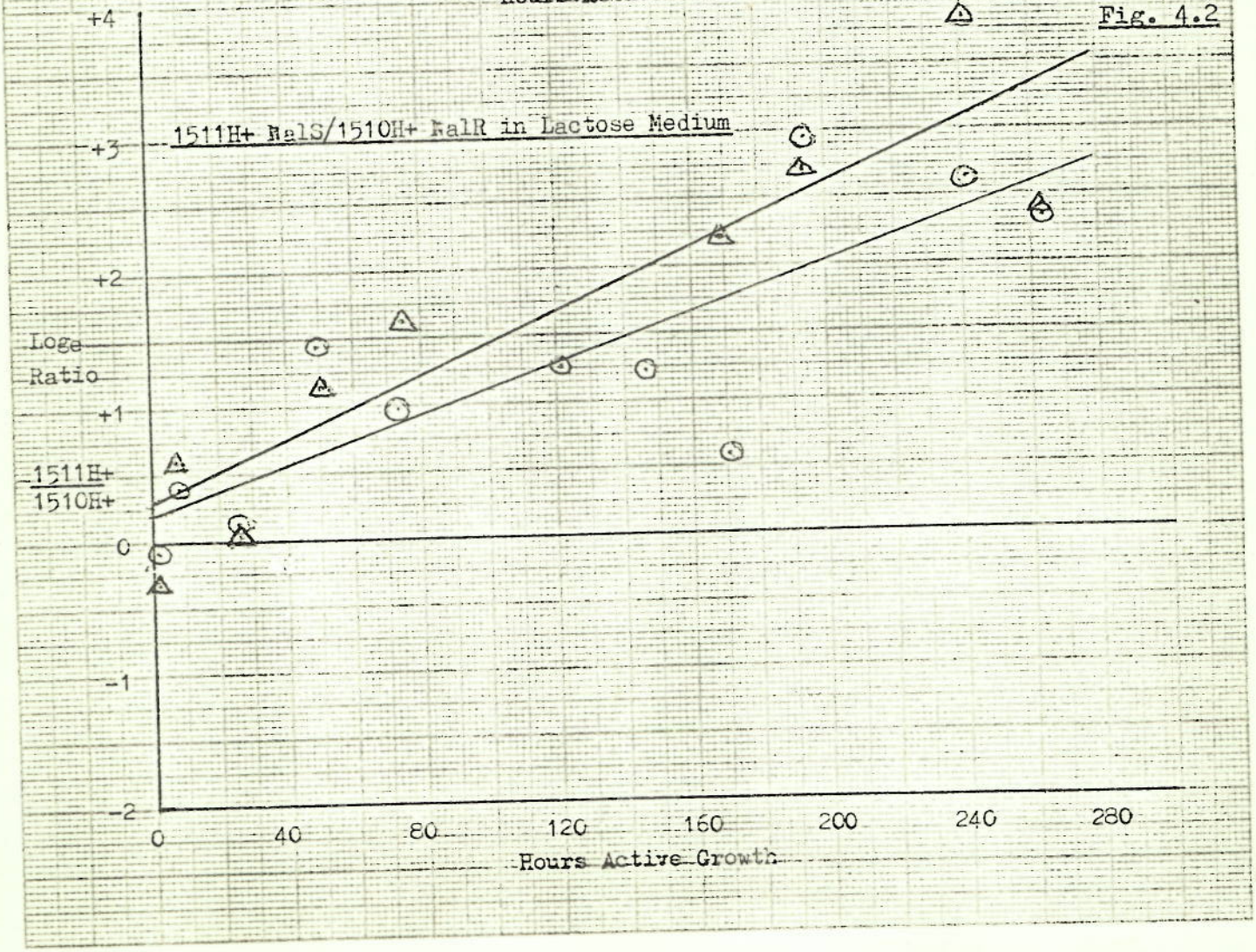


Fig. 4.3

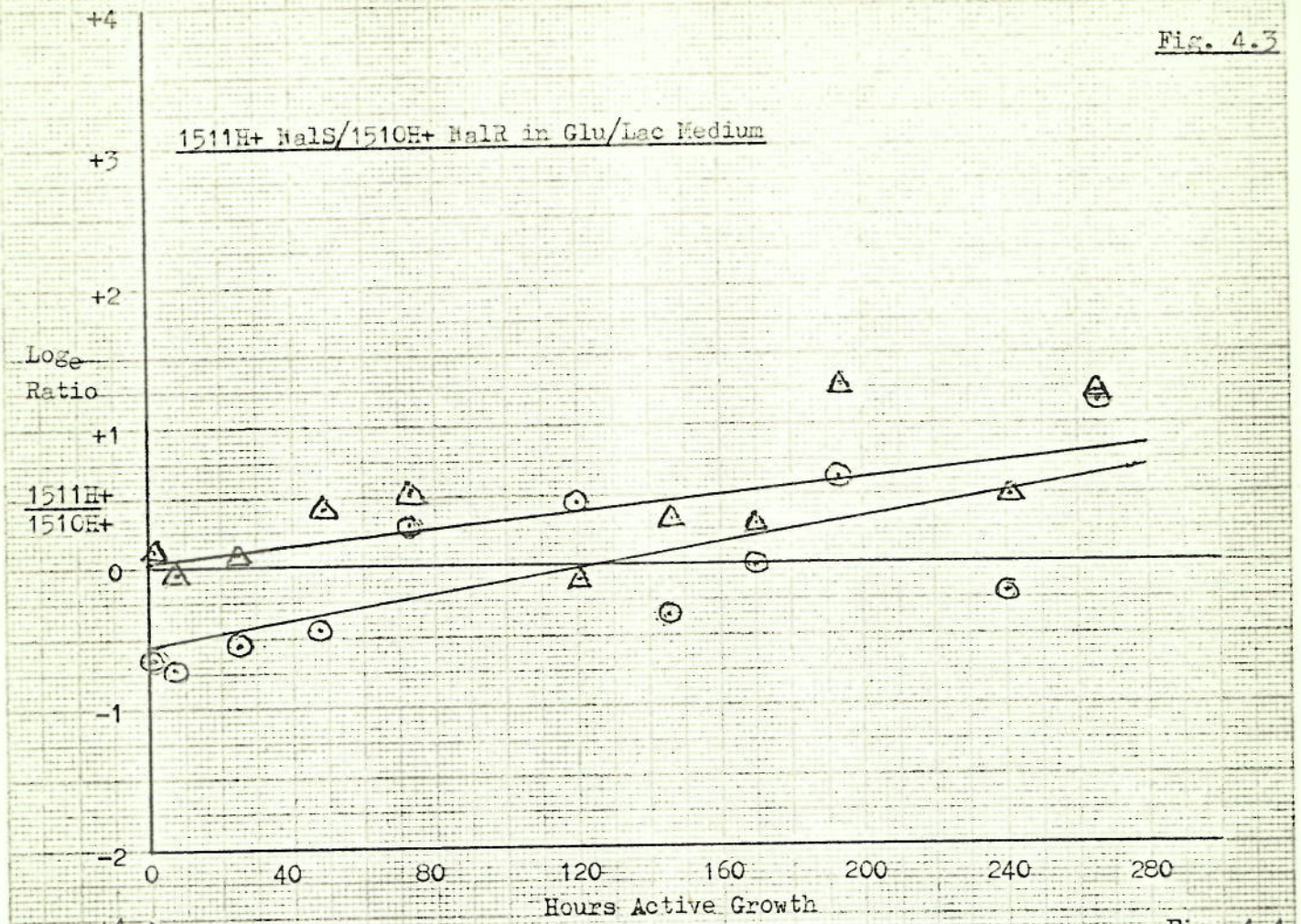

1511H+ NalS/1510H+ NalR in Glu/Lac Medium
+4
+3
+2
Log$_e$ Ratio
+1
1511H+ / 1510H+
0
-1
-2
0
40
80
120
160
200
240
280
