THE PRODUCTION AND ISOLATION OF MUTANTS OF *Acinetobacter calcoaceticus* NCIB 8250

by

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Thesis Presented for the Degree of

Master of Science,

The

University of Glasgow

September, 1974

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ACKNOWLEDGEMENTS

I would like to thank Professor R.M.S. Smellie for the opportunity to work in the Department of Biochemistry. I extend my sincere gratitude to Dr C.A. Fewson, who has encouraged me over the last two years with his enthusiasm and advice, and by his example. I also thank Dr Fewson for providing continuous-culture samples and Mr I.C. Matson for the *in vivo* mandelate dehydrogenase assays. I would like to thank Dr D.A. Ritchie of the Institute of Virology for his help in establishing a system of genetic recombination for this organism and for his valuable comments during the preparation of this thesis. I am grateful to Dr W.H. Holms and my colleagues in this laboratory for discussion and suggestions arising from this work.

Some of the equipment and materials used in this research were purchased with grants to Dr Holms and Dr Fewson from the Royal Society, the Medical Research Council and the Science Research Council.

Finally, I am grateful to Miss M.T. Emerson for her painstaking work in typing this thesis.

ABBREVIATIONS

With the exception of those listed below, all abbreviations used in this thesis conform to the Biochemical Journal "Policy of the Journal and Instructions to Authors" (1973):

NTG	-	N-methyl-N'-nitro-N-nitrosoguanidine
EMS	-	ethyl methanesulphonate
DCIP	-	phenol-indo-2:6-dichloro-phenol
Carboxy-Q	-	N-ethyl-2-carboxy-7-chloro-4-quinolone

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SUMMARY

The work described in this thesis was undertaken in an attempt to establish systems for the induction, selection and isolation of mutants for the bacterium Acinetobacter calcoaceticus NCIB 8250. This study formed part of a general biochemical and genetic investigation of the control of aromatic carbon compound catabolism and involved a systematic analysis of mutagenesis, and the development of methods for the enrichment and isolation of both non-specific mutants (e.g. amino acid auxotrophs) and specific mutants affecting enzymes catalysing particular reactions in the catabolism of aromatic carbon compounds.

A study was made of the mutagenic and lethal effects of N-methyl-N'-nitro-N-nitrosoguanidine, ultraviolet light irradiation, ethyl methanesulphonate and near ultraviolet light irradiation with 8-methoxypsoralen. In each case the percentage of auxotrophs obtained following exposure to these treatments was used as the measure of their mutagenic efficiencies. All four agents were found to be mutagenic, however, ethyl methanesulphonate and N-methyl-N'-nitro-N-nitrosoguanidine, although more effective than irradiation, did not cause such a high frequency of mutation as has been observed with other bacteria.

A range of antibiotics was screened for their suitability for use in the selection of mutants. Vancomycin and penicillin V, which both inhibit bacterial wall synthesis, proved most suitable particularly when used together. Vancomycin causes a much greater inactivation of

growing bacteria than non-growing bacteria, and penicillin V was found to protect non-growing bacteria from even the small effects of vancomycin. The various parameters of a selection system, such as substrate and antibiotic concentration, length of antibiotic treatment and ratio of live to inactivated bacteria during antibiotic treatment, were tested. Using reconstruction experiments the vancomycin and vancomycin/penicillin V selection systems were further developed for the isolation of auxotrophs and for mutants blocked in the catabolism of aromatic compounds. Cysteine, tyrosine, isoleucine and isoleucine/valine requiring auxotrophs and mutants blocked at "benzoate oxidase", between catechol 1,2-oxygenase and β-ketoadipate enol-lactone hydrolase and at or below β-ketoadipate succinyl-CoA transferase were isolated and partially characterised by growth experiments.

Streptomycin-resistant mutants were obtained by a positive selection method. Positive selection of blocked aromatic mutants with p-fluoro-analogues of substrates was, however, not successful.

A continuous-culture method was used for isolating a mutant strain mesoconstitutive for L-mandelate dehydrogenase but failed to select for magnoconstitutive mutants.

Multiple mutants were constructed from existing single mutants by genetic transformation. One class of these grew on D-mandelate but not on L-mandelate: this gave proof that the selection of mutant strains of A. calcoaceticus able to

grow on D-mandelate is due to the presence of a D-mandelate dehydrogenase and not to the presence of a mandelate racemase.

The results of this work provide general methods suitable for the isolation of various types of mutant for use in the genetic analysis of this organism.

INTRODUCTION

1. The Organism

Happold and Key (1932) isolated from sewage a Gram-negative vibrio which was capable of catabolising monohydroxyphenols. This organism, named "Vibrio O1", was used in early work on the metabolism of aromatic compounds (e.g. Evans, 1947; Dagley *et al.*, 1952). However, Fewson (1967a) found that the "Vibrio O1" strain stored by the National Collection of Industrial Bacteria (as NCIB8250) was basically different from the bacterium isolated by Happold and Key and NCIB8250 was subsequently placed in the *Acinetobacter*-*Moraxella* group (Sebald and Véron, 1963; Fewson, 1967a). Baumann *et al.* (1968) later described it as a biotype of the species *Acinetobacter calcoaceticus*.

A. calcoaceticus is a non-flagellated, non-sporeforming bacterium, rod shaped during exponential growth and becoming smaller and more spherical in stationary phase. Cells usually occur in pairs during exponential phase but single cells may be found in stationary phase. The species has the following characteristic properties: generally Gram-negative but sometimes "Gram-variable"; does not accumulate polyhydroxybutyrate or glycogen as a reserve material; is obligately aerobic, chemoorganotrophic and incapable of denitrification; will grow in mineral media with single carbon sources and either ammonia or nitrate as sole nitrogen source and is commonly isolated from soil and fresh water (Baumann, 1968). The species contains a wide spectrum of strains, some of which can utilize a wide

variety of organic compounds as carbon sources (Baumann et al., 1968; Fewson, 1967b; Grant, 1973) including hydrocarbons, aliphatic acids and alcohols, amino acids, pentose sugars and aromatic compounds and for this reason they are being increasingly studied. They cannot use methanol, formate, glycerol, sugar-alcohols, disaccharides or polysaccharides.

Fewson (1967b) showed that strain NCIB8250 of A. calcoaceticus was capable of growth on over 100 carbon compounds which served as carbon and energy sources, 30 of which were aromatic, and could use almost 50 compounds as carbon and nitrogen sources. Cook and Fewson (1973) showed that this strain was unable to utilize carbohydrates. Experiments on the carboxylic acid metabolism of this organism have shown the importance of the Krebs cycle in A. calcoaceticus (Dagley and Patel, 1955; Dagley, 1956; Callely et al., 1958; Chapman and Duggleby, 1967). Studies have been made of its ability to oxidise liquid paraffin (Linday and Donald, 1961), ubiquinone biosynthesis (Whitstance et al., 1970) and of its electron transport system (Whittaker, 1967). This strain has also been used for studies on the metabolism of aromatic compounds (e.g. Kilby, 1951; Kennedy and Fewson, 1968a+b; Livingstone et al., 1972; Livingstone and Fewson, 1972; Cook and Fewson, 1972; Callely and Jones, 1965).

It is largely because of their metabolic versatility that strains of A. calcoaceticus are being increasingly studied, especially their metabolism of aromatic compounds.

In Pseudomonas spp. many similar compounds are oxidised and passed into the Krebs cycle by sequentially inducible and converging enzyme pathways (e.g. Gunsalus et al., 1953a, b; Stanier et al., 1953; Hegeman, 1966a,b,c; Ornston and Stanier, 1966; Cánovas et al., 1967). A. calcoaceticus provides another system for the study of inducible complex enzyme systems and is also of interest for the comparisons which can be drawn between it and the other organisms which utilize the same substrates (see Introduction, section 5).

2. Mutation in Acinetobacter

Bacterial mutants are not only necessary for genetic analysis but have proved to be invaluable for the investigation of metabolic processes. Several authors (Cánovas and Stanier, 1967; Herman and Juni, 1974; Sawula et al., 1972; Livingstone and Fewson, 1972; Livingstone et al., 1972) have obtained various classes of Acinetobacter mutants induced by treatment with NTG (100 μg/ml) in 10 mM-citrate buffer, pH 6.0. At this pH NTG decomposes to diazomethane which is now considered to be the main agent of mutagenesis by NTG (Cerdá-Olmedo and Hanawatt, 1968). Diazomethane exerts its mutagenic effect by methylation of all four DNA bases at several positions (Holy and Scheit, 1966). At this concentration NTG halts the movement of the DNA replication region in Escherichia coli, therefore mutation occurs selectively at the replication point and when multiple mutations occur they are closely linked (Cerdá-Olmedo et al., 1968; Geurola et al., 1971). Misleading information could be obtained from genetic crosses involving

mutants with unidentified extra mutations in one gene or closely-linked genes. For this reason it is advisable to use NTG at levels low enough to avoid the induction of closely-linked multiple mutations.

Ultraviolet radiation has been used without success in this bacterium by Livingstone (1970) at a surviving fraction of 0.1%. No other mutagen appears to have been tried with this organism and no mutagen has ever been systematically studied in Acinetobacter. In all the above-mentioned papers on Acinetobacter the mutagenic techniques were taken directly from those used with other bacteria. Delić et al. (1970) showed, with their study of the effect of NTG on Streptomyces spp., that methods devised for one organism will not necessarily give the same results in another organism. Because of this potential variability it was thought that a systematic study of mutagenesis in A. calcoaceticus was desirable.

Other possible mutagens include ionizing radiations, nitrous acid, alkylating agents, base analogues, frame-shift mutagens (acridines and ICR compounds) and the photo-sensitizing compound 8-methoxypsoralen. The use of all but 8-methoxypsoralen have been reviewed by Hopwood (1970). The choice of mutagen to be studied in Acinetobacter depended on the convenience of the mutagenic technique and on the effectiveness of the mutagen in other organisms. In addition to u.v. and NTG it was decided to study EMS, an alkylating agent which has been used successfully in a number of bacteria (Loveless and Haworth, 1959;

Zamenhof and Arikawa, 1970; Nečásek et al., 1966), and 8-methoxypsoralen which has recently been used to obtain mutants in actinomycetes and fungi (Scott and Alderson, 1971; Townsend et al., 1971).

3. Selection of Mutants

Having found a suitable mutagen the next problem is selection of the desired mutant from large numbers of wild-type bacteria, general methods of selection have been reviewed by Schlegel and Jannasch (1967). To isolate mutants unable to grow on certain media the penicillin technique (Davis, 1948; Lederberg and Zinder, 1948), and modifications of it, has been widely used. The technique exploits the fact that some antibiotics will kill growing bacteria to a much greater extent than non-growing bacteria. The addition of antibiotics to media in which the desired mutant cannot grow will therefore produce an enrichment of the mutants relative to the wild-type. Antibiotics which interfere with bacterial-wall synthesis, such as bacitracin, vancomycin, ristocetin A and B and the penicillins, are particularly suitable for this technique. However, other types of antibiotic have also been used successfully. Actinobacillus mallei mutants have been selected with Kanamycin (Evans, 1966) which interferes with RNA metabolism and Pseudomonas aeruginosa mutants have been selected with the protein synthesis inhibitor dihydrostreptomycin (Ishida et al., 1966). One drawback to this technique is cross-feeding which can occur if the antibiotic lyses growing bacteria thereby releasing nutrients which may allow growth of mutants, particularly

auxotrophs. Growing mutants are then killed by the antibiotic. This kind of cross-feeding can be reduced by lowering the bacterial density; Davis (1950) recommended that the density should not be more than 10^7 bacteria/ml. If the mutants are defective in substrate catabolism, difficulties arise if the growing bacteria accumulate substrates on which the mutants can grow. It is doubtful whether reducing the bacterial density would be helpful in such a case.

Very little has been published on selection methods for Acinetobacter. Livingstone (1970) attempted to isolate mandelate pathway mutants using penicillin V (1,000 μg/ml) or Carboxy-Q (90 μM) as selective agents, but did not obtain many mutants and did not assess the degree of enrichment obtained. Herman and Juni (1974) used a combination of cycloserine (400 μg/ml) and ampicillin (2,000 μg/ml) to select auxotrophs, but gave no indication of the degree of enrichment. The first step in developing a selection system for A. calcoaceticus would be a survey of its sensitivity to antibiotics since no such study has been published.

Mutants which have gained a property, e.g. antibiotic resistance or the ability to utilize a new substrate, can be selected from very large numbers of wild-type since the addition of the antibiotic or substrate will permit the mutants to overgrow the wild-type. This method has been used to isolate D-mandelate utilizing mutants of NCIB 8250 (Lancaster, 1970). A similar kind of positive selection

can be obtained using toxic growth substrate analogues as selective agents. If the analogue prevents the growth of the majority of organisms in the population but does not inhibit the desired mutants then the mutants will overgrow the other bacteria. Clarke and Tata (1973) used fluoroacetamide to isolate amidase-negative mutants of Pseudomonas aeroginosa from a population of constitutive amidase mutants. This method has not yet been used for the isolation of Acinetobacter mutants.

The alternate-culture technique (Cohen-Bazire and Jolit, 1953) has been used successfully for the isolation of constitutive mutants in some bacteria. This method is based on the growth-lag exhibited by inducible wild-type bacteria during the induction of enzymes necessary for growth. Bacteria are passaged in medium for which the wild-type must induce enzymes, then in medium in which wild-type bacteria lose their complement of induced enzymes. Constitutive mutants, having neither to induce the necessary enzymes nor to lose them in the absence of inducer, do not have a growth-lag when put into the inducing medium and eventually overgrow the wild-type after several alternating cycles in the two media. This method was used by Livingstone (1970) to isolate mesoconstitutive L-mandelate dehydrogenase mutants of A. calcoaceticus though it failed to select magnoconstitutive mutants.

Two similar methods for isolating constitutive mutants make use of non-inducing substrates and anti-inducers (e.g. Buttin, 1963). An anti-inducer will prevent the growth of

uninduced wild-type bacteria in the presence of the substrate but will not affect the growth of a constitutive mutant. Similarly a non-inducing substrate will only allow the growth of constitutive mutants. Neither non-inducing substrates nor anti-inducers are known for the aromatic catabolism enzymes of A. calcoaceticus.

A fourth method for the isolation of constitutive mutants is continuous-culture when the appropriate substrate is chosen as the growth-limiting factor. At low substrate concentrations the growth rate of constitutive mutants is proportional to the substrate concentration. Inducible bacteria require the substrate for induction as well as growth and their growth rate is comparatively low at low substrate concentrations. In continuous-culture with the growth-limiting substrate at low concentration the constitutive bacteria overgrow the inducible bacteria (Novick and Horiuchi, 1961; Horiuchi et al., 1962). This method has not yet been used for isolating constitutive A. calcoaceticus mutants and it would be interesting to try it.

4. Genetic Recombination in Acinetobacter

Genetic analysis is facilitated if a system of genetic recombination is available. Therefore, as well as finding ways to make and isolate mutants it is also necessary to identify and develop a method of recombination for A. calcoaceticus. Three systems for genetic analysis are known in bacteria:

(i) direct transfer of DNA from one bacterium to another (conjugation), (ii) transfer of bacterial DNA by a bacteriophage vector (transduction) and (iii) uptake of naked DNA from solution (transformation). No evidence for conjugation in Acinetobacter and related genera has been published. A preliminary search for conjugation in strain NCIB 8250 failed to produce any recombinants and an attempt to make F^- strains, as found in coliform bacteria, with acridine orange was also unsuccessful in producing recombinants (E.F. Ahlquist, unpublished work). Transduction has been reported for a limited number of Acinetobacter strains (Herman and Juni, 1974; Twarog and Blouse, 1968), however, all Acinetobacter phage isolated so far have been strain-specific and could not be used with NCIB 8250. Searches for NCIB 8250-specific phage have been unsuccessful so far (E.F. Ahlquist, unpublished work) but there is no reason to suppose that such a phage does not exist.

Transformation has been shown to be a common form of genetic exchange in Acinetobacter. Juni (1972), using a plate transformation assay in which bacterial paste was streaked on agar with transforming DNA, reported transformation between many Acinetobacter strains. Transformation has also been demonstrated in the related genus Moraxella; Bøvre and Henriksen (1962) and Frøholm and Bøvre (1973) showed that fimbriated moraxellas had some advantage for transformation over non-fimbriated moraxellas. Sawula et al (1972) developed a quantitative transformation

assay with A. calcoaceticus BD 413 and used it to map the tryptophan genes. Stationary-phase bacteria were inoculated into broth and grown for 2 h to develop competence for transformation, they were then washed and spread on selective media with transforming DNA. A similar system has been developed for NCIB 8250 (E.F. Ahlquist, unpublished work); stationary-phase bacteria growing in stirred nutrient broth attained a peak of competence after 6½ h incubation at 30°C, this time corresponding to the end of exponential-phase. By concentrating lag-phase bacteria before plating, Podmore (unpublished results) found a short-lived peak of high competence after 1½ h incubation. The numbers of transformants obtained during these two peaks of competence make this system suitable for genetic analysis. It would be interesting to see if transformation could be used to construct multiple A. calcoaceticus mutants from existing single mutants.

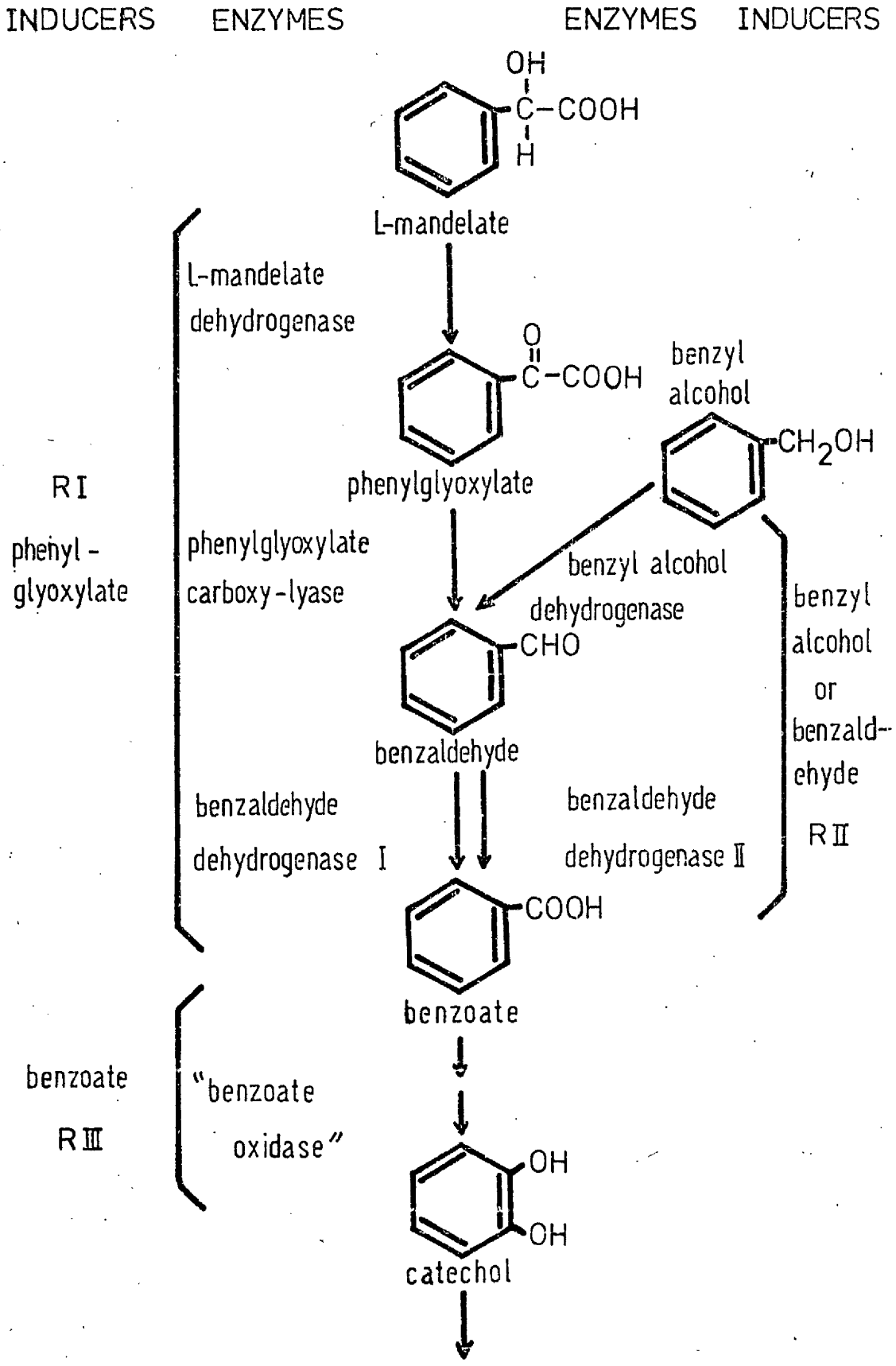
5. Metabolism of Aromatic Compounds by A. calcoaceticus

The aromatic catabolism of A. calcoaceticus NCIB 8250 is being studied in this laboratory and part of this thesis is concerned with the selection of mutants of aromatic catabolism. Therefore, the following summary has been included to describe current knowledge of this field in Acinetobacter.

In the breakdown of an aromatic compound by any micro-organism the aromatic ring has to be opened to allow complete metabolism. Ring cleavage is effected through either ortho- or meta-dihydroxyphenols, therefore the initial stages

Fig.1

Regulation of the mandelate and benzyl alcohol pathways in A.calcoaceticus NCIB 8250

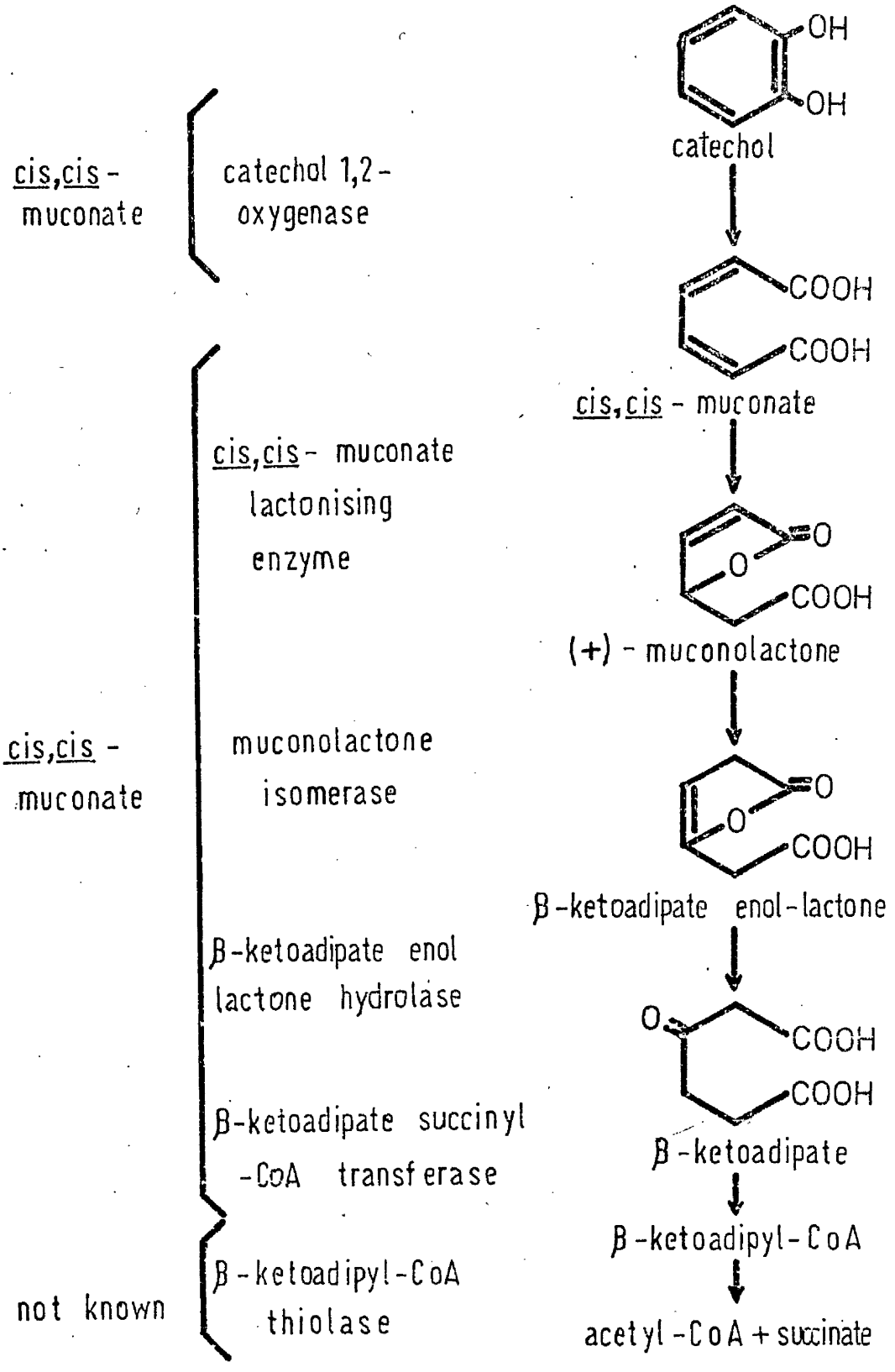


of the breakdown pathway must be directed towards the production of such a compound (Ribbons, 1965). A variety of microorganisms can oxidise aromatic compounds, the list includes Pseudomonas spp. (Stanier et al., 1966), A. calcoaceticus (Cánovas and Stanier, 1967; Fewson, 1967b; Grant, 1973), Alcaligenes eutrophus (Johnson and Stanier, 1971) and Norcardia (Rann and Cain, 1969). Members of all four fungal groups also metabolise aromatic compounds.

The breakdown of the aromatic compounds L (+) - mandelic acid and benzyl alcohol have been studied in this laboratory. In both A. calcoaceticus (Kennedy and Fewson, 1968a,b) and Pseudomonas putida (Gunsalus et al., 1953a,b; Stanier et al., 1953) L(+)-mandelate is dehydrogenated to phenylglyoxylate which is then decarboxylated to benzaldehyde (Fig. 1). Benzaldehyde is converted to benzoate in A. calcoaceticus by two NAD^+-linked benzaldehyde dehydrogenases, while in P. putida there is one NAD^+-linked and one $NADP^+$-linked benzaldehyde dehydrogenase. Benzoate is then converted in both organisms to catechol. P. putida can metabolise D(-)-mandelic acid by converting it to the L(+) isomer with a racemase, however, wild-type A. calcoaceticus cannot use D(-)-mandelate although a D(-)-mandelate utilizing strain has been isolated (Lancaster, unpublished results). In A. calcoaceticus benzyl alcohol is dehydrogenated to benzaldehyde via benzyl alcohol dehydrogenase (Fig. 1) and the benzaldehyde is then oxidized.

Fig. 2

Regulation of the catechol pathway in A. calcoaceticus



Ortho-dihydroxyphenols can be split between two adjacent hydroxylated carbon atoms (ortho-cleavage) or between a hydroxylated and a non-hydroxylated carbon atom (meta-cleavage). Ortho-cleavage leads to the formation of a muconic acid which is then metabolised to β-ketoadipate and cleaved to succinate and acetyl-CoA. In A. calcoaceticus, as in P. putida, ortho-cleavage of catechol occurs (Canovas et al., 1967) to cis,cis-muconate then (+)-muconolactone (Evans and Smith, 1951; Evans et al., 1951) (Fig. 2). The next intermediate is β-ketoadipate enol-lactone (Ornston and Stanier, 1966) and finally β-ketoadipate (Kilby, 1948; Stanier et al., 1950).

In A. calcoaceticus the enzymes L-mandelate dehydrogenase, phenylglyoxylate carboxy-lyase and benzaldehyde dehydrogenase I are coordinately regulated (Livingstone and Fewson, 1972), phenylglyoxylate being the primary inducer of the regulon (known as the RI regulon; Fig. 1). Benzaldehyde dehydrogenase II and benzyl alcohol dehydrogenase are also coordinately regulated (Livingstone et al., 1972), both benzyl alcohol and benzaldehyde acting as inducers of the regulon (known as the RII regulon). The term operon is not used (Maas and McFall, 1964) (Livingstone, 1970) since as yet there is no evidence as to the relative location of these genes. "Benzoate oxidase" is induced by benzoate or 2-hydroxybenzoate and is controlled by a separate regulatory unit from the RI and RII regulons (Kennedy and Fewson, 1968b) which is referred to as the RIII regulon. "Benzoate oxidase" may not be a single enzyme, the reaction may involve the intermediate formation of 3,5-cyclohexadiene-1,2-diol-

1-carboxylate (Reiner, 1971; Reiner and Hegeman, 1971). Very few data have been obtained on the regulation of enzymes below "benzoate oxidase" in this strain of A. calcoaceticus, however, Cánovas and Stanier (1967) studied the regulation of the catechol pathway in a related organism, A. calcoaceticus strain ATCC 23393. They found that catechol oxygenase formed a regulatory unit induced by cis,cis-muconate and that muconate lactonising enzyme, muconolactone isomerase, β-ketoadipate enol-lactone hydrolase and β-ketoadipate succinyl-CoA transferase formed a second regulatory unit induced by cis,cis-muconate. Unlike ATCC 23393, NCIB 8250 can grow on β-ketoadipate (Dr C.A. Fewson, unpublished work) and similar behaviour is shown by the closely related strain NCIB 10553 (Grant, 1973). These two strains must be permeable to β-ketoadipate which probably acts as an inducer of at least β-ketoadipate succinyl-CoA transferase. NCIB 8250 has also been shown to possess a catechol 1,2 oxygenase rather than a catechol 2,3 oxygenase (Kennedy and Fewson, 1968b).

6. The Scope of this Thesis

The aims of the work presented in this thesis were as follows:

(i) To make a systematic study of mutagenesis in A. calcoaceticus.

(ii) To find suitable antibiotics for use in a selection system of the "penicillin technique" type.

(iii) To develop selection systems for the isolation of (a) auxotrophs for use in the development of a system for genetic analysis and in physiological experiments, (b) blocked and constitutive mutants of aromatic catabolism for use in genetic mapping and physiological experiments and (c) antibiotic resistant mutants for use in the construction of multiple mutants (see iv) and in genetic mapping.

(iv) To investigate the possibility of constructing double and triple mutants by transformation for use in genetic mapping and physiological experiments.

MATERIALS AND METHODS

1. Bacteriological Methods

1.i. Bacterial strains

Acinetobacter calcoaceticus strain NCIB 8250 was obtained originally from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen. The mutant strains used in this work, and their derivation, are listed in Table 1.

1.ii. Nomenclature of mutant strains

Mutants isolated during this work have been given a three component name. The first letter indicates either the number of known genetic defects that the strain contains or if there is only one mutation this letter indicates the origin of the mutation:

single mutation induced by u.v. irradiation	-	U
single mutation induced by EMS	-	E
single mutation induced by NTG	-	N
single spontaneous mutation	-	S
double mutation	-	D
triple mutation	-	T

The number denotes the number of the experiment in which the mutant was isolated and the final letter distinguishes mutants isolated in the same experiment.

Abbreviations for phenotypic characters are listed in Table 1.