Hours Active Growth

Fig. 4.4

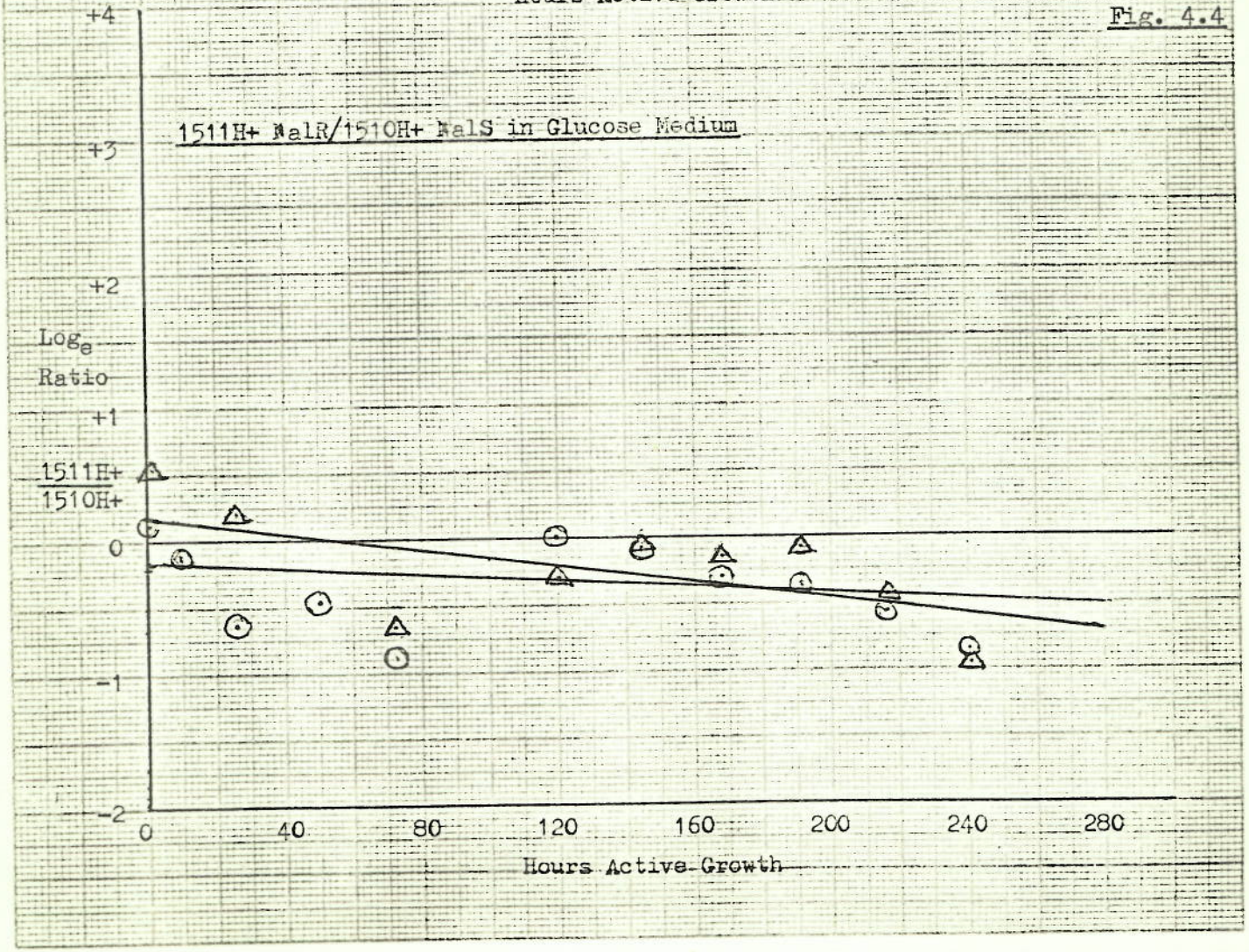

1511H+ NalR/1510H+ NalS in Glucose Medium
+4
+3
+2
Log$_e$ Ratio
+1
1511H+ / 1510H+
0
-1
-2
0
40
80
120
160
200
240
280
Hours Active Growth