Table 1 Mutants of *A. calcoaceticus* NCIB 8250

Mutant strain	Description and abbreviations used in text		Source, if not isolated in this work
U4K	requires tyrosine for growth	Tyr^-	
U4A	requires isoleucine for growth	$Ileu^-$	
NF1408	lacks L-mandelate dehydrogenase	$L\text{-}madh^-$	Livingstone (1970)
NA36	lacks phenylglyoxylate carboxy-lyase	PgC^-	Livingstone (1970)
NA37	lacks benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II	Bdh^-, $BadhII^-$	Livingstone (1970)
41 41Z 41Y	possess D-mandelate dehydrogenase	$D\text{-}mdh^+$	Beggs (1970)
D40E D40F D40G	possess D-mandelate dehydrogenase, lack L-mandelate dehydrogenase	$D\text{-}mdh^+$, $L\text{-}mdh^-$	
D35A	streptomycin resistant, lacks phenylglyoxylate carboxy-lyase	SmR, PgC^-	

Mutant strain	Descriptions and abbreviations used in text		Source, if not isolated in this work
T36A	lack L-mandelate dehydrogenase		
T36B	and phenylglyoxylate carboxy-lyase	L-mdh$^-$, PgC$^-$	
E48F	unidentified impairment of benzyl alcohol metabolism	—	
E59M	presumed block at "benzoate oxidase"	—	
E59L E59N E59P E59Q E59R E59S E59T	blocked between catechol 1,2 oxygenase and β-ketoadipate enol-lactone hydrolase blocked at or below β-ketoadipate succinyl -CoA transferase	— —	
S25F	constitutive L-mandelate dehydrogenase		
C04211	mesoconstitutive L-mandelate dehydrogenase	—	Livingstone (1970)

1.iii. Storage of bacteria

Bacterial strains were maintained in cooked meat medium (10 ml) in rubber-sealed McCartney bottles stored at 4°C. Cultures to be used as starting inocula were subcultured into nutrient broth (10 ml), incubated overnight at 30°C, and stored at 4°C for 4 to 6 months.

1.iv. Growth media

Bacteria in liquid culture were grown in nutrient broth or in minimal salts medium [basal medium containing $MgSO_4.7H_2O$ (0.04% w/v) plus a carbon and energy source]. Basal medium consisted of KH_2PO_4 (2 g/l) and $(NH_4)_2\ SO_4$ (1 g/l) dissolved in glass-distilled water and adjusted to pH 7.0 with 5M-NaOH. After sterilization of basal medium 20 ml/l of $MgSO_4.7H_2O$ (2% w/v) was added (Fewson, 1967).

Solid media were nutrient agar, or minimal salts media solidified with agar (7.5 g/l). All media not used immediately were stored at 4°C.

1.v. Sterilization

Nutrient agar, nutrient broth, basal medium and apparatus were steam sterilized at 120°C. Basal medium containing heat-stable carbon sources and $MgSO_4.7H_2O$ (2% w/v) were separately steam sterilized at 109°C. Heat-labile carbon sources, antibiotics and amino acids were filter sterilized through Millipore filters (GSWP 04700, 0.22 μ) in Sterifil units (xxl10 4700, Millipore Corp.,

Bedford, Mass., USA).

1.vi. Measurement of bacterial growth and viability

The titre of viable bacteria in liquid cultures was determined by spreading samples diluted in basal medium onto nutrient agar plates. The Miles and Misra colony counting technique (Mackie and McCartney, 1953) was used where only a rough estimate of viable bacteria was required. Plates were incubated at 30°C.

For rapid estimations of bacterial numbers cell turbidity was measured at 500 nm in a Spectronic 20 colorimeter (Bausch and Lomb Inc., Rochester, New York). Curves relating numbers of viable bacteria to E500 were constructed for three growth media (Fig. 3).

1.vii. Growth of bacteria for mutation and antibiotic treatment

0.1 ml of a nutrient broth culture of A. calcoaceticus was inoculated into 100 ml of nutrient broth in a 500 ml Erlenmeyer flask plugged with a disposable bung (Dispo Plugs, T.1385, American Hospital Supply Corp., Edison, New Jersey, USA) and incubated at 30°C for 24 h without shaking. 0.1 ml of this culture was then used to inoculate 50 ml of 5mM-L-glutamate or 10 mM-succinate salts medium in a 250 ml Erlenmeyer flask which was shaken for 16 to 17 h on a rotary shaker (L.H. Engineering Co., Bells Hill, Stoke Poges, Bucks., Mk V) moving at about 180 oscillations/min. From this culture a 4 ml aliquot was inoculated into 50 ml of the same medium in a 250 ml Erlenmeyer flask and grown to a

Fig. 3

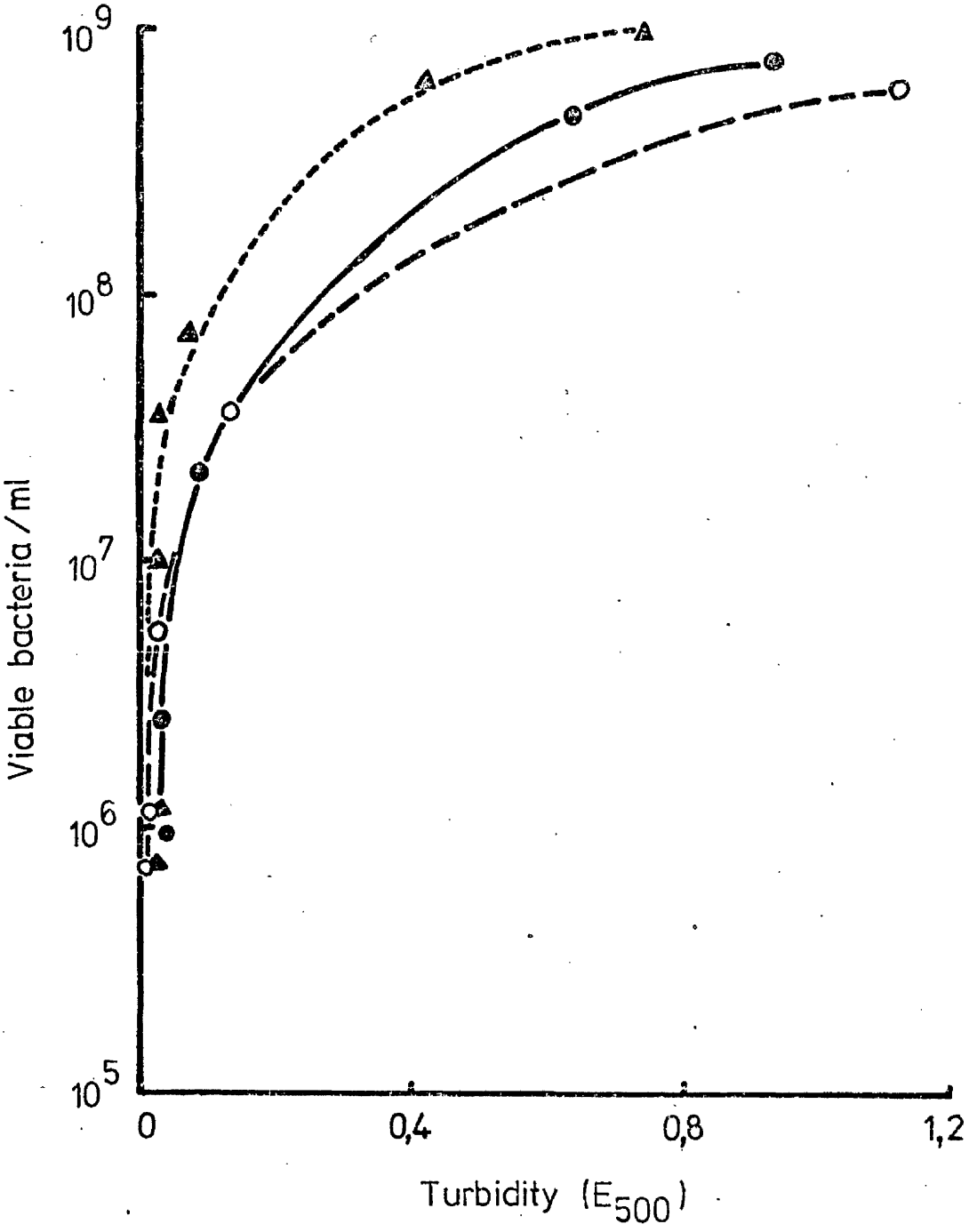
The relationship between turbidity (E_{500}) and viable count for bacteria growing on three different media.

500 ml volumes of nutrient broth, 10 mM-succinate-salts medium and 10 mM-D,L-mandelate-salts medium in 2 l side-arm flasks were each inoculated with 0.5 ml of stationary phase bacteria (grown in nutrient broth (50 ml) with shaking for 17 h at 30°C). The three cultures were stirred rapidly at 30°C and at intervals samples were assayed for turbidity and viable count.

● - nutrient broth

○ - 10 mM-succinate-salts medium

▲ - 10 mM-D,L-mandelate-salts medium


10^9
10^8
10^7
10^6
10^5
Viable bacteria / ml
0
0,4
0,8
1,2
Turbidity (E_{500})

density of 10^8 bacteria/ml by shaking at 30°C for 4½ h.

For larger cultures, 1 ml of a 24 h nutrient broth culture, prepared as described above, was inoculated into 750 ml of 5mM-L-glutamate-salts medium in a 2 l side-arm flask and aerated at a rate of 200 ml/min. The medium was stirred by means of a 45 mm magnetic stirring bar so that the vortex produced by stirring extended to the bottom of the flask and was disturbed by the bar, causing bubbles to enter the body of the medium (Harvey et al., 1968). The medium was incubated for 13 to 14 h at 30°C, at which time the concentration of bacteria was 1 to 5 x 10^8/ml. Four litre cultures were similarly prepared except that 10 l flasks were used, the cultures were inoculated with 5 ml of bacteria and incubation was for 20 h.

1.viii. Harvesting bacteria by centrifugation

Unless otherwise stated, bacteria were centrifuged in sterile polycarbonate tubes (50 ml) for 15 min at 12,000 g (10,000 rpm) in the 8 x 50 ml angle rotor of an MSE 18 centrifuge operating at 4°C.

1.ix. Growth of bacteria competent for transformation

Three millilitres of a stationary phase nutrient broth culture was added to 100 ml of nutrient broth in a 500 ml Erlenmeyer flask equipped with a stirring bar. The flask was incubated in a water bath with a magnetic stirring-drive assembly (Harvey et al., 1968) in which the temperature was maintained at 30°C by a Circotherm IIa constant temperature unit (Shandon Scientific Co. Ltd, London).

The broth was stirred so that a vortex formed but did not break over the stirring bar. Peaks of competence occurred after 1 h and 6½ h incubation. Bacteria competent at 6½ h could be used for transformation directly from the flask (E.F. Ahlquist, unpublished work), those competent at 1 h were concentrated by centrifugation before use (Podmore, 1974).

For transformation, 0.1 ml of DNA (50 to 100 μg/ml) was mixed with 0.1 ml of competent bacteria on the desired selective medium.

1.x. Isolation of transforming DNA

DNA was isolated by a modification of the Marmur (1961) method. Bacteria were grown in 4 l of 5mM-benzyl alcohol-salts medium and 600 ml amounts were harvested in 750 ml polythene centrifuge bottles at 5,200 rpm in an MSE 6L centrifuge operating at 4°C for 30 min. For each DNA extraction 1.5 g wet weight of bacteria were used, equivalent to 1,800 ml of the culture. The bacteria were washed in 20 ml of saline-EDTA (0.15 M-NaCl + 0.1 M-EDTA, pH 8.0), centrifuged, resuspended in 25 ml of saline EDTA in a stoppered conical flask (100 ml) and lysed by incubation for 10 min at 60°C following the addition of 2.0 ml of sodium lauryl sulphate (25% w/v). Six millilitres of 5M-sodium perchlorate and 30ml of isoamyl alcohol/chloroform (1/24 v/v) were then added and the flask was gently shaken by hand for 30 min. This mixture was centrifuged in Corex-glass centrifuge tubes (30 ml) at 10,000 rpm in the 8 x 50 ml angle rotor of an MSE 18 centrifuge, the upper aqueous fraction was removed by pipette into a beaker and the

nucleic acid was precipitated by the slow addition of 2 volumes of absolute ethanol. The mixture was stirred slowly with a glass rod onto which the DNA spooled. The DNA was redissolved in 4.5 ml of dilute saline sodium citrate (0.015 M-NaCl + 0.0015 M-sodium citrate, pH 7.0) in a McCartney bottle and 0.5 ml of concentrated saline sodium citrate (1.5 M-NaCl + 0.15 M-sodium citrate, pH 7.0) was added. Finally the bottle of DNA was heated at 60°C for 10 min to kill any remaining bacteria.

DNA concentrations were determined by comparison of the adsorption at 260 nm with that of a standard curve of calf-thymus DNA dissolved in standard saline citrate (0.15 M-NaCl + 0.015 M-sodium citrate, pH 7.0). This extraction technique gave DNA at concentrations of about 0.5 to 1.0 mg/ml.

1.xi. Assay for auxotrophs

To assay all classes of auxotrophs in a population of wild-type bacteria, samples were diluted and spread on nutrient agar to give between 100 and 500 colonies/plate. To assay specific auxotrophs the samples were plated on 10 mM-succinate-salts agar supplemented with the appropriate growth factor (amino acids were used at a concentration of 100 μg/ml). In both cases the colonies were replicated with sterile velvet (Lederberg and Lederberg, 1952) onto 10 mM-succinate-salts agar on which auxotrophs are unable to grow. Non-replicating colonies were streaked on 10 mM-succinate-salts agar as a final check. Auxotrophs were further characterised by the pool-plate method of Holliday (1956).

1.xii. Enzymological techniques

The DCIP tube-test (Livingstone, 1970) was used to screen bacteria for L-mandelate dehydrogenase activity. Individual colonies were picked into 3 ml of 5 mM-L-glutamate-salts medium in test tubes and grown for 48 h at 30°C. The bacteria were centrifuged, washed, recentrifuged, resuspended in 3 ml of basal medium and decryptified (cell membrane perforated) with 0.5 ml of toluene in ethanol (2% v/v). L-mandelate (500 μM) and DCIP (67 μM) were then added and the tubes left for 3 h at room temperature to allow the reaction to develop. Decolourization (reduction) of DCIP was examined visually with reference to induced and uninduced wild-type controls.

Enzyme activities were assayed *in vivo* with an oxygen electrode (Rank Brothers, Bottisham, Cambridge, UK). Reaction mixtures (3 ml) contained bacteria suspended in basal medium at a turbidity of 0.5 to 1.0. Substrates were added to a final concentration of 0.5 mM and rates of O_2 uptake were corrected by subtraction of endogenous respiration rates.

L-mandelate dehydrogenase, benzaldehyde dehydrogenase II and benzyl alcohol dehydrogenase activities were measured spectrophotometrically by the methods of Beggs (1974). Assays were conducted in 10 mm path-length silica or quartz cuvettes and extinction measurements were made with a Unicam SP800 spectrophotometer (Pye Unicam Instruments Ltd, Cambridge, UK) connected to a servoscribe chart recorder (Smiths Industries Ltd, Wembley, Middlesex, UK). Bacteria,

decryptified with toluene, were added to a final reaction mixture of 3 ml and reactions were started by the addition of substrate. In the L-mandelate dehydrogenase assay the reduction of DCIP was followed at 600 nm. For the other two assays NAD^+ reduction was followed at 340 nm.

1.xiii. Characterisation tests for A. calcoaceticus mutants

Tests for carbon-source utilization and bacteriological tests were from Fewson (1967). The pattern of utilization of the ten carbon sources listed in Table 2 is unique to wild-type A. calcoaceticus NCIB 8250 (Fewson, 1967b; Baumann et al., 1968).

The other bacteriological tests were:

(i) production of acid and gas from glucose, lactose, sucrose and dulcitol in peptone water + phenol red. The wild-type does not produce acid or gas from these sugars;

(ii) acetoin production (Voges-Proskaur test), the wild-type is V.P. negative;

(iii) Koser's citrate test, the wild-type slowly uses citrate as a carbon source;

(iv) indole production, the wild-type is indole negative;

(v) Christensen's urease test, the wild-type is urease negative;

Table 2

The pattern of utilization of the test carbon sources by wild-type *A. calcoaceticus* NCIB8250.

The carbon sources, dissolved in 50 ml of salts medium in Erlenmeyer flasks (250 ml), were inoculated with 0.1 ml of a 24 h nutrient broth culture. The flasks were shaken at 30°C for three days, the turbidity in each flask being recorded every day.

Carbon source	Conc. of carbon source (mM)	Growth after 3 days
L(+) mandelic acid	2.5	+
D(-) mandelic acid	2.5	-
phenylglyoxylate	2.0	+
benzoic acid	2.0	+
benzyl alcohol	5.0	+
benzaldehyde	2.0	+
p-hydroxybenzoic acid	0.5	+
m-hydroxybenzoic acid	0.5	-
salicylic acid	0.5	+
L-arginine mono HCl	1.5	-

(vi) Kovac's oxidase test, the wild-type is oxidase negative.

2. Mutagenesis

Bacteria for mutagenic treatments were grown in 5mM-L-glutamate-salts medium as described in Methods, section 1.vii, and were concentrated by centrifugation to a density of about 3 x 10^9/ml after resuspension.

2.i. Mutagenesis by ultraviolet light irradiation

Ten millilitre samples of bacteria suspended in basal medium were irradiated in a glass petri dish using a Hanovia model 13 bactericidal lamp (Engelhard Hanovia Lamps, Slough, Bucks., UK) as the u.v. source. During irradiation the bacteria were magnetically stirred to facilitate mixing. Irradiation was started by removal of the petri dish lid and stopped by its replacement. To prevent photoreactivation all manipulations were carried out in the light of a sodium lamp.

2.ii. Mutagenesis by EMS

Millipore-filtered 625 mM- EMS was added to the bacteria suspended in 4 to 8 ml of basal medium in polycarbonate centrifuge tubes. The bacteria were incubated at 30°C (length of incubation time depending on the final EMS concentration and the desired level of mutation) then centrifuged, washed and resuspended in basal medium to remove the EMS.

2.iii. Mutagenesis by NTG

Bacteria were suspended in 7.2 ml of 10 mM-sodium citrate buffer, pH 6.0 in a sterile Erlenmeyer flask (50 ml) and 0.8 ml of NTG, dissolved in 10 mM-sodium citrate buffer, was added at the desired concentration. The bacteria were incubated at 30°C for varying times and were then diluted in citrate buffer, centrifuged, washed and resuspended in basal medium to stop the reaction.

2.iv. Mutagenesis by near ultraviolet light irradiation with 8-methoxypsoralen

Bacteria were suspended in 9 ml of basal medium in polycarbonate centrifuge tubes, 1 ml of 8-methoxypsoralen (1.0 or 0.1 mg/ml) was added to each tube and the bacteria were left at room temperature for 45 min to take up the 8-methoxypsoralen. 8-methoxypsoralen-treated bacteria were irradiated, in a matt-black painted petri dish, with near u.v. light from a 125 W G.E.C. black lamp at a distance of 20 cm from the dish, and were magnetically stirred during irradiation to facilitate mixing. Irradiation was started by removal of the petri dish lid and stopped by its replacement. All manipulations were carried out in the light of a sodium lamp to prevent extra irradiation by fluorescent lamps.

3. Materials

All reagents were the best grade which could be obtained commercially and with the exception of those listed below were supplied by British Drug Houses, Poole, Dorset, UK.

All amino acids except serine, L-tryptophan, D,L-ornithine, L-aspartic acid and L-lysine: T.J. Sas and Son, Ltd, London, WC1.

L-mandelate, D-mandelate and phenylglyoxylate: Fluka A.G., Buchs, Switzerland.

NTG and D,L-ornithine: Koch-Light Laboratories Ltd, Colnbrook, Bucks., UK.

Neomycin , EMS and 8-methoxypsoralen: Sigma London Chemical Co. Ltd, London.

Streptomycin: Glaxo Laboratories Ltd, Greenford, UK.

Vancomycin and D-cycloserine: Eli Lilly and Co. Ltd, Basingstoke, Hants., UK.

Carbenicillin: Beecham Research Laboratories, Brentford, UK. (Gift of the Department of Bacteriology and Immunology, Western Infirmary, Glasgow)

Penicillin V and Carboxy-Q were a gift to Dr W.H. Holms of this laboratory from I.C.I. (Pharmaceuticals Division) Ltd, Alderley Park, Cheshire, UK.

Cooked meat medium (CM82), nutrient broth (CM1), nutrient agar (CM3) and antibiotic discs (multodisk): Oxoid Ltd, London, SE1.

Bacto-agar (B140): Difco Laboratories, West Molesey, Surrey, UK.

RESULTS

1. Mutagenesis

A systematic study was made of four mutagens: ultraviolet light irradiation, NTG, EMS and near u.v. light irradiation in the presence of 8-methoxypsoralen.

1.i. Inactivation and mutagenesis by ultraviolet light irradiation

Loss of viability was exponential at all positions of the petri dish, inactivation proceeding more rapidly the nearer the bacteria were to the u.v. source (Fig. 4). The position of the petri dish made little difference to the amount of mutation observed. A few auxotrophs were isolated, at various positions of the petri dish, but only from surviving fractions of 0.1 to 0.01% (Table 3). Three samples were treated with photoreactivating light for 50 min and in all three the surviving fraction was raised from approximately 0.005% to 0.15% (Table 4). Since no auxotrophs were isolated from the unreactivated samples no data were obtainable on possible changes in the number of auxotrophs following photoreactivation; though in other bacteria where photoreactivation occurs, loss of u.v.-induced mutants occurs to the same extent (Rupert and Harm, 1966). It is therefore necessary to avoid exposing u.v. irradiated A. calcoaceticus to photoreactivating light.

Fig. 4

The inactivation of A. calcoaceticus by ultraviolet irradiation.

Suspensions (10 ml), containing 5×10^9 bacteria/ml in basal medium, were irradiated in glass petri dishes at varying distances from a u.v. lamp. Each suspension was stirred continuously during irradiation. Irradiation was stopped at intervals, by replacement of the petri dish lid, and samples were removed for viable counting.

Distances from u.v. lamp (cm):

● - 80.8

○ - 40.4

▲ - 26.9

△ - 20.2

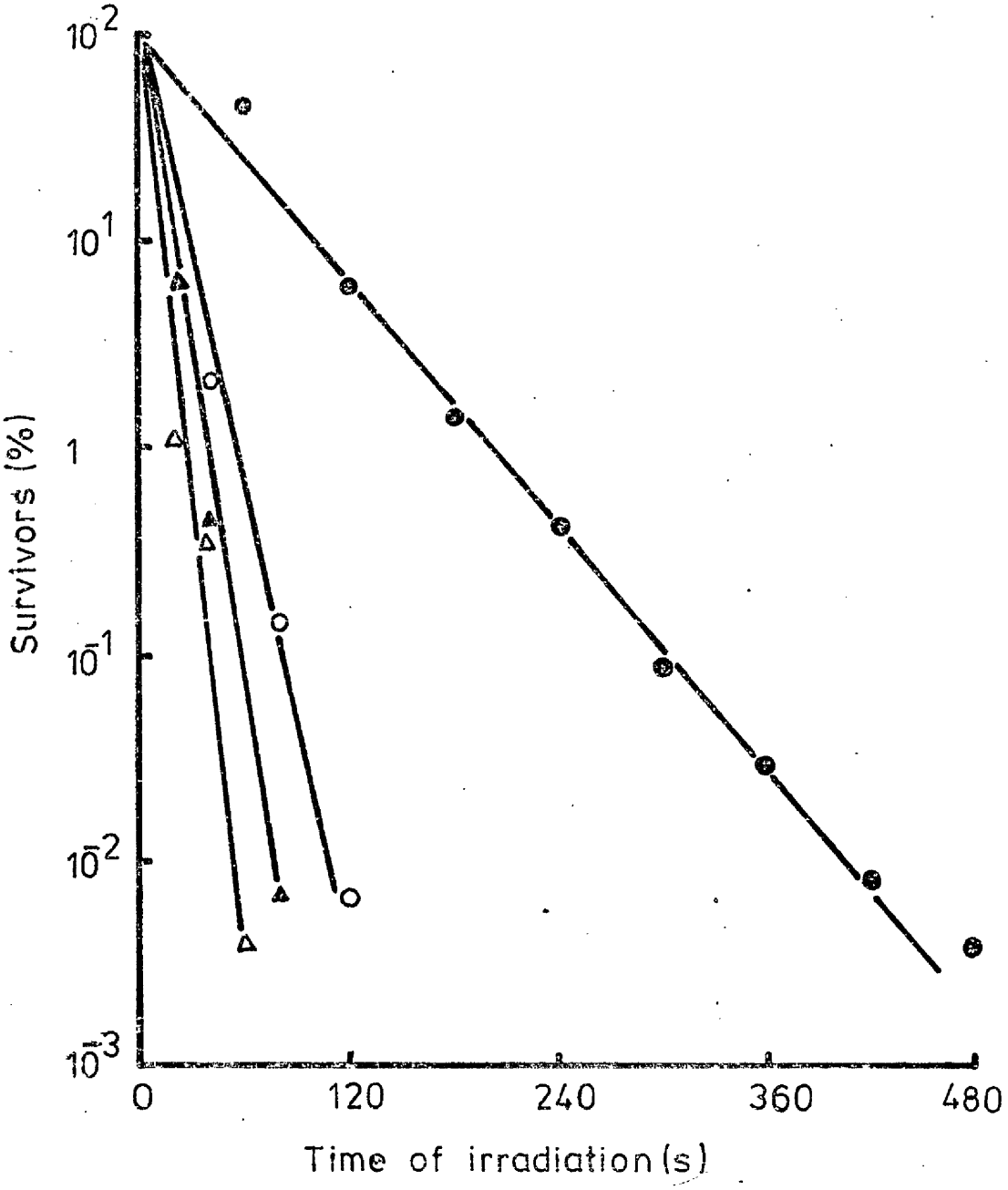

10^2
10^1
1
10^{-1}
10^{-2}
10^{-3}
Survivors (%)
0
120
240
360
480
Time of irradiation (s)

Table 3

Numbers of auxotrophs obtained from the treatment of *A. calcoaceticus* with u.v. irradiation.

Bacteria, irradiated in the experiment described in Fig. 4, were assayed for auxotrophs.

Distance from u.v. lamp (cm)	Time of irradiation (s)	% survivors	No. of auxotrophic colonies / No. of colonies tested	% auxotrophs
80.0	0	100	0/746	0
	60	48	0/369	0
	120	6.1	0/701	0
	180	1.4	0/1033	0
	240	0.42	0/473	0
	300	0.09	0/700	0
	360	0.03	2/338	0.6
	420	0.008	0/605	0
	480	0.004	0/505	0
40.4	0	100	0/1255	0
	40	2.2	0/2760	0
	80	0.14	2/1778	0.1
	120	0.007	0/940	0
26.9	0	100	0/1153	0
	20	6.5	0/767	0
	40	0.46	0/539	0
	80	0.007	1/798	0.1

Table 4

Photoreactivation of u.v. irradiated *A. calcoaceticus*.

Wild-type bacteria, at a concentration of 5×10^9/ml, were u.v. irradiated at various distances from the u.v. source for times calculated to give 0.001 to 0.009% survivors. The bacteria were then exposed to photoreactivating light for 50 min and assayed for viable bacteria and auxotrophs. Photoreactivating light was provided by 3 tungsten bulbs (40 W) and 6 fluorescent tubes (250 W) situated within 40 cm of the petri dishes.

Conditions of irradiation		% survival before photo-reactivation	% survival after photo-reactivation
Distance from u.v. lamp (cm)	Time of u.v. irradiation (s)		
80.8	480	0.004	0.14
40.4	120	0.007	0.16
20.2	60	0.004	0.10

Fig. 5

The inactivation of A. calcoaceticus by NTG.

Suspensions (8 ml), containing 5×10^9 bacteria/ml in 10 mM-sodium citrate buffer pH 6.0, were treated in Erlenmeyer flasks (50 ml) with 10,100 or 1,000 µg/ml NTG by incubation at 30°C. Samples were removed at intervals for viable counting.

Concentrations of NTG (µg/ml):

▲ - 10

● - 100

■ - 1,000

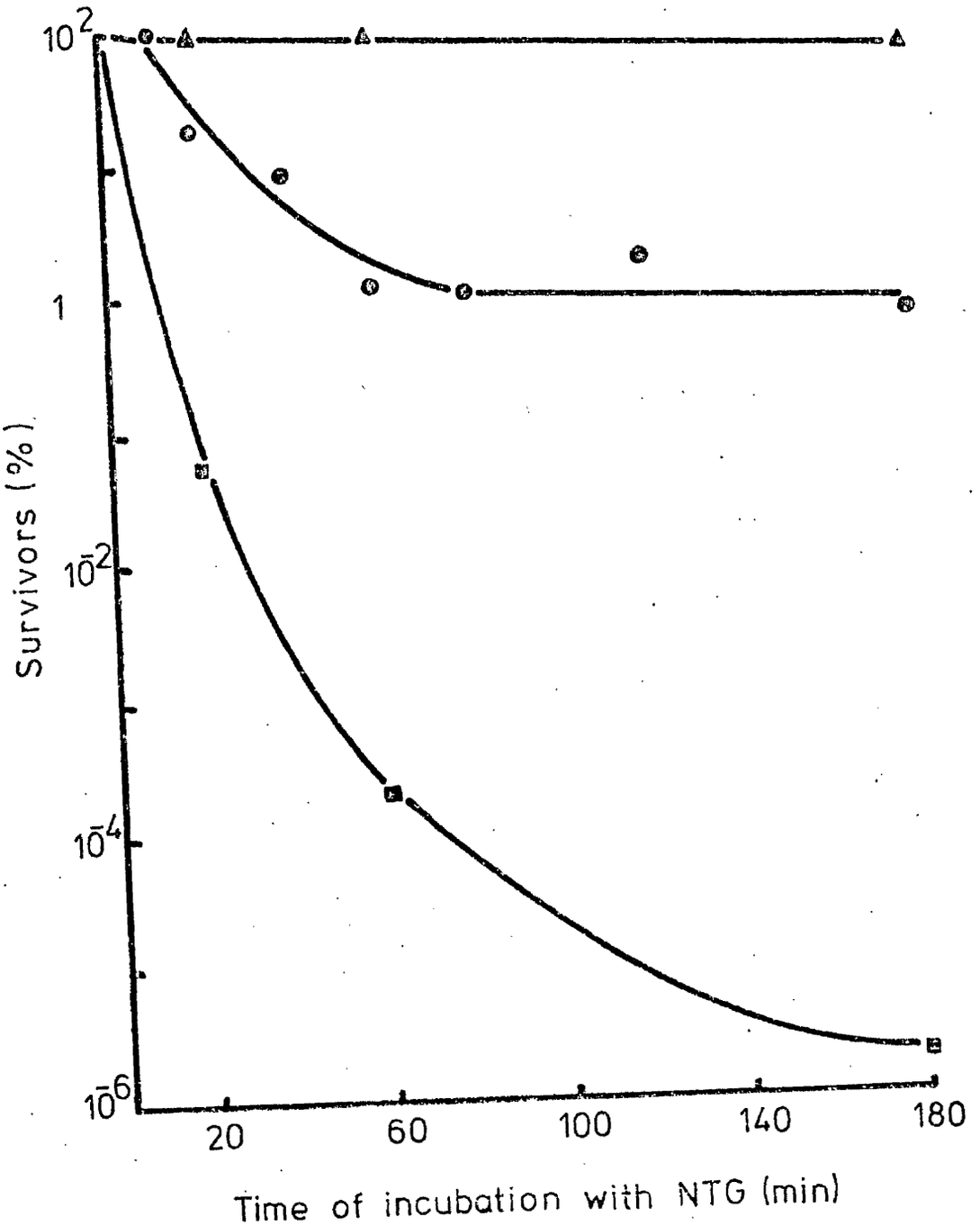

10^2
1
10^{-2}
10^{-4}
10^{-6}
Survivors (%)
20
60
100
140
180
Time of incubation with NTG (min)

Table 5

Numbers of auxotrophs obtained from the treatment of A. calcoaceticus with NTG.

Bacteria, treated with NTG in the experiment described in Fig. 5, were assayed for auxotrophs.

NTG conc. (μg/ml)	Treatment time (min)	% survivors	No. of auxotrophic colonies / Total no. of colonies tested	% auxotrophs
10	0	100	0/1139	0
	20	89	0/1018	0
	60	86	0/983	0
	180	49	1/557	0.2
100	0	100	0/1073	0
	10	100	1/1729	0.06
	20	18	4/2075	0.19
	40	8.3	4/910	0.43
	60	1.1	3/1246	0.24
	80	1.0	4/1091	0.36
	120	1.8	9/3282	0.27
	180	0.62	7/677	1.0
1,000	20	0.05	0/615	0
	60	0.0002	11/282	3.9

1.ii. Inactivation and mutagenesis by NTG

Inactivation of cell viability was greater the higher the NTG concentration, but as the length of NTG treatment increased the rate of inactivation levelled off (Fig. 5). The production of auxotrophs increased as the length of treatment and the concentration increased (Table 5).