Fig. 4.5

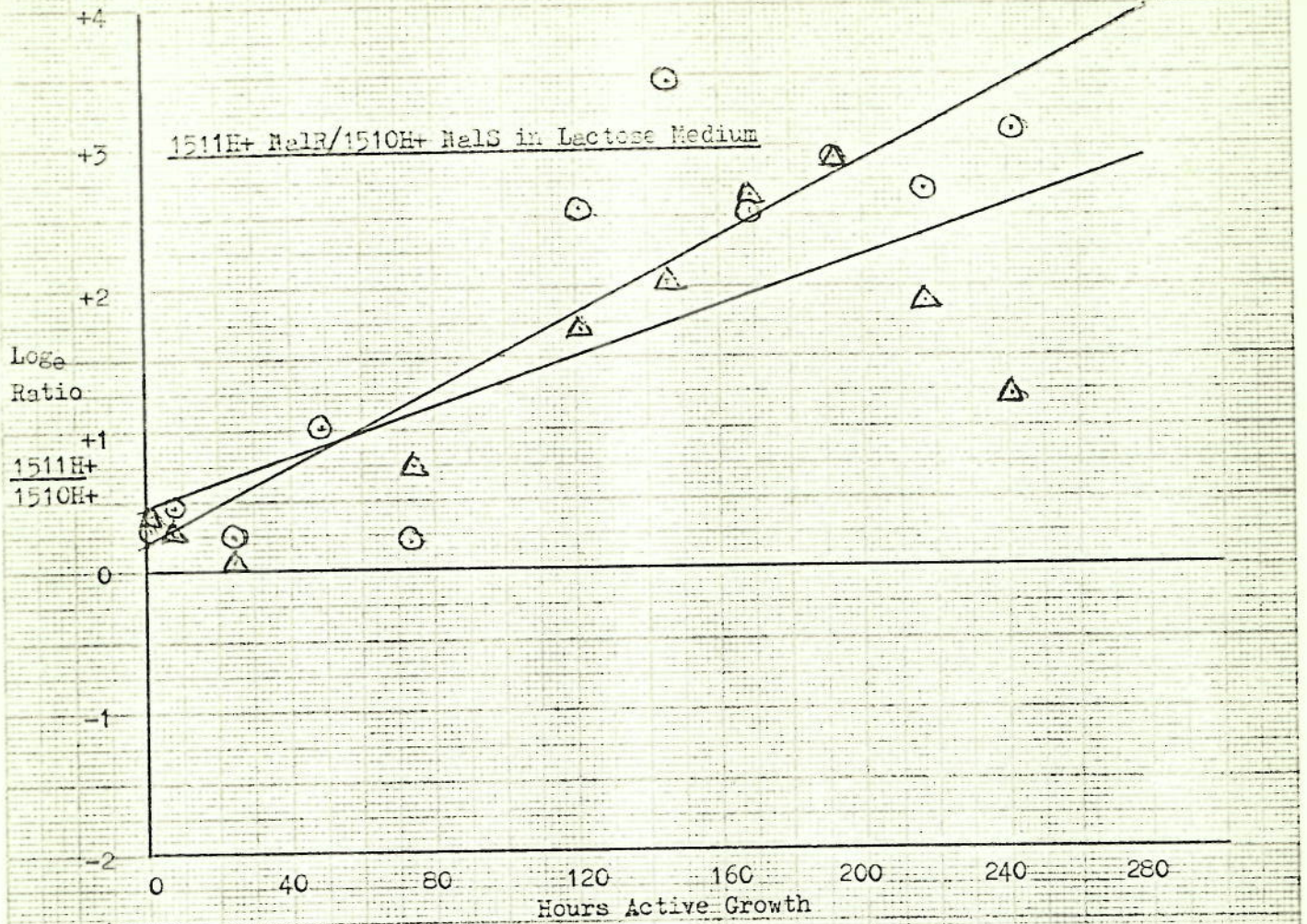
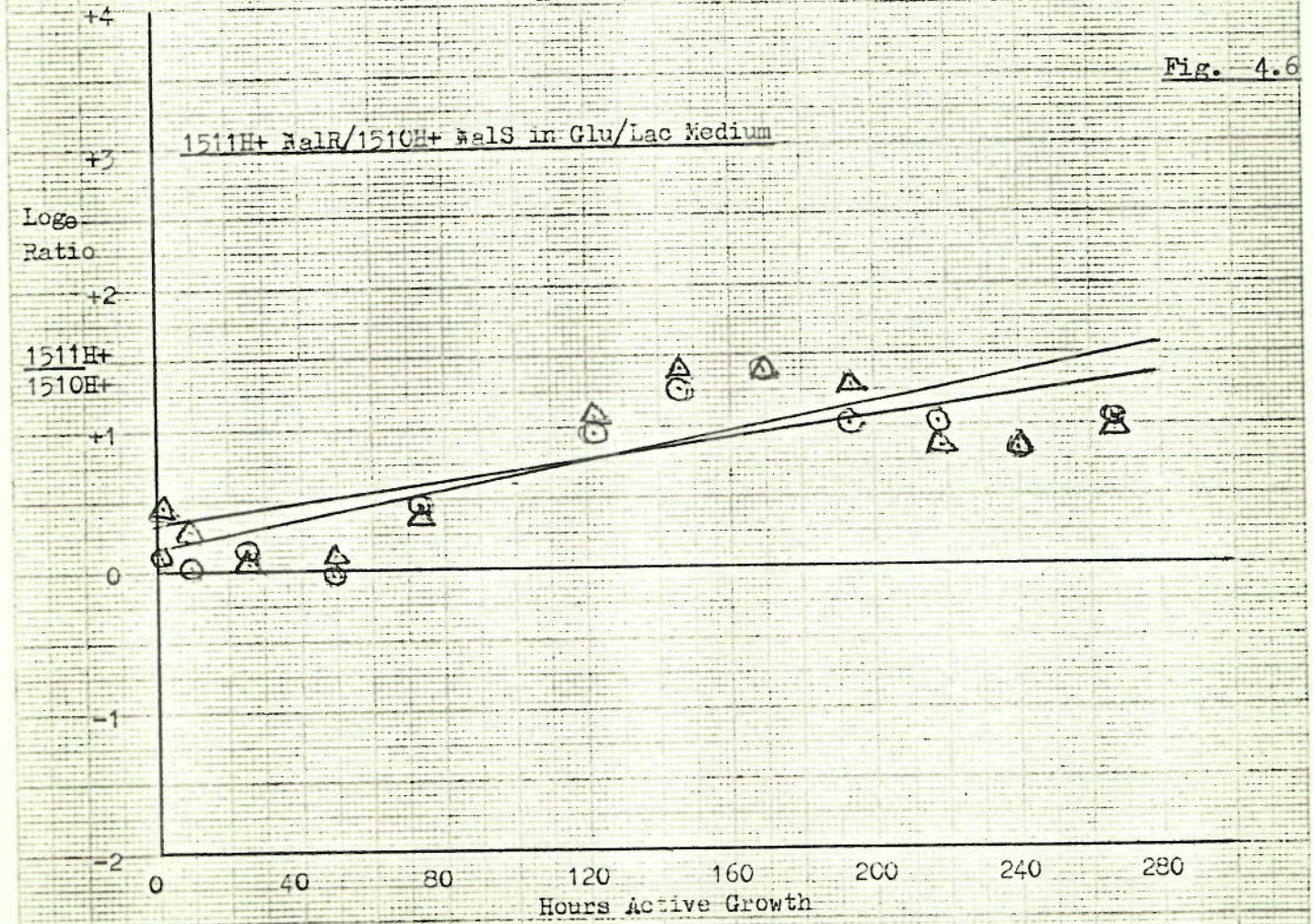

1511H+ MalR/1510H+ MalS in Lactose Medium
+4
+3
+2
Log$_e$ Ratio
1511H+ / 1510H+
+1
0
-1
-2
0
40
80
120
160
200
240
280
Hours Active Growth