1.iii. Inactivation and mutagenesis by EMS

There was no detectable inactivation after 90 min incubation with EMS at concentrations below 8 mM, but at concentrations above 80 mM inactivation proceeded rapidly (Fig. 6). The rate of inactivation over long periods increased as the EMS concentration increased (Fig. 7). Auxotrophs appeared earlier the higher the EMS concentration being detectable after 8 h in 70 and 95 mM-EMS but not until after 25 h in 20 and 45 mM-EMS (Table 6). At all concentrations of EMS the production of auxotrophs increased as the time of incubation increased. After 25 h at 30°C there was no loss in viability in bacteria incubated with basal medium alone.

1.iv. Inactivation and mutagenesis by near ultraviolet light in the presence of 8-methoxypsoralen

Inactivation by near u.v. irradiation and 8-methoxypsoralen (0.1 and 0.01 mg/ml) proceeded exponentially, after a small shoulder at the beginning of the curve, then rapidly declined (Fig. 8). Auxotrophs were only detectable at low frequencies in the 0.1 mg/ml-8-methoxypsoralen sample, though failure to detect them after 200s irradiation may be

Fig. 6

The effect of EMS concentration on the survival of A. calcoaceticus.

Suspensions (8 ml), containing 3 x 10^9 bacteria/ml in basal medium, were treated with various concentrations of EMS for 90 min at 30°C. Samples were removed from each suspension before and after this period for viable counting. (At concentrations higher than 160 mM no survivors were detectable.)

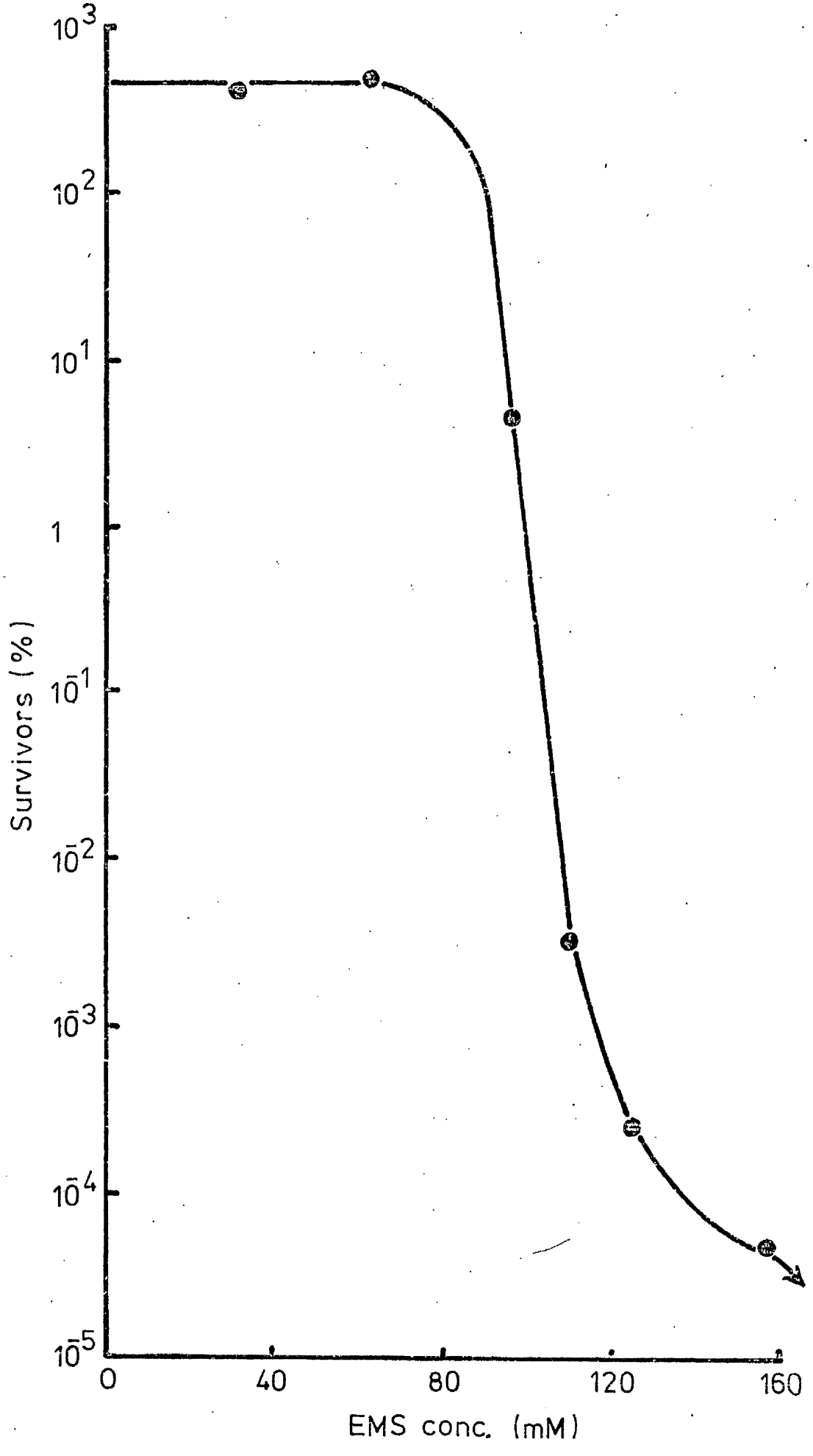

10^3
10^2
10^1
1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
Survivors (%)
0
40
80
120
160
EMS conc. (mM)

Fig. 7

The effect of long exposure time on the inactivation of A. calcoaceticus by EMS.

Suspensions (8 ml), containing 5×10^9 bacteria/ml in basal medium, were treated with various concentrations of EMS at 30°C and sampled for viable counting at intervals.

EMS concentrations (mM):

● - 20

□ - 45

▲ - 70

○ - 95

(Arrows indicate that no survivors were detectable after the last times shown on the graph.)

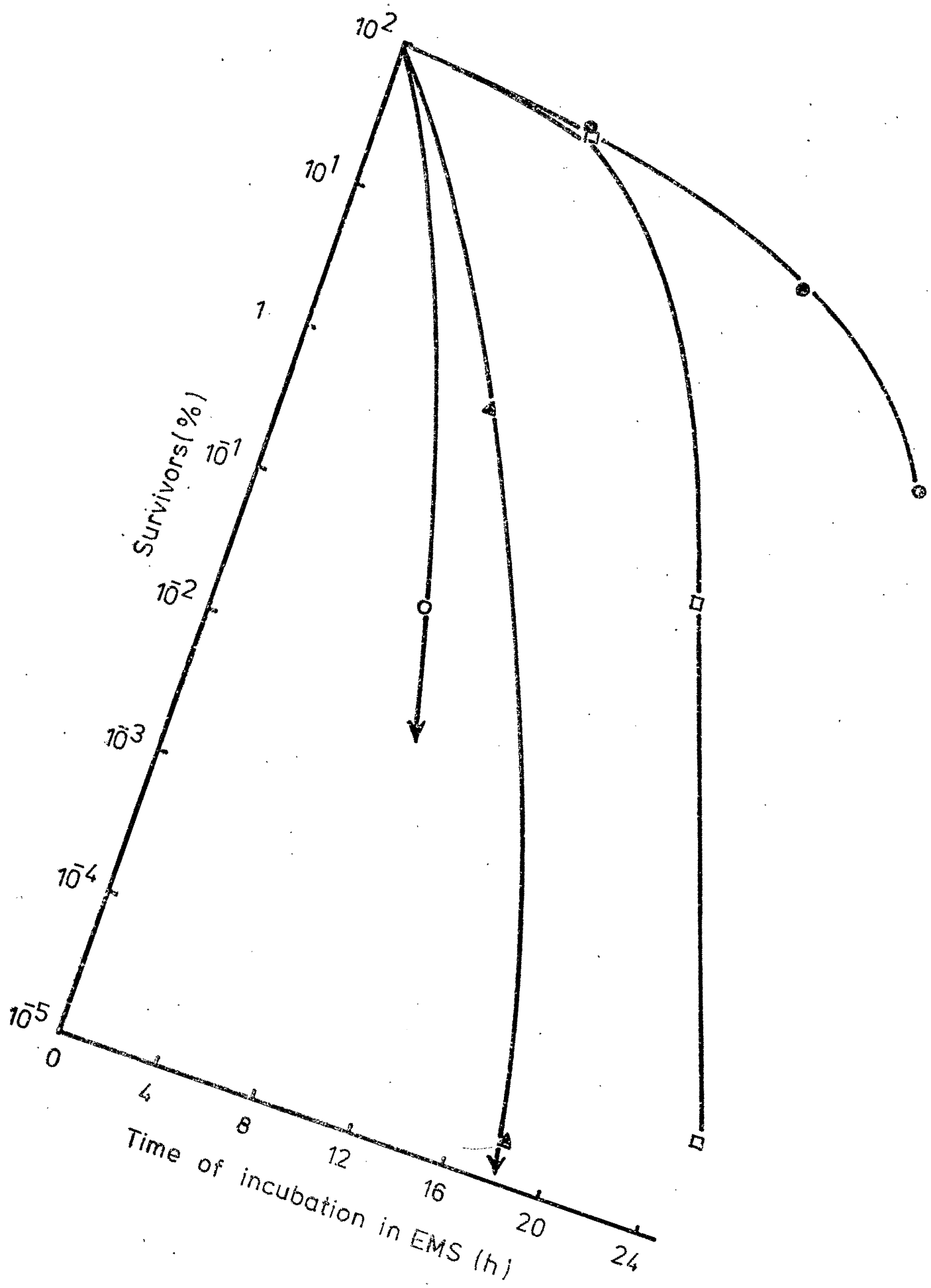

10^2
10^1
1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
Survivors (%)
0
4
8
12
16
20
24
Time of incubation in EMS (h)

Table 6

Numbers of auxotrophs obtained from the treatment of *A. calcoaceticus* with EMS

Bacteria, treated with EMS in the experiment described in Fig. 7, were assayed for auxotrophs.

EMS conc. (mM)	Time of treatment (h)	% survivors	No. of auxotrophic colonies / Total no. of colonies tested	% auxotrophs
20	0	100	0/991	0
	8	67	0/657	0
	18	20	0/208	0
	25	2	1/201	0.5
45	0	100	0/896	0
	8	60	0/545	0
	18	0.13	0/1186	0
	25	0.00005	13/499	2.6
70	0	100	0/917	0
	8	0.84	3/775	0.4
	18	0.00002	25/163	15.3
95	0	100	0/964	0
	8	0.03	3/297	1.0

Fig. 8

The inactivation of A. calcoaceticus by near ultraviolet irradiation + 8-methoxypsoralen.

Suspensions (10 ml), containing 5 x 10^9 bacteria/ml in basal medium, were treated with 0.1 or 0.01 mg/ml of 8-methoxypsoralen for 45 min. Each suspension was then irradiated in a matt-black painted petri dish 20 cm from a 125 W G.E.C. black lamp. Irradiation was stopped at intervals, by replacement of the petri dish lid, to remove samples for viable counting.

Concentration of 8-methoxypsoralen (mg/ml):

● - 0.01

○ - 0.1

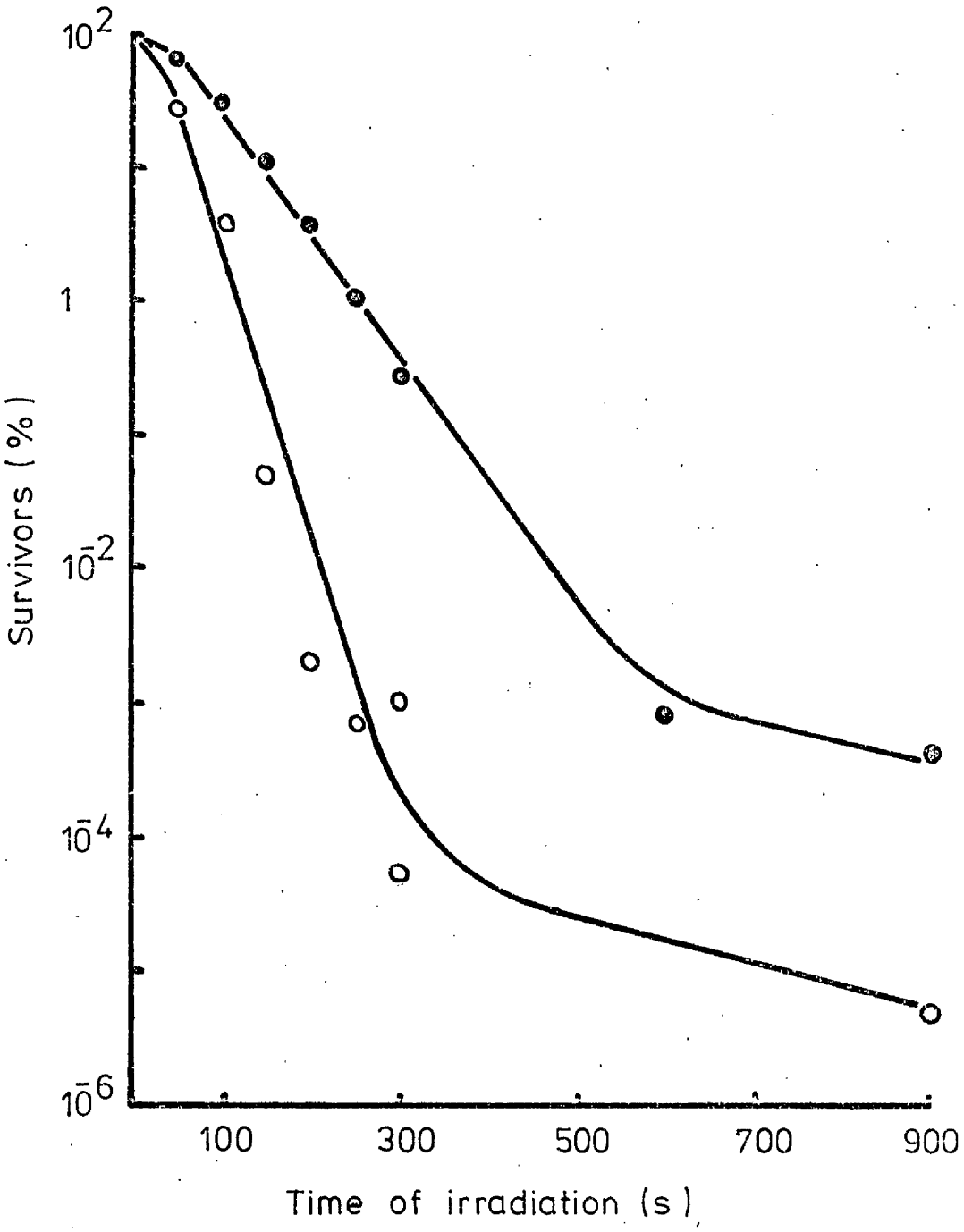

10^2
1
10^{-2}
10^{-4}
10^{-6}
Survivors (%)
100
300
500
700
900
Time of irradiation (s)

Table 7

Numbers of auxotrophs obtained from the treatment of *A. calcoaceticus* with near u.v. irradiation and 8-methoxypsoralen (0.1mg/ml).

Bacteria, treated with 8-methoxypsoralen and irradiated with near u.v. light in the experiment described in Fig. 8 were assayed for auxotrophs.

Time of irradiation (s)	% survivors	No. of auxotrophic colonies / Total no. of colonies tested	% auxotrophs
0	100	0/940	0
50	25	0/2396	0
100	3.6	2/3400	0.06
150	0.05	1/549	0.2
200	0.002	0/188	0
250	0.0004	0/349	0
300	0.00005	0/571	0

due to the small number of colonies assayed (Table 7). Neither inactivation nor mutagenesis was observed in cultures exposed separately to 8-methoxypsoralen or near u.v. irradiation.