Fig. 4.6


1511H+ MalR/1510H+ MalS in Glu/Lac Medium
+4
+3
+2
Log$_e$ Ratio
1511H+ / 1510H+
+1
0
-1
-2
0
40
80
120
160
200
240
280
Hours Active Growth

Computer simulations based on fitted parameters

These were undertaken out of curiosity rather than optimism, in order to test whether the parameters fitted to growth data from the four strains in pure culture would be sufficient to predict the results of the competition experiments, qualitatively if not quantitatively. They were based on a stepwise approximate numerical solution to the equations

$$\frac{dB_1}{dt} = B_1 r_{max1} \frac{c}{K_1 + c} \qquad (5.1)$$

$$\frac{dB_2}{dt} = B_2 r_{max2} \frac{c}{K_2 + c} \qquad (5.2)$$

$$\frac{dc}{dt} = -\frac{1}{y_1} \frac{dB_1}{dt} - \frac{1}{y_2} \frac{dB_2}{dt} \qquad (5.3)$$

$$\ln R = \ln \frac{B_1}{B_2} \qquad (5.4)$$

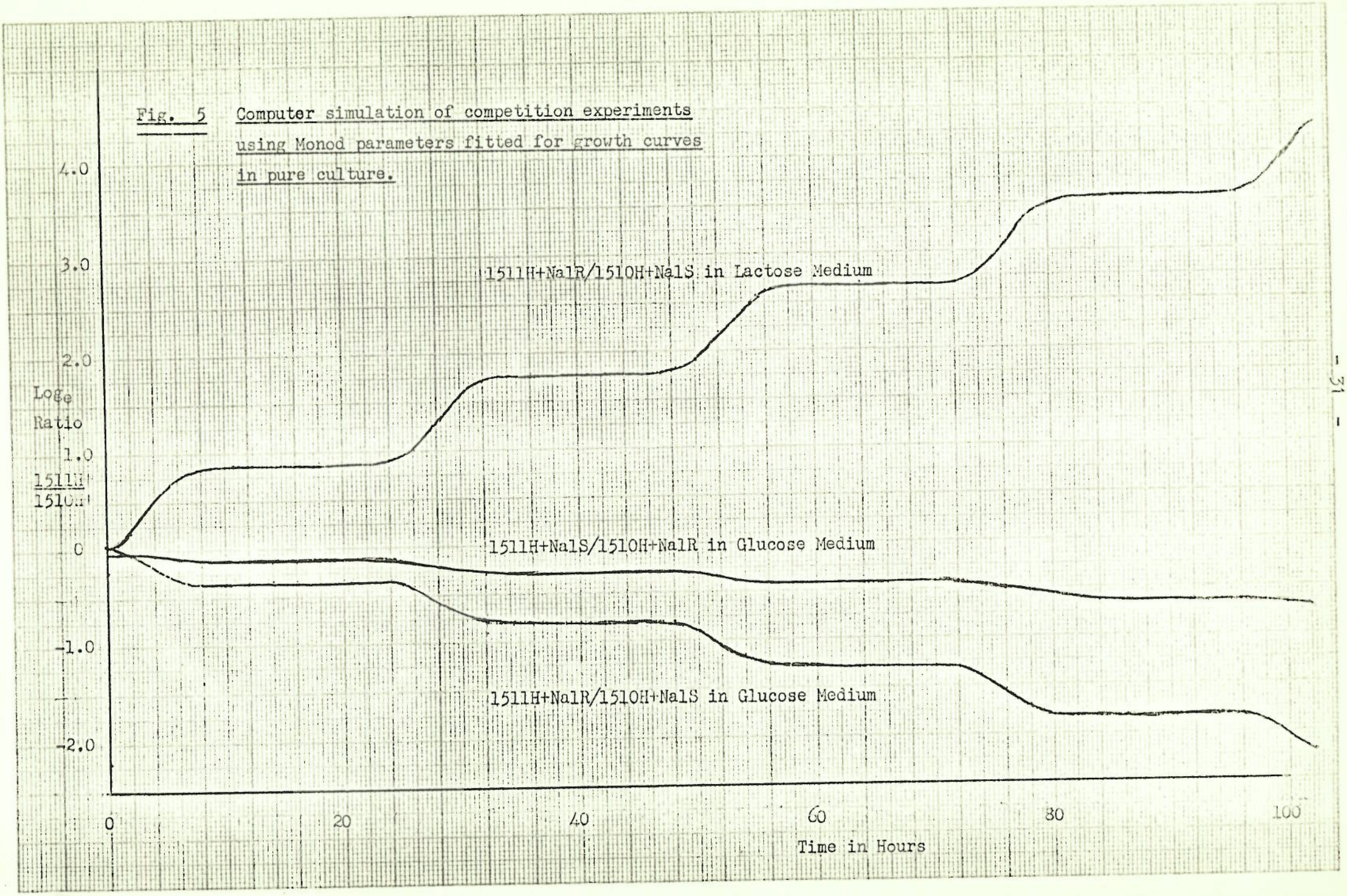
where B_1 is the density of the constitutive mutant strain, B_2 is the density of the operon-controlled strain, the $r_{max\,1}$, y_1 and K_1 are the growth parameters of the appropriate strains (see equation (1)), R is the ratio of the density of the constitutive mutant to the density of the operon controlled strain and c is the concentration of the carbohydrate source. The latter was not directly measurable with the available techniques, but was included in the simulation because there was no easy way of eliminating it from the equations (3.1), (3.2), and (3.3), although for pure culture it can be eliminated from the Monod equations to give equation (1). It was reckoned as a proportion of the initial concentration for simulation purposes. The lack of availability of data on levels of carbohydrate concentration may introduce some error into the fitted estimates of the parameters of equation (1).

For purposes of the simulation, initial bacterial densities at the beginning of the experiment simulated were assumed to be equal to that which would result from a 0.2 cm^3 innoculation from pure culture of a density equal to the fitted final concentration $\hat{B}$ of a pure culture of the strain. It was assumed that, as in the real-life experiment,

a 0.2 cm^3 innoculum from the competition culture was transferred each day to a fresh tube of medium. A lag phase lasting one hour was assumed for both strains for simplicity.

The results of the simulations (see Fig. 5), as might be expected from the error-proneness of results from only one replicate of each treatment, were not identical to the results of the competition experiments. Of the three competitions simulated (1511 H^+NalS/1510 H^+NalR in glucose, 1511 H^+NalR / 1510 H^+NalS in glucose, 1511 H^+NalR/1510 H^+NalS in lactose), only for 1511 H^+NalS / 1510 H^+NalR in glucose did the rate of selection quantitatively fit the results of the competition experiments, giving a slope in between the regression slopes fitted for the two replicates. The other two gave general slopes of far greater magnitude than occurred in the real-life experiments. However, in all three the same strain tended to be selected as in the real-life experiments, with selection taking place a lot faster in the lactose simulation than in either of the two glucose simulations, as also happened in the real-life experiments. While none of this conclusively proves anything positive, it indicates that the differences in growth rate observed in pure culture are not too small to account for the population changes observed in mixed culture. It follows that either the differences in growth rate detected by curve fitting are meaningful, and subject to a relatively small amount of error, or the differences are due to chance, and subject to errors so large that differences in the intrinsic growth rate of the right magnitude to account for the results of the competition experiments would be very hard to detect by looking at the population kinetics of pure cultures.