2. Development of Antibiotic Selection Methods

Since there is no published report on the comparative effects of antibiotics on NCIB 8250, the development of an antibiotic selection system required the identification of a suitable antibiotic (Results, section 2.i). Having found a suitable antibiotic the next stage (Results, sections 2.ii to 2.v) was a study of its effects on *A. calcoaceticus* under various conditions.

In a number of experiments the effects of the antibiotic were studied in non-growing as well as growing bacteria. Non-growing bacteria were either wild-type in basal medium or auxotrophs in minimal salts media and in both cases addition of antibiotic was preceded by two hours' incubation to allow cessation of growth.

2.i. Screening antibiotics

Antibiotic discs were used to screen a variety of antibiotics for use in a selection system (Table 8). *A. calcoaceticus* was sensitive to gentamycin, 10 μg; neomycin, 10 μg; Streptomycin, 10 μg; tetracycline, 50 μg; terramycin, 30 μg and carbenicillin, 100 μg. It was moderately resistant to erythromycin, 10 μg; chloramphenicol, 10 μg; vancomycin, 30 μg; colistin sulphate, 10 μg and

Table 8

The sensitivity of A. calcoaceticus to antibiotic discs.

The antibiotics were tested as Oxoid 'Multodisks' placed on 10 mM-succinate salts agar which had been spread with approximately 10^8 bacteria. The plates were incubated at 30°C for 17 h.

R - resistant, no zone of sensitivity or zone less than 2 mm wide.

MR - moderately resistant, zone of sensitivity between 2 and 4 mm wide.

S - sensitive, zone of sensitivity over 4 mm wide.

*U = antibiotic unit

Antibiotic	Antibiotic action	Antibiotic conc. (μg)	Reaction
gentamycin	cause misreading in translation	10	S
kanamycin		5	R
neomycin		10	S
streptomycin		10	S
tetracycline	inhibit protein synthesis	50	S
terramycin		30	S
erythromycin		10	MR
chloramphenicol		10	MR
cloxacillin	inhibit bacterial wall synthesis	5	R
ampicillin		25	R
methecillin		10	R
penicillin		1.5U*	R
carbenicillin		100	S
vancomycin		30	MR
cephaloridine		25	R
bacitracin		10U*	MR

Antibiotic	Antibiotic action	Antibiotic conc. (μg)	Reaction
colistin sulphate	attacks bacterial membrane	10	MR
novobiocin	inhibits growth by combining Mg^{++}	5	R
sulphurazole	not known	500	R
sulphamethoxazole/ trimethoprim		25	R
fusidic acid		10	R

bacitracin, 10 U and resistant to all the other compounds tested.

2.ii. The effects of various antibiotics in liquid culture

Some antibiotics which gave a sensitive reaction in the disc test, and some others which were already available in the laboratory, were tested singly and in combinations for their effects on growing and non-growing bacteria in liquid media (Table 9). Vancomycin and neomycin gave the greatest inactivation of growing bacteria, however, neomycin gave the same inactivation with non-growing bacteria. Vancomycin gave a much lower inactivation of non-growing bacteria and thus had the desired characteristics of an antibiotic for mutant selection. The two penicillins, carbenicillin and penicillin V, gave approximately the same amount of inactivation of growing bacteria. When used in conjunction with vancomycin, carbenicillin blocked the action of vancomycin on growing and non-growing bacteria whereas penicillin V only blocked its action on non-growing bacteria. Therefore, penicillin V could be used to increase the selective effect of vancomycin.

As a preliminary to the development of a selection system with vancomycin a study was made of the effect of vancomycin concentration on growing bacteria (Fig. 9). Growing bacteria were sensitive to concentrations above 10 μg/ml, 1,000 μg/ml inactivating 99.992% in 3 h. In all subsequent experiments with vancomycin a concentration of 500 μg/ml was used. Livingstone (1970) had already shown

Table 9

The inactivation of A. calcoaceticus by various antibiotics.

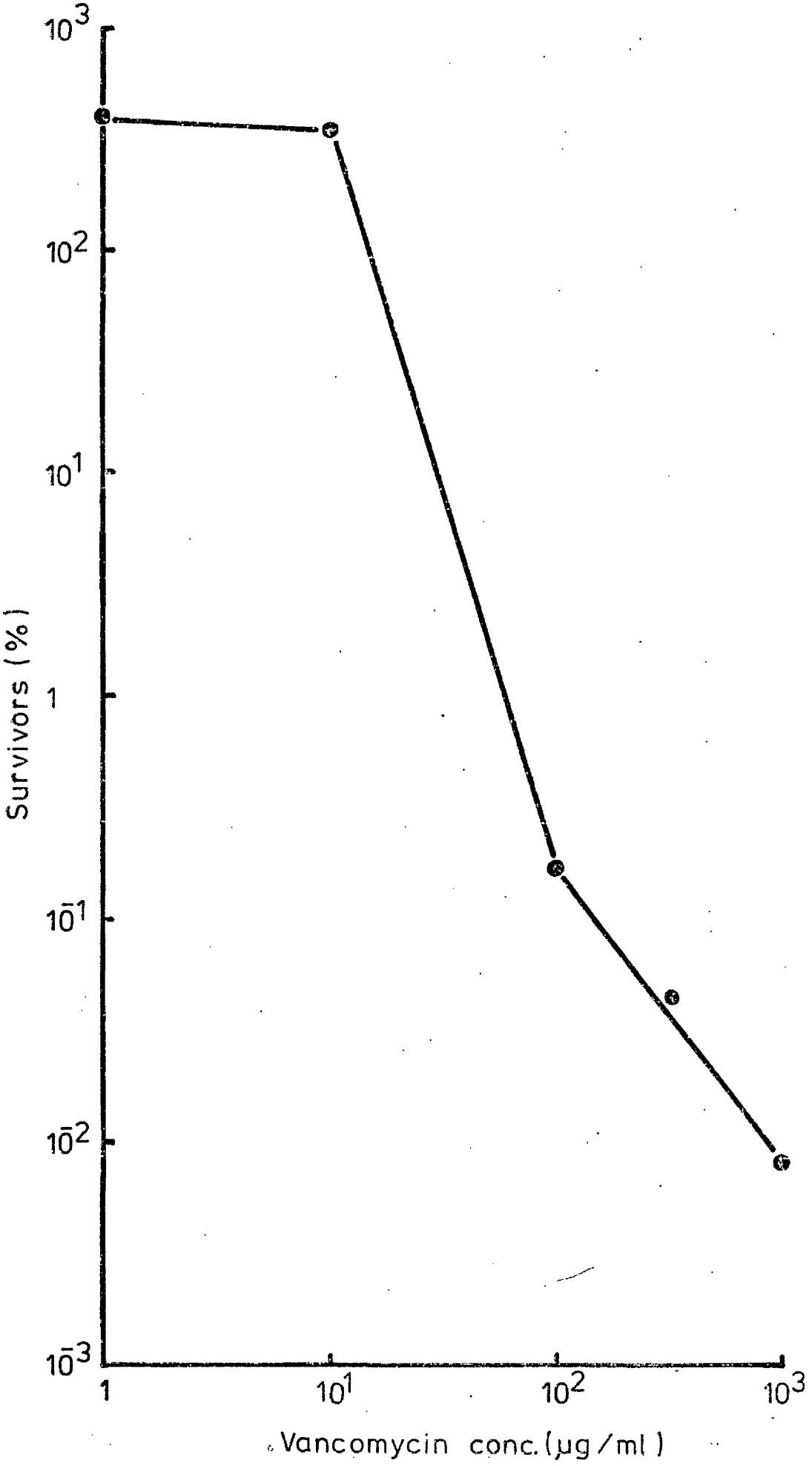
Twelve Erlenmeyer flasks (250 ml) contained 50 ml of 5 mM-benzyl alcohol-salts medium and wild-type bacteria at a concentration of 7 x 10^7/ml. Twelve similar flasks contained strain U4K (Tyr^-). The cultures were shaken for 2 h at 30°C then various antibiotics and combinations of antibiotics were added. Shaking was continued for 17 h, samples being assayed for surviving bacteria at the beginning and end of this period.

Antibiotic	Conc. of antibiotic (μg/ml)	% wild-type survivors (a)	% U4K survivors (b)	Relative % survival (b/a)
vancomycin	500	0.004	0.8	200
penicillin V	1,000	0.6	100	166
carbenicillin	1,000	0.3	14	46
D-cycloserine	500	34	100	3
carboxy-Q	2,500	1.4	100	71
neomycin	1,000	0.005	0.006	1.2
vancomycin/ penicillin V	500 100	0.002	18.7	9,350
vancomycin/ D-cycloserine	500 500	4.1	27.2	7
vancomycin/ carboxy-Q	500 2,500	0	0	-
carbenicillin/ vancomycin	1,000 500	0.2	58	290
carbenicillin/ neomycin	1,000 1,000	0.003	0.001	0.3
neomycin/ vancomycin	1,000 500	0	0	-

Fig. 9

The effect of vancomycin concentration on *A. calcoaceticus*.

Erlenmeyer flasks (250 ml) containing 50 ml of 10 mM-succinate-salts medium were incubated with 0.1 ml of a 10 mM-succinate-salts culture in stationary phase. The cultures were shaken at 30°C till they had reached a turbidity of 0.2, various concentrations of vancomycin were then added. The cultures were incubated for a further 3 h and were sampled at the beginning and end of this period for viable counting.


10^3
10^2
10^1
1
10^{-1}
10^{-2}
10^{-3}
Survivors (%)
1
10^1
10^2
10^3
Vancomycin conc. (μg/ml)

that 1,000 μg/ml was a suitable concentration for penicillin V.

2.iii. The effect of incubation time and different substrates on inactivation by vancomycin and vancomycin + penicillin V

Some preliminary experiments were made with vancomycin alone to study the effect of L-mandelate and benzyl alcohol as substrates during vancomycin inactivation since it was likely that these compounds would be used in attempts to isolate mutants of aromatic catabolism pathways (Fig. 10). Over 8 h there was very little difference in the amount of inactivation obtained in 5 mM-benzyl alcohol and 5 mM-L-mandelate-salts medium. With both substrates the surviving fraction of growing bacteria was approximately 0.1%. In the same time the surviving fraction of non-growing bacteria was between 10 and 40%.

Since penicillin V exerts a protective effect on non-growing bacteria during vancomycin treatment (Table 9) it was thought that vancomycin + penicillin V treatments could be used for selection without too great a loss of non-growing mutants. An experiment was done to study the inactivation of growing and non-growing bacteria, in 5 mM-benzyl alcohol-salts- medium, by vancomycin (500 μg/ml) and penicillin V (1,000 μg/ml) over 17 h (Fig. 11). After 17 h the surviving fraction of non-growing bacteria was 18% and of growing bacteria 0.002%. With growing bacteria, vancomycin + penicillin V showed the same type of

Fig. 10

The effect of incubation time and different substrates on the inactivation of *A. calcoaceticus* by vancomycin.

Wild-type bacteria, previously grown in 5 mM-L-glutamate-salts medium, were suspended (10^8 bacteria/ml) in 50 ml of (i) 5 mM-L-mandelate, (ii) 5 mM-benzyl alcohol-salts medium and (iii) basal medium, all in 250 ml Erlenmeyer flasks. The bacteria were shaken for 2 h at 30°C then vancomycin (500 μg/ml) was added to each. Incubation was continued for a further 8 h, samples being removed at intervals for viable counting.

Strain U4K (Tyr^-) was similarly treated in 50 ml of 5 mM-L-mandelate-salts medium.

■ - U4K in 5 mM-L-mandelate

○ - Wild-type in basal medium

△ - Wild-type in 5 mM-benzyl alcohol

● - Wild-type in 5 mM-L-mandelate

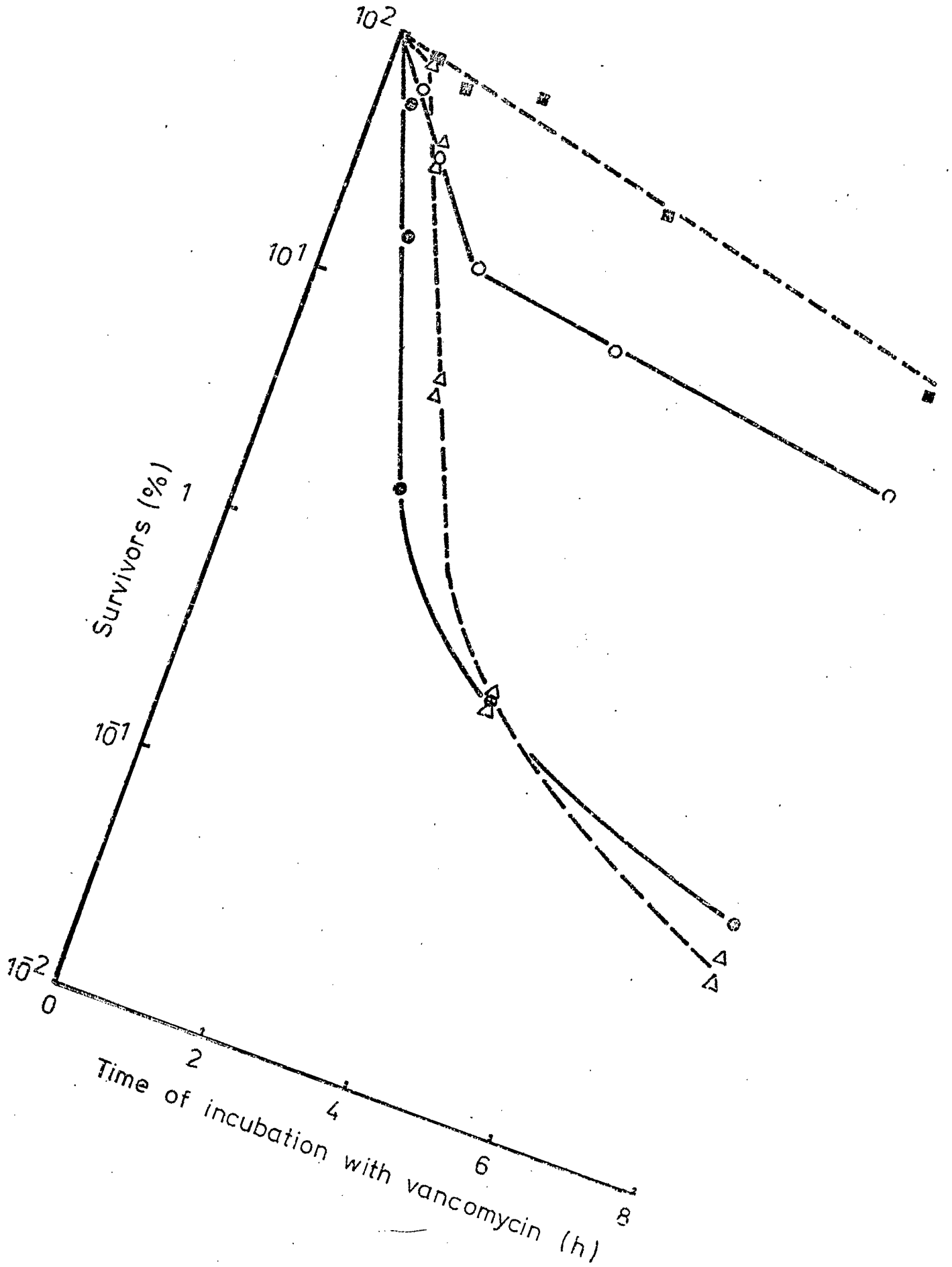

10²
10¹
1
10⁻¹
10⁻²
Survivors (%)
0
2
4
6
8
Time of incubation with vancomycin (h)

Fig. 11

The inactivation of *A. calcoaceticus* by vancomycin and penicillin V.