Fig. 5 Computer simulation of competition experiments using Monod parameters fitted for growth curves in pure culture.



Discussion

The above work seems to have shown that a definite selective pressure in favour of control of the lactose operon exists for strains grown in batch culture in a glucose medium. It has not shown what brings this selection pressure about, although it has manifestly failed to show that it is inexplicable in terms of intrinsic differences which also exist in pure culture, and, by extension, it has failed to disprove the hypothesis that the selection pressure is due to intrinsic economy of resources.

Competition experiments using various auxotrophs for tryptophan differing in the point in the tryptophan synthesis pathway at which the deficiency exists (Zamenhof and Eichhorn, 1967) have shown that not only do tryptophan auxotrophs in Bacillus subtilis have a selective advantage against prototrophs in the presence of tryptophan, but strains auxotrophic owing to deficiencies further back along the pathway compete successfully against strains auxotrophic owing to deficiencies affecting subsequent steps on the pathway. This tends to confirm the hypothesis that the advantage enjoyed by the auxotroph arises from resource economy, since less energy would presumably be needed to carry out the first few steps of the pathway than to complete a larger number of steps. An equivalent experiment for the case of the lactose operon in E. coli is probably feasible in principle. Constitutive mutants such as 1511 and other i^- mutants differ from the 'wild type' owing to point mutations affecting the repressor for the lactose operon by lowering its affinity for the operator region in the absence of inducer. Operon control, of course, is not an all-or-nothing control system, at least on the population level. The binding of repressors to operators is a reversible chemical reaction denoted by the equation

$$O + R \rightleftharpoons OR \qquad (6)$$

where O is the operator, R is the repressor and OR is the operator-repressor complex. It follows that an individual operator region has a nonzero probability of being repressed at any time, assuming the presence of effective repressor molecules. On the population level, this means that, in a population of cells able to produce repressor with nonzero affinity for the operator region, a non-zero proportion of genomes will be repressed, the exact proportion

depending on concentration of the inducer and the concentration and chemical properties (including affinity for the operator) of the repressor. Presumably, therefore, it should be possible in principle to produce a number of mutant strains carrying various point mutations in the i gene, which produces the lactose repressor, and having a wide range of different repressor affinities for the operator region, and thus a range of different rates of β-galactosidase and lactose permease production for most concentrations of inducer (including zero). Such strains could be competed pairwise in a lactose-free medium, and also grown in pure culture to evaluate growth parameters and find out if maximal reproductive rate/or yield in glucose was positively related to repressor affinity for the operator region or negatively related to any enzyme assay in a given concentration of carbohydrate, as would be predicted by the hypothesis that the selective advantage of operon control arises simply from resource economy.

A similar set of experiments would be informative in investigating the nature of the selective advantage enjoyed by the constitutive mutants in the lactose medium. The obvious hypothesis that would explain this is that the operon-controlled strain, whose repressor has a far higher affinity for the operator region than that of the constitutive mutant, is liable at any one time to have a small but nonzero proportion of its population repressed, even in the presence of an inducer. The mode of action of the inducer is to bind to the repressor moiety of the operator-repressor complex and decrease its affinity for the operator, causing the complex to fly apart with a very high probability. The chemical equation describing the inducer-repressor-operator system can thus be described by the equations

$$O + R + I \rightleftharpoons OR + I \rightleftharpoons ORI \rightleftharpoons O + RI \qquad (7)$$

As indicated, the inducer does not bring the affinity of the repressor down to zero. The residual affinity, in the presence of excess inducer, is sufficient, in the 'wild type' E.coli cell, to effect repression 5% of the time if there is enough repressor present to effect repression 99.9% of the time in the total absence of inducer (Gilbert and Müller-Hill, 1970). This level of repression is of the right order of magnitude to account for the observed differences in growth rate and fitness in pure and mixed cultures, respectively, in lactose medium, assuming that maximal

reproductive rate is proportional to the amount of lactose permease and/or β-galactosidase present, and that the amount of enzyme present is roughly proportional to the amount of time spent in an induced state by the operon. Further work on the relationship between percentage induction and maximal growth rate would be necessary to clarify this issue and shed light on the tenability of the hypothesis that the observed advantage of the constitutive mutants in lactose medium is caused by residual repression in the operon-controlled strain.

Another mechanism which could be proposed to account for the selective advantage of the constitutive mutant is induction lag. There are reasons to expect that, when placed in fresh medium after being in stationary phase, the operon-controlled strain might take longer to start growing at a rate appropriate to the concentration of the new medium than the constitutive mutant. We might expect that, even after probably twelve hours in stationary phase, the average density of residual lactose permease in the cell membrane might be very slightly greater in the constitutive mutant than in the operon-controlled strain, making for a slight difference in average permeability of the cell membrane to lactose. It might also be expected that, even once the lactose was inside the cell, its digestion would begin sooner in the constitutive mutant than in the operon-controlled strain, where some of it would have to be turned into inducer and the inducer would have to start attacking repressor-operator complices before synthesis could begin. However, work carried out on induction lag (Keps, 1963, mentioned in Contesse, Crépin and Gros, 1970) indicates that, of the time (3-4 minutes) elapsed between addition of excess inducer and production of β-galactosidase at the full rate, most can be accounted for by the time taken for transcription and translation, which presumably takes as long in constitutive mutants as in operon-controlled strains. In these experiments, of course, the inducer must first be made from the lactose, but it is rather doubtful that this occupies very much extra time. The lag phase in these experiments lasts around two hours (see Figs. 1, 2 and 3), and the speed with which it is broken is therefore probably not appreciably limited by the rate of activation of the lactose operon. However, it would be necessary to do a lot more

work, preferably in continuous culture, where, of course, there is no daily lag phase, to be certain that differences in induction lag had no significant effect.

In the mixed medium it is, of course, impossible with the available data to assess the contribution of diauxy to the selective advantage of the constitutive mutant. Generally, the rates of selection in the mixed medium are lower than those in lactose, suggesting that the presence of glucose has, on the whole, benefited the constitutive mutant more by compensating for its lower production of lactose enzymes than it has disadvantaged it by depressing its uptake of lactose. Considering that there was a 3:1 ratio, by mass, of lactose to glucose, this would seem to suggest that the effect of diauxy has been relatively slight. Here, as in the question of the causative agent bringing about the selective advantage of the constitutive mutant in lactose, enzyme assay techniques would have been useful, in this case to assess the extent of the glucose effect in the four strains.

An interesting effect observed in some of the competition experiments (see Figs. 4.4 and 4.6) is the tendency of the natural log of the observed ratio to fluctuate fairly consistently around the regression line. It is impossible, with the data available, to determine what causes this.

The proportional differences in maximal reproductive rates observed in these experiments, both those fitted to the data from pure culture and those which would be necessary in theory to produce the results in mixed culture, are of similar order of magnitude to the proportional differences in growth rates estimated by Baich and Johnson (1968) in competition experiments using strains of E. coli with and without end-product inhibition for the proline synthesis pathway. In the latter experiments, bath culture was used, in flasks containing 1 dm^3 of medium.

The idea that redundant structures or activities tend to bring about a selective disadvantage in the organism possessing them tends to be confirmed by the work presented here. Where effective operon control was itself redundant, as in the lactose medium, it was itself selected against. In the process of evolution of E. coli outside the laboratory,

of course, populations in the human gut, at any rate, are not often exposed for long periods either to a zero-lactose environment or to an environment where lactose is the sole energy source. The presence of lactose would probably be an intermittent feature of the environment, with the result that selection for efficient utilisation of lactose would alternate with selection against the waste of resources associated with the superfluous production of the enzymes needed to make efficient use of lactose. Those strains ancestral to K12 seem to have hit upon an optimal strategy permitting the opportunistic exploitation of both intermittent situations by retaining the ability to produce the enzymes associated with lactose utilisation while producing a repressor which will inhibit their synthesis when lactose is not present. It would be interesting to know whether strains of E. coli in the guts of animals where the presence of lactose is less intermittent, as in the case of young animals still on a milk diet or older animals which drink little or no milk, tend to adopt different strategies.

APPENDIX I The Monod equations describing growth of bacterial cultures in minimal-based media

Consider the case of a culture of bacteria growing in a relatively simple medium in which all nutrients are present in excess except one, which is likely to become limiting. The relative (or logarithmic) growth rate of the culture can be represented as a function of the concentration of the nutrient which is likely to become limiting:

$$\frac{dB}{dt} = r(c)B \qquad (8)$$

$$\frac{d}{dt}(\ln B) = r(c) \qquad (9)$$

where B is the bacterial density and r(c) is the relative growth rate expressed as a function of concentration.

It is necessary to find a form which such a function might take for a model of this form to be considered accountable. Monod ( ), observing that the limiting nutrient became less limiting as its concentration increased, proposed a description of relative growth rate as a function of concentration of limiting nutrient to explain this phenomenon:

$$r(c) = r_{max} \frac{c}{K + c} \qquad (10)$$

where r is the maximal reproductive rate approached asymptotically for very high concentrations where the 'limiting' nutrient ceases to be limiting, and K is a constant equal to the concentration of limiting nutrient when the relative growth rate is equal to half the maximal growth rate. The equation is based on the assumptions that growth rate is limited by the rate of active uptake of the limiting nutrient, and that the rate of active uptake as a function of concentration of nutrient in the medium follows a Michaelis-Menten curve, so that the maximal reproductive rate r_{max} is interpreted as being proportional to the maximal rate of the enzymatic uptake of nutrient from the medium, and the constant K is interpreted as the Michaelis constant of the enzyme responsible for this uptake.

Having provided a description of growth rate as a function of concentration,

it remains to define a formula describing change in concentration; this is especially necessary in batch culture, for which the equation usually used is

$$\frac{dc}{dt} = -\frac{1}{y}\frac{dB}{dt} \qquad (11)$$

where y is a yield constant representing the amount of bacterial biomass produced per unit limiting nutrient consumed. The growth of bacterial culture is here looked upon much as a chemical reaction turning the medium into bacterial biomass and catalysed by existing bacterial biomass.

It follows from equation (11) that the amount of bacterial biomass produced is proportional to the amount of limiting nutrient consumed, and that total bacterial biomass at a given time is linearly related to concentration:

$$B - B_o = y(c_o - c) \qquad (12)$$

where B_o is bacterial density at zero time and c_o is concentration of the limiting nutrient at zero time.

It follows from equation (10) that, for nonzero r_{max} and B, the bacterial population is stationary if and only if concentration is zero. The equilibrium concentration is then equal to

$$\hat{B} = B_o + yc_o \qquad (13)$$

from (12).

The above equations are obviously inaccurate where relative growth rate is not a function of concentration, as is the case, for instance, in very concentrated broths where contact inhibition causes a stationary phase to be reached before the limiting nutrient is exhausted.

The usual procedure for the evaluation of the parameters r_{max}, k and y of the above equations for a particular strain in a particular medium is to grow specific cultures of the strain in the medium, take values for concentration of limiting nutrient and bacterial density for different times, fit a value for y from the relationship

between bacterial density and substrate concentration, and then use other numerical methods to fit the other parameters, having calculated a value for y (see, for instance, Lee, Frederickson and Tsuchiya, 1974).

In the absence of any immediately available method of assaying for the limiting nutrient, the above equations, as they stand, are obviously useless. It was therefore deemed necessary to eliminate c from equation (10). This can be done by multiplying both numerator and denominator by y:

$$r(c) = r_{max} \frac{yc}{Ky + yc} \tag{14}$$

Since, from (12) and (13), $yc = B_o + yc_o - B = \hat{B} - B$, we have

$$r(c) = r_{max} \frac{(\hat{B} - B)}{Ky + \hat{B} - B} \tag{15}$$

and therefore, from (8),

$$\frac{dB}{dt} = Br_{max} \frac{(\hat{B} - B)}{Ky + \hat{B} - B} \tag{16}$$

This differential equation is soluble by the method of separation of variables together with partial fractions:

$$r_{max}t = \int \frac{Ky + \hat{B} - B}{B(\hat{B} - B)} \, dB$$

$$\therefore \quad r_{max}t\hat{B} = \int \frac{(Ky + \hat{B})}{B} \, dB + \int \frac{Ky}{(\hat{B} - B)} \, dB$$

$$= (Ky + \hat{B}) \ln B - Ky \ln (\hat{B} - B) + C$$

where C is an integration constant.