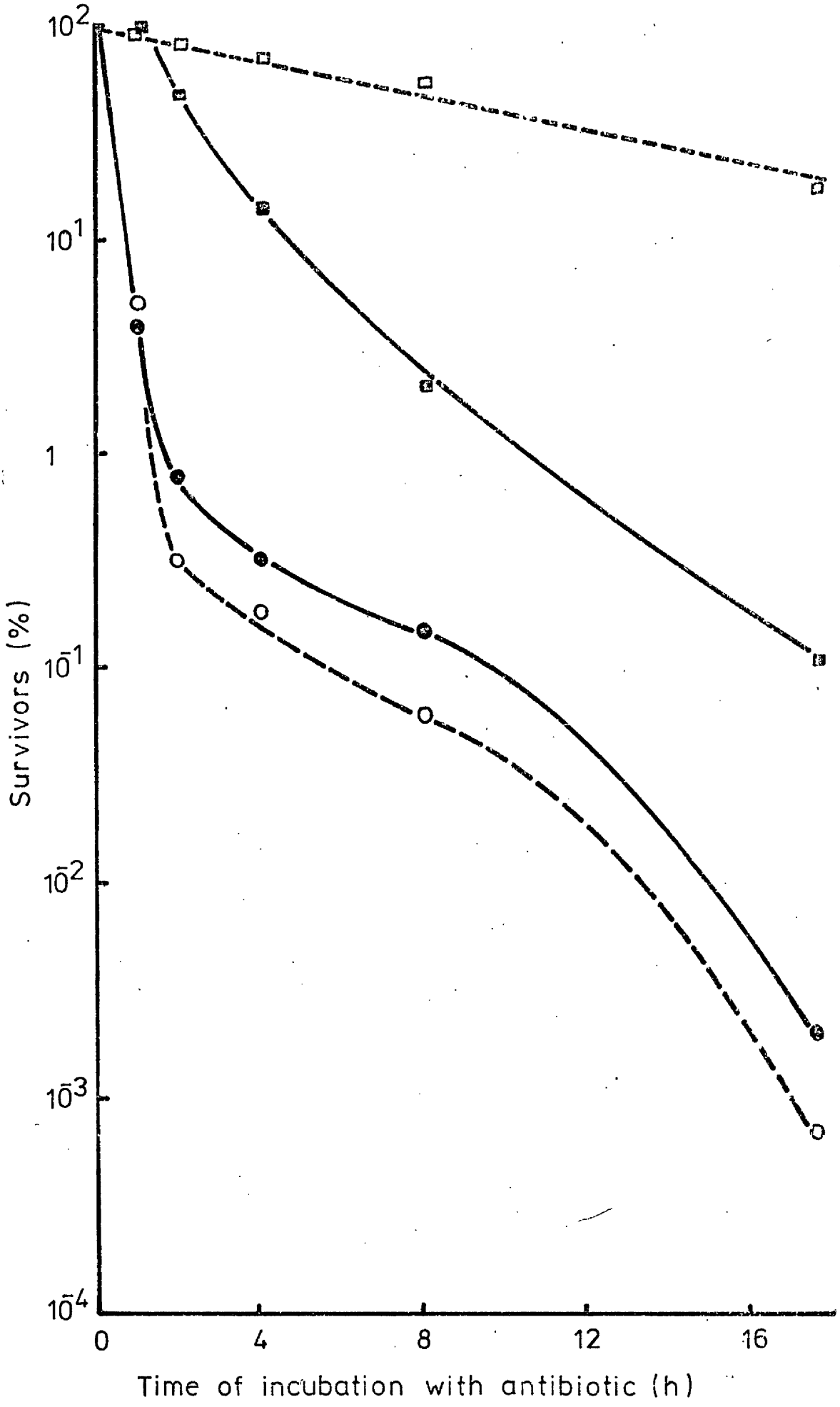
Wild-type bacteria, previously grown on 5 mM-L-glutamate + 1.5 mM-benzoate-salts medium, were suspended (10^8 bacteria/ml) in three Erlenmeyer flasks (250 ml) containing 50 ml of 5 mM-benzyl alcohol-salts medium. The bacteria were shaken for 2 h at 30°C then the following antibiotics were added:

flask (i) vancomycin (500 μg/ml);
flask (ii) vancomycin (500 μg/ml) + penicillin V (1,000 μg/ml);
flask (iii) penicillin V (1,000 μg/ml).

Incubation was continued for a further 18 h, samples being removed at intervals for viable counting.

Strain U4K was similarly treated with vancomycin (500 μg/ml) + penicillin V (1,000 μg/ml).

□ - U4K + penicillin V/vancomycin
■ - Wild-type + penicillin V
● - Wild-type + penicillin V/vancomycin
○ - Wild-type + vancomycin


10^2
10^1
1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
Survivors (%)
0
4
8
12
16
Time of incubation with antibiotic (h)

inactivation curve as vancomycin alone.

It was concluded that vancomycin on its own may be used as a selective agent over short incubation periods but a greater enrichment may be achieved by using vancomycin + penicillin V over 17 h.

2.iv. The growth of vancomycin-treated bacteria in nutrient broth and 5 mM-L-glutamate-salts medium

Some Gram-positive bacteria, which have been treated with vancomycin and then transferred to fresh medium, are unable to grow (Reynolds, 1966). Therefore, one experiment was done to test the growth of vancomycin-treated *A. calcoaceticus* in nutrient broth and 5 mM-L-glutamate-salts medium. In both media, vancomycin treated bacteria always exhibited a longer growth-lag than non-treated bacteria. The period of growth-lag increased as the length of vancomycin treatment increased, but growth was always faster in nutrient broth than in L-glutamate. Growth of bacteria which were not growing during vancomycin treatment was faster than the growth of bacteria which were growing during treatment. Therefore, if surviving bacteria are to be grown after vancomycin treatment nutrient broth should be used in preference to minimal-salts media.

2.v. The effect of different numbers of live and inactivated bacteria on vancomycin + penicillin V-inactivation

In a number of early unsuccessful aromatic selection experiments it was noticed that the inactivation by vancomycin was rarely as much as that achieved in the

Fig. 12

The effect of different numbers of live and inactivated bacteria on the inactivation of A. calcoaceticus by vancomycin + penicillin V.

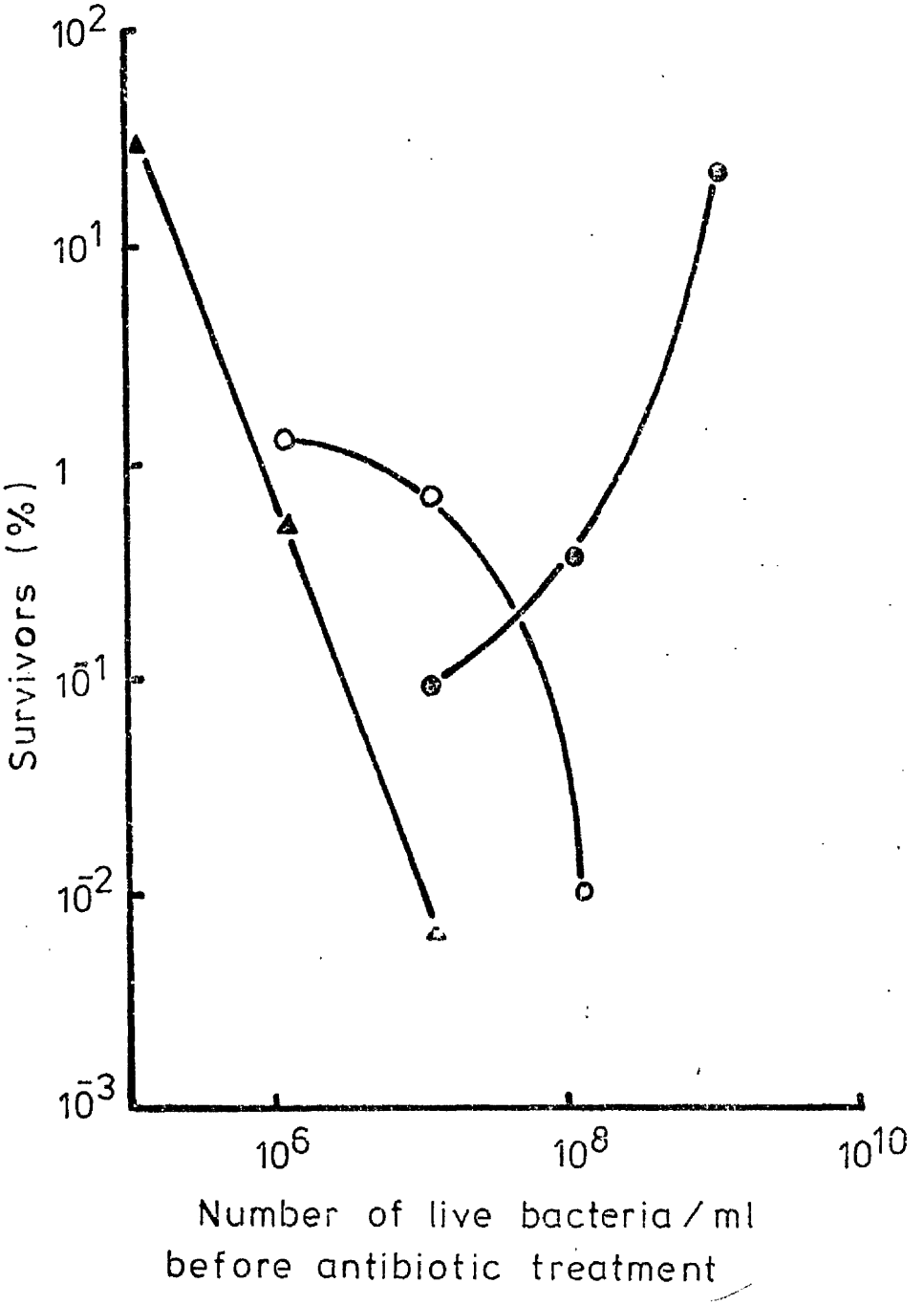
A series of Erlenmeyer flasks (250 ml) contained 40 ml of 2 mM-benzyl alcohol-salts medium and various ratios of live and inactivated (by long exposure to EMS) wild-type bacteria. The flasks were shaken for 2 h at 30°C then vancomycin (500 μg/ml) and penicillin V (1,000 μg/ml) were added to each flask. The flasks were shaken for a further 17 h, samples being removed at the beginning and end of this period for viable counting.

Numbers of inactivated bacteria/ml before antibiotic treatment:

● - 10^9

○ - 10^8

▲ - 10^7


10^2
10^1
1
10^{-1}
10^{-2}
10^{-3}
Survivors (%)
10^6
10^8
10^{10}
Number of live bacteria / ml
before antibiotic treatment

development experiments (Fig. 11). In the selection experiments varying numbers of mutagen-inactivated bacteria were present, giving rise to the possibility that mutagen-inactivated bacteria might be exerting an effect on vancomycin-inactivation, therefore, an experiment was done to test this (Fig. 12). When the culture initially contained 10^9 mutagen-inactivated bacteria/ml a high vancomycin-inactivation was achieved if the initial number of live bacteria was 10^7/ml. If the number of live bacteria was 10^9/ml then a much lower vancomycin inactivation was obtained. Curiously, an opposite effect was observed if the culture initially contained 10^7 or 10^8 mutagen-inactivated bacteria/ml. In these cases the highest vancomycin-inactivation was observed when the intial number of live bacteria was equivalent to the initial number of mutagen-inactivated bacteria. In subsequent experiments the number of mutagen-inactivated bacteria was kept below 10^9/ml and the number of live bacteria was equivalent to or greater than the number of inactivated bacteria.

3. Isolation of Mutants by Antibiotic Selection

3.i. Selection of auxotrophs with vancomycin

When vancomycin was identified as a suitable antibiotic for selection purposes an experiment was designed in which it was used to select auxotrophs. The selection procedure was as follows: samples of u.v.-irradiated bacteria were grown in nutrient broth (50 ml) for 16 h to

Table 10

Numbers of A. calcoaceticus auxotrophs isolated during two cycles of enrichment in 10 mM-succinate-salts medium + vancomycin (500 μg/ml).

A suspension (10 ml) of wild-type bacteria was u.v. irradiated. Samples (1 ml) removed at 98% and 99.9% inactivation were grown to stationary phase in 50 ml of nutrient broth. They were subsequently enriched in 10 mM-succinate-salts medium with vancomycin (500 μg/ml) for 3 h (see Results 3.i), two cycles of this enrichment were performed and at the end of each cycle the surviving bacteria were grown into stationary phase in nutrient broth then assayed for auxotrophs.

These results, and those in Table 11, probably reflect the repeated isolation of the same mutants.

% u.v. inactivation	Cycles of enrichment	% survivors of vancomycin treatment	No. of auxotrophic colonies / Total no. of colonies tested	% auxotrophs
98	1	0.014	10/2850	0.35
	2	0.60	146/1950	7.4
99.9	1	0.014	14/2250	0.62
	2	0.45	57/1950	2.9

Table 11

Numbers and classes of *A. calcoaceticus* auxotrophs isolated during two cycles of enrichment in 10 mM-succinate-salts medium + vancomycin (500 μg/ml).

The nutritional requirements of some of the auxotrophs isolated in the experiment described in Table 10 were identified using the pool plate method of Holliday (1956).

In each class the mutants could be identical though no genetic analysis was done to confirm this.

% u.v. inactivation	Cycle of enrichment	No. of auxotrophs tested	Classes of auxotroph obtained	No. of each class obtained
98	1	10	tyrosine$^-$	3
			isoleucine/ valine	3
			multiple (unidentified)	2
			cysteine$^-$	1
			isoleucine$^-$	1
	2	22	isoleucine/ valine	20
			isoleucine$^-$	1
			multiple (unidentified)	1
99.9	1	14	tyrosine$^-$	14
	2	22	tyrosine$^-$	22

Table 12

Numbers of strains U4A and U4K recovered during a reconstruction of the 10 mM-succinate + vancomycin (500 μg/ml) selection system.

Strains U4A ($Ileu^-$) and U4K (Tyr^-), grown into stationary phase in nutrient broth, were each added to a stationary phase culture of wild-type bacteria to give a final concentration of 0.02% auxotrophs. The wild-type + auxotroph mixtures were then subjected to three cycles of enrichment in 10 mM-succinate-salts medium + vancomycin (500 μg/ml) for 3 h (see Results, section 3.i). Bacteria surviving vancomycin treatment were grown into stationary phase in 50 ml of nutrient broth and assayed for auxotrophs.

*These percentages were obtained from viable counts of the original nutrient broth cultures.