Setting $t = 0$, we have

$$C + (Ky + \hat{B}) \ln B_o - Ky \ln(\hat{B} - B_o) = 0$$

$$\therefore \quad C = Ky \ln(\hat{B} - B_o) - (Ky + B) \ln B_o$$

$$\therefore \quad r_{max}t\hat{B} = (Ky + \hat{B}) \ln \left(\frac{B}{B_o}\right) - Ky \ln \left(\frac{\hat{B} - B}{\hat{B} - B_o}\right)$$

$$\therefore \quad B^{\hat{B}+Ky}\,(\hat{B}-B)^{-Ky} = B_o^{\hat{B}+Ky}(\hat{B}-B_o)^{-Ky}\,e^{(r_{max}t\hat{B})}$$

$$\therefore \quad B^{\hat{B}+Ky}\,(\hat{B}-B)^{-Ky} - B_o^{\hat{B}+Ky}(\hat{B}-B_o)^{-Ky}\,e^{(r_{max}t\hat{B})} = 0 \qquad (17)$$

It was thought that iterative solution of this equation would be very time-consuming on the computer for curve-fitting purposes. The curves on the graphs in Figs. 1, 2 and 3 were accordingly fitted using a simple stepwise numerical solution of (16), treating it, in fact, as a difference equation with half-minute time intervals. This was time-consuming, but, it was suspected, not as time-consuming as fitting an analytical solution derived from (17) would be. Since all numerical approximations to (16) converge to B_o at one end of the curve and $\hat{B}$ at the other, it was thought that the cumulative errors usually associated with numerical solutions to differential equations in general, and stepwise solutions in particular, would not be as serious as for some differential equations.

If a solution of (16) is fitted to a set of real-world data, in the absence of data recording change in concentration, there is, of course, very little justification for thinking of the parameters as having any rigorous biochemical significance. It is more pertinent to think of $\hat{B}$ as a final concentration and Ky as a measure of how soon the growth of the culture starts to top out from logarithmic phase.

In mixed culture of two types with densities B_1 and B_2 and separate growth parameters r_{max1}, r_{max2}, y_1, y_2, K_1 and K_2, equation (10) gives rise to

$$\frac{dr}{dt} = Rc\left(r_{max1}\frac{c}{K_1+c} - r_{max2}\frac{c}{K_2+c}\right) \qquad (18)$$

$$\left(\text{where } R = \frac{B_1}{B_2}\right)$$

$$\therefore \quad \frac{d}{dt}\ln R = c\left(r_{max1}\frac{c}{K_1+c} - r_{max2}\frac{c}{K_2+c}\right).$$

In a chemostat, where concentration of substrate is approximately constant, (19) approximates to a linearisation of (18). In batch culture on a daily basis where the yield constants y_1 and y_2 are not vastly different, concentration approximates to a periodic function of

time, and (19) again approximates to a linearisation when R is measured over a period of days.

A consequence of (19) is that the rate of replacement of one strain by the other is a function of the r_{max_i}, K_i and c. The yield constants, y_i, can affect the rate of replacement only in as far as they affect concentration. Changes in concentration, although they are likely to affect the magnitude of population tendencies, are unlikely to change their direction. (The conditions under which variation in concentration might, possibly, affect the direction of population changes are defined by

$$\frac{r_{max_1}K_2 - r_{max_2}K_1}{r_{max_2} - r_{max_1}} > 0 \qquad (20)$$

and, even then, it is not certain that it will.) It is therefore likely that, if superior resource economy on the part of one strain affects the direction of the population change, it will do so through creating a difference in the maximal reproductive rates rather than in yield. A non-mathematical interpretation of this statement is that, if two species are competing in a culture, it is not as advantageous for species A to produce a large amount of biomass per unit concentration (i.e. to have a high yield constant) as it is to produce a large amount of biomass per unit time (i.e. to have a high maximal reproductive rate), because the more strain A eats, the less strain B will get. For this reason, differences in maximal reproductive rate r_{max} are likely to be more important in competition culture than differences in y .

Appendix II Computational methods used in fitting the growth curves for pure culture

The growth curves were produced by fitting a simple stepwise numerical solution of equation (16) to each set of data points. It was considered that the use of a simple stepwise solution consumed less computer time than any other (see Appendix I).

The initial estimate for r_{max} was derived from the regression line fitted logarithmically to the first five data points, whereas the initial estimates for B_0 and $\hat{B}$ were set equal to the first and last data points, respectively, while Ky was initially estimated to be equal to $\frac{1}{2}\hat{B}$.

Owing to lack of access to library programs such as E04FBF/A, for fitting of a form of the user's choice to a set of data, it was necessary when writing a program for fitting the growth curves, to write a new routine for the actual fitting. The segment, written in FORTRAN since that is the programming language in which the writer is most fluent, was based on a simple steepest-descent least-squares minimisation method, taking steps in the direction of steepest descent of a size scaled to take account of the respective sizes of the parameters being fitted and decreasing in size as required. (For the principles on which such curve-fitting is based, see Rosenbrock and Storey (1966), which is a recommended text on computational methods for microbiology.) The text of the curve-fitting subroutine is reproduced overleaf.

```
      SUBROUTINE RBN(NDAT,NPAR,PAR,RES,F,ACC,DELTA,STEPMX,NITMAX,IFAIL)
      DIMENSION PAR(4)
      DIMENSION PAR1(4)
      DIMENSION DRIVE(4)
      DIMENSION DIREC(4)
      DIMENSION RES(18)
      DIMENSION RES1(18)
      DO 4 I=1,NPAR
      PAR1(I)=PAR(I)
    4 CONTINUE
      STEP=STEPMX
      CALL RESID(NDAT,NPAR,PAR,RES)
      F=0.0
      DO 1 I=1,NDAT
      RSQ=RES(I)*RES(I)
      F=F+RSQ
    1 CONTINUE
      DO 2 NIT=1,NITMAX
      DO 3 I=1,NPAR
      DPAR=PAR(I)*DELTA
      PAR1(I)=PAR(I)+DPAR
      J=I-1
      IF(J.GT.0)PAR1(J)=PAR(J)
      CALL RESID(NDAT,NPAR,PAR1,RES1)
      F1=0.0
      DO 5 J=1,NDAT
      RSQ=RES1(J)*RES1(J)
      F1=F1+RSQ
    5 CONTINUE
      DF=F1-F
      DRIVE(I)=DF/DPAR
    3 CONTINUE
      SUM=0.0
      DO 6 I=1,NPAR
      DSQ=DRIVE(I)*DRIVE(I)
      SUM=SUM+DSQ
    6 CONTINUE
      B=SQRT(SUM)
   10 DO 7 I=1,NPAR
      DIREC(I)=-DRIVE(I)/B
      DPAR=PAR(I)*STEP
      DPAR=DIREC(I)*DPAR
      PAR1(I)=PAR(I)+DPAR
    7 CONTINUE
      CALL RESID(NDAT,NPAR,PAR1,RES1)
      F1=0.0
      DO 8 J=1,NDAT
      RSQ=RES1(J)*RES1(J)
      F1=F1+RSQ
    8 CONTINUE
      IF(F1.GT.F.AND.STEP.LT.ACC)GOTO 9
      IF(F1.GE.F)STEP=STEP*0.5
      IF(F1.GT.F)GOTO 10
      F=F1
      DO 11 I=1,NPAR
      PAR(I)=PAR1(I)
   11 CONTINUE
    2 CONTINUE
    9 IF(NIT.EQ.NITMAX.AND.STEP.GE.ACC)IFAIL=2
      IF(IFAIL.NE.0)RETURN
      CALL RESID(NDAT,NPAR,PAR,RES)
      RETURN
      END
```

Appendix III Derivation of Equation (21)

Consider the situation of two strains of bacteria growing together in a culture. Their growth will be described by some system of equations of the form

$$\frac{dB_1}{dt} = r_2(t)B_1 \qquad (24)$$

$$\frac{dB_2}{dt} = r_2(t)B_2 \qquad (25)$$

where the B_i are the densities of the two strains and the $r_i(t)$ are logarithmic growth rates considered as some function of time.

Add the constraine that that ratio of the two logarithmic growth rates is constant (although the rates themselves may still vary). This is a property of many models of mixed-culture bacterial growth, including solutions of the Monod equations where the constant K is equal to both strains and also models based on the Pearl-Verhulst logistic equation where the equilibrium density in pure culture is equal for both strains. (Where the conditions for constant growth ratio are 'almost' obeyed, it is likely that the ratio of the two growth rates will be 'roughly' constant.) Equation (25) can then be re-written

$$\frac{dB_2}{dt} = r_1(t)B_2(1 - s) \qquad (26)$$

where $s = 1 - \frac{B_2}{B_1}$.

If the exact form of the functions $r_i(t)$ is uncertain, it will obviously be convenient to eliminate them from (24) and (25). This can be done as follows.

$$\frac{d}{dt} \ln B_1 = r_1 \quad ; \quad \frac{d}{dt} \ln B_2 = r_1(1 - s)$$

$$\therefore \quad \ln\left(\frac{B_2}{B_{2,0}}\right) = \left(\ln\left(\frac{B_1}{B_{1,0}}\right)\right)(1 - s)$$

$$\therefore \quad \frac{B_2}{B_{2,0}} = \left(\frac{B_1}{B_{1,0}}\right)^{1-s} \qquad (27)$$

In the competition experiments, the relative frequencies of the two strains in each culture were expressed as the natural logarithm of the ratio R. This was done because, since

$$\frac{d}{dt}\ln R = \frac{d}{dt}\ln \frac{B_1}{B_2} = r_1(t) - r_2(t) \qquad (28)$$

and in batch culture on a daily basis, where the r_i can be expected to approximate to a periodic function of time, ln R can therefore be expected to be roughly linear with respect to time if measurements are taken regularly on time each day.

It is therefore necessary to transform (27) to be expressed in terms of the ratio and the total bacterial density:

$$B_1 = \frac{BR}{1+R} \quad ; \quad B_2 = \frac{B}{1+R}$$

(where $B = B_1 + B_2$)

$$\therefore \quad \left(\frac{B(1+R_o)}{B_o(1+R)}\right) = \left(\frac{RB(1+R_o)}{R_oB_o(1+R)}\right)^{1-s} \qquad \text{(from (27))}$$

$$\therefore \quad \frac{R}{R_o}\left(\frac{RB(1+R_o)}{R_oB_o(1+R)}\right)^{-s} = 1$$

$$\therefore \quad \ln\left(\frac{R}{R_o}\right) - s\ln\left(\frac{RB(1+R_o)}{R_oB_o(1+R)}\right) = 0$$

$$\therefore \quad s = \frac{\ln\left(\frac{R}{R_o}\right)}{\ln\left(\frac{RB(1+R_o)}{R_oB_o(1+R)}\right)} \qquad (29)$$

or, more simply,

$$\frac{R^{1-\frac{1}{s}}}{1+R} - \left(\frac{B_o}{B}\right)\frac{R_o^{1-\frac{1}{s}}}{1+R_o} = 0 \qquad (30)$$

These equations hold for any pair of strains in batch culture where the ratio between the two logarithmic growth rates is constant. Their pertinence in a given case, therefore, does not depend on the strict

pertinence of the Monod equations to the case in question.

When the competition mixture is inoculated into new medium each day, bacterial density is only a continuous function of time over the interval between inoculations. Equation (30) then becomes a difference equation describing the value of R at inoculation time on day t+1 as a function of the value of R at inoculation time on day t:

$$\frac{R_{t+1}^{1-\frac{1}{s}}}{1+R_{t+1}} - \left(\frac{B_o}{\hat{B}}\right)\frac{R_t^{1-\frac{1}{s}}}{1+R_t} = 0 \qquad (31)$$

assuming that the bacterial density reaches the stationary value B within the day. Where the levels of population at stationary phase are not considerably different for the two strains, $\frac{B_o}{\hat{B}}$ can be reckoned as the ratio of inoculum volume to culture volume.

References

Baich, A. and Johnson, M. (1968). Evolutionary advantage of control of a biosynthetic pathway. Nature, 218, pp. 464-5.

Brown, J.W. (1965). Bacterial Genetics, Saunders.

Contesse, G., Crepin, M. and Gros, F. (1970). Transcription of the lactose operon in E. Coli. In Beckwith, J. R. and Zipser, D., The Lactose Operon, Cold Spring Harbor.

Gilbert, W. and Muller-Hill, B. (1970). The lactose repressor. In Beckwith and Zipser, op.cit.

Leahy, J. J. (1969) Cobaltous Ion Effect on Diauxie. J. Bacteriol, 100 (3), pp. 1194-1197.

Lee, I. H., Fredrickson, A. G. and Tsuchiya, H.M. (1974). Diauxic growth of Propionibacterium shermanii. Appl. microbiol. 28 (5), pp. 831-835.

Magasanik, B. (1970). Glucose effects: inducer exclusion and repression. In Beckwith and Zipser, op.cit.

Monod, J. (1949) The growth of bacterial cultures. Ann. Rev. Microbiol., 3, pp. 371-393.

Rosenbrock, H. H. and Storey, C. (1966). Computational Techniques for Chemical Engineers, Pergamon, Oxford.

Zamenhof, S., and Eichhorn, H. H. (1967). Study of microbial evolution through loss of biosynthetic functions: establishment of 'defective' mutants. Nature, 216, pp. 456-458

Zamenhof, S., Heldenmuth, L. H. and Zamenhof, P.J. (1966) . Studies on mechanisms for the maintenance of constant mutability: mutability and the resistance to mutagens. Proc. Nat. Acad. Sci. U.S.A., 55, p. 50.