Cycle of enrichment	% survivors of vancomycin treatment		No. of auxotrophic colonies / Total no. of colonies tested		% auxotrophs	
	U4A	U4K	U4A	U4K	U4A	U4K
0	no treatment		-	-	0.02	0.02*
1	0.09	0.11	3/410	5/880	0.7	0.6
2	0.11	0.43	11/429	9/533	2.6	1.7
3	2.7	0.54	527/830	182/470	63.5	38.7

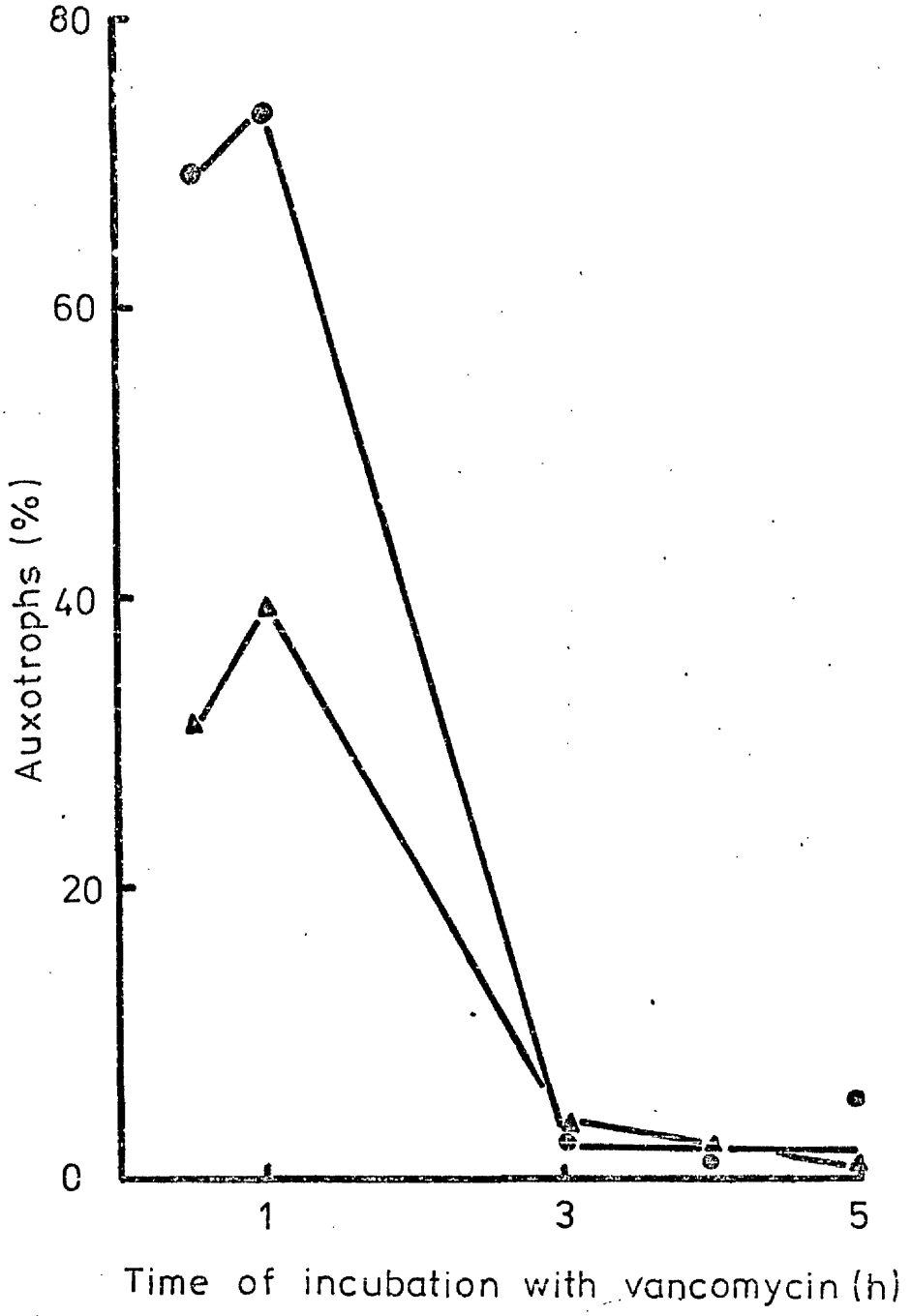
Fig. 13

The effect of incubation time on the selection of auxotrophs by vancomycin.

Wild-type bacteria grown in nutrient broth, + 0.6% of strain U4A ($Ileu^-$) or U4K (Tyr^-), were resuspended in 10 mM-succinate-salts medium (50 ml Erlenmeyer flasks) to a final concentration of 10^8 bacteria/ml. After shaking for 1 h at 30°C 2 ml was transferred from each cutlure to five flasks of fresh 10 mM-succinate-salts medium to give a concentration of 5×10^6 bacteria/ml. Vancomycin (500 μg/ml) was then added to each flask and the bacteria were shaken at 30°C for various times. They were then washed in basal medium, resuspended in 50 ml of nutrient broth and grown to stationary phase. Samples from each broth culture were then assayed for auxotrophs.

● - U4K (Tyr^-)

▲ - U4A ($Ileu^-$)


80
60
40
20
0
Auxotrophs (%)
1
3
5
Time of incubation with vancomycin (h)

allow phenotypic expression. Nutrient broth cultures were then washed and resuspended in 10 ml of basal medium and 1 ml of these suspensions transferred to 10 mM-succinate-salts medium (50 ml). These cultures were shaken at 30°C for 2 h to allow auxotrophs to stop growing then 2 ml of each culture was transferred to 10 mM-succinate-salts + vancomycin (500 μg/ml) medium. Shaking was continued for 3 h, the bacteria were then harvested and resuspended in 50 ml of nutrient broth. After overnight incubation samples were removed from each flask to assay auxotrophs and the bacteria in the broth cultures were passed through an identical cycle of enrichment. A number of different classes of auxotroph were obtained (Table 11) and it was noticed that the fraction of auxotrophs rose by approximately tenfold during the second cycle of enrichment (Table 10).

As a test of this system the auxotrophs U4K (Tyr^-) and U4A ($Ileu^-$) were added at low concentration to wild-type bacteria and put through three cycles of enrichment (Table 12). A 5 to 10-fold enrichment was obtained during each cycle. During the second cycle the incubation times with vancomycin were varied (Fig. 13) and it was found that after only 1 h incubation an enrichment of approximately 100-fold was obtained.

3.ii. Selection of aromatic catabolism mutants with vancomycin or vancomycin/penicillin V

Attempts to obtain aromatic mutants by plating and replication of unenriched EMS-treated bacteria were

unsuccessful. This demonstrated the necessity of having a selection system for such mutants.

Early attempts were made to isolate aromatic mutants with 1 h and 3 h vancomycin and 17 h vancomycin/penicillin V treatments using both benzyl alcohol and L-mandelate as substrates. No aromatic mutants were obtained but observations made in these experiments led to the development experiments discussed in Results, section 2.

To try and prevent the possibility of the growth of mutants on accumulated intermediates of metabolism it was decided to induce, before the addition of vancomycin, the enzymes which would remove these intermediates. Accordingly, bacteria were preinduced with 1.5 mM-benzoate before addition to benzyl alcohol-salts + vancomycin medium.

To optimise the substrate concentration for vancomycin-inactivation an experiment was done to test a range of L-mandelate and benzyl alcohol concentrations. In both media a concentration of 10 mM gave the best vancomycin-inactivation and this concentration was used in subsequent enrichment experiments.

A selection procedure was finally devised which was successful for the enrichment of RIII and catechol pathway mutants. For this procedure wild-type bacteria were treated with 45 mM-EMS (5 lots of 4 ml) for 25 h, then washed and resuspended in 5 ml of basal medium. Two millilitres from each sample was divided between two flasks of 5 mM-L-glutamate (40 ml) which were then shaken for

17 h at 30°C to allow phenotypic expression. Basal medium (20 ml) was then added to each flask with benzoate and glutamate at final concentrations of 1.5 mM and 5 mM respectively. Incubation was continued for 2½ h to allow preinduction with benzoate. Forty millilitres of each culture was then harvested, washed, resuspended in 10 ml basal medium then 1 ml of each suspension was added to 10 mM-benzyl alcohol-salts medium (40 ml). Vancomycin (500 μg/ml) and penicillin V (1,000 μg/ml) were then added to each culture and shaking was continued for 17 h. After this, 40 ml of each culture was harvested, washed in chilled nutrient broth to remove antibiotics, recentrifuged and resuspended in 5 ml of chilled nutrient broth. Exploratory viable counts were made for each culture during which they were kept at 4°C to prevent further growth. Finally, suitable dilutions of each culture were plated on nutrient agar and assayed for aromatic mutants by replication onto 10 mM-benzyl alcohol-salts agar. Non-replicating colonies were cloned for further testing; being inoculated into 1.5 mM-benzoate, 2 mM-catechol and 2 mM- β-ketoadipate-salts media and divided into classes on the basis of their growth in these media after 20 h. Those growing on none of these substrates contain lesions at β-ketoadipate succinyl-CoA transferase or enzymes below it (see Fig. 2). Those growing only on β-ketoadipate contained lesions between catechol 1,2 oxygenase and β-ketoadipate enol-lactone hydrolase. Those growing only on catechol and β-ketoadipate were "benzoate oxidase" mutants. All these mutants were capable of growth on

succinate and therefore contained no lesions below the level of succinate.

4. Isolation of Mutants by Methods Other than Antibiotic Selection

4.i. Isolation of streptomycin-resistant mutants

Streptomycin-resistant mutants were required for the isolation of multiple mutants and also to show that antibiotic resistant mutants of A. calcoaceticus could be selected by a positive method. The technique of isolation was as follows: nutrient broth-grown bacteria were spread (10^8/plate) on 10 mM-succinate or 1.5 mM-benzoate-salts agar (+ amino acids if the bacteria were auxotrophs) with the addition of streptomycin (10 μg/ml). Plates were incubated for 4 days at 30°C and the largest colonies were cloned for further testing. With this method, spontaneous mutations to streptomycin resistance were isolated from wild-type and a variety of mutant strains. No strain that was tested failed to give rise to such mutants.

In liquid culture the wild-type was sensitive to concentrations of streptomycin above 0.1 μg/ml whereas strain D35A (derived from strain NA36), resistant to 10 μg/ml-streptomycin in solid media, was sensitive to concentrations above 1.0 μg/ml (Fig. 14). Subsequently, when growing D35A in bulk for DNA extraction, 1 μg/ml-streptomycin was added to the medium to prevent the growth of streptomycin-sensitive revertants.

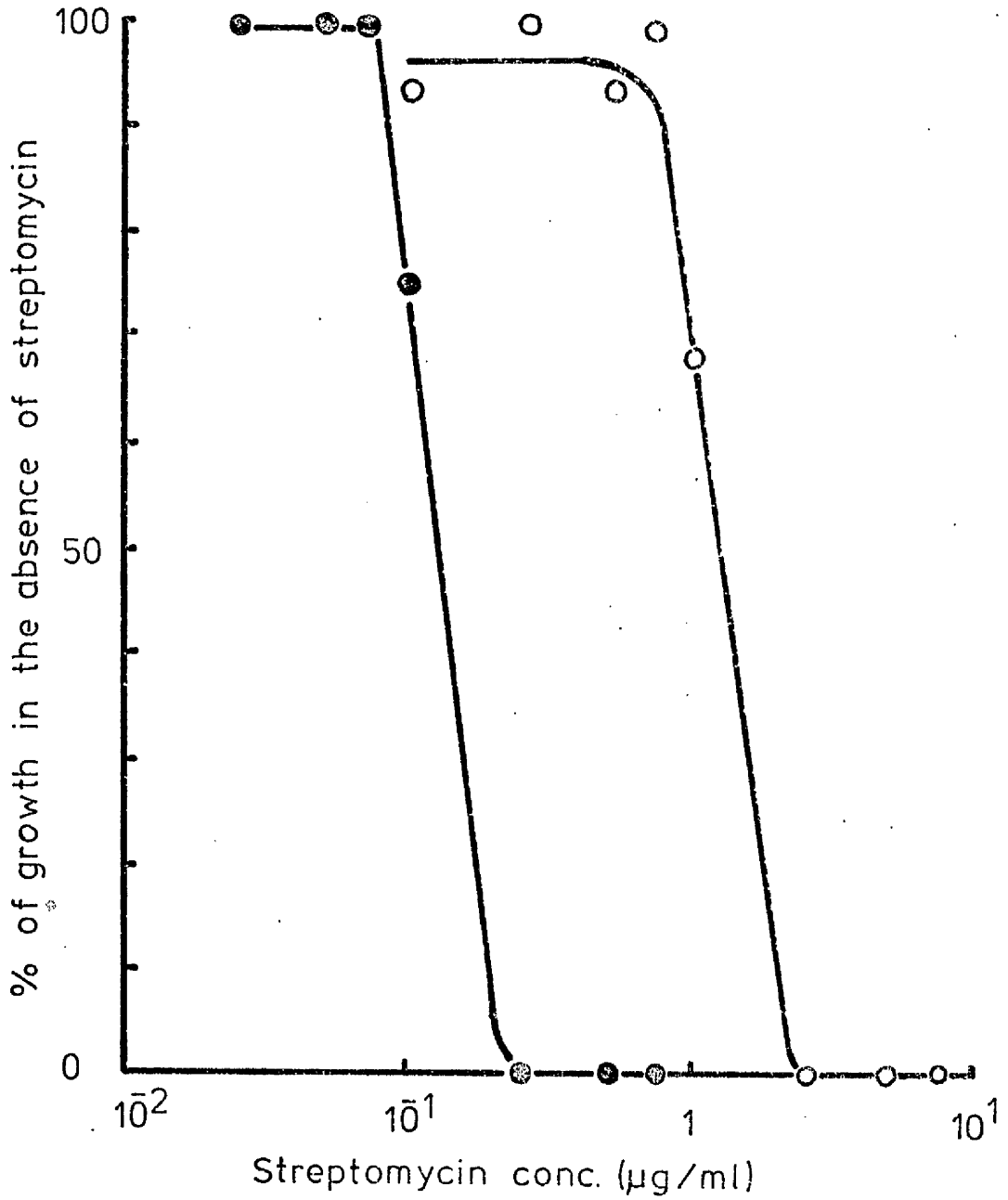
Fig. 14

Sensitivity to streptomycin of wild-type *A. calcoaceticus* and strain D35A grown in liquid culture.

Erlenmeyer flasks (250 ml) containing 50 ml of 1.5 mM-benzoate-salts medium with various concentrations of streptomycin were inoculated with 0.1 ml of a 24 h nutrient broth culture. The flasks were then shaken for 18 h at 30°C and the turbidity of each culture measured.

● - wild-type

○ - strain D35A


100
50
0
% of growth in the absence of streptomycin
10^{-2}
10^{-1}
1
10^{1}
Streptomycin conc. (µg/ml)

4.ii. Construction of multiple mutants by transformation

4.ii.1. Construction of phenylglyoxylate carboxy-lyase$^-$, L-mandelate dehydrogenase$^-$ mutants

The isolation of PgC$^-$, L-mdh$^-$ mutants was done in order to test the feasibility of using transformation for the construction of multiple mutants (Table 13). Their isolation involved the use of streptomycin resistance as the selected marker. Out of 555 transformant colonies 18 PgC$^-$, L-mdh$^-$ mutants were obtained, of which two were retained (T36A and T36B).

4.ii.2. Construction of D-mandelate dehydrogenase$^+$, L-mandelate dehydrogenase$^-$ mutants

To examine whether the D-mandelate utilizing enzyme of strain 41 (Lancaster, unpublished results) could support growth in the absence of L-mandelate dehydrogenase it was necessary to try and construct a D-mdh$^+$, L-mdh$^-$ mutant. The existence of such a mutant would prove that the D-mandelate utilizing enzyme was a dehydrogenase and not a racemase (see Discussion, section 3.ii).

Strain NF1408 (L-mdh$^-$, D-mdh$^-$) was grown to competence for transformation and 0.1 ml aliquots were then spread with 0.1 ml of strain 41 (L-mdh$^+$, D-mdh$^+$) DNA (100 μg/ml) on 5 mM-D-mandelate and 5 mM-D-mandelate + 0.05 mM-phenylglyoxylate-salts agar. The plates were incubated for 4 days at 30°C then replicated onto 5 mM-L-mandelate and 5 mM-D-mandelate-salts agar. No transformants were obtained when strain NF1408 was transformed on 5 mM-D-mandelate agar, but

Table 13

Numbers and phenotypes of bacteria isolated from the transformation of strain NF1408 with DNA from strain D35A.

NF1408 (an L-mdh$^-$, PgC$^+$, SmS strain) (Livingstone, 1970) was transformed with DNA from strain D35A (L-mdh$^+$, PgC$^-$, SmR; derived from the parent strain NA36, L-mdh$^+$, PgC$^-$, SmS) (Livingstone, 1970). The transformation was done on 1.5 mM-benzoate + 10 μg/ml-streptomycin agar. Transformant colonies were distinguished by replica plating on 10 mM-D,L-mandelate agar and 1.5 mM-phenylglyoxylate agar. L-mdh$^+$, PgC$^-$ and L-mdh$^-$, PgC$^-$ colonies were distinguished by the DCIP reduction test (see Methods, section 1.xii).

Note: all classes of transformant could include spontaneous SmR mutants for which no controls were included.

Phenotype	Number of bacteria with this phenotype
L-mdh$^+$ PgC$^-$ SmR	385
L-mdh$^+$ PgC$^-$ SmR	1
L-mdh$^-$ PgC$^+$ SmR	143
L-mdh$^-$ PgC$^-$ SmR	18

Table 14

Activities of L-mandelate dehydrogenase and D-mandelate dehydrogenase in strains 41Z (L-mdh$^+$, D-mdh$^+$) and D40G (L-mdh$^-$, D-mdh$^+$).

Bacteria were grown in 5 mM-L-glutamate-salts medium to stationary phase. Extra glutamate and the listed inducers were added and the bacteria were incubated for a further $2\frac{1}{2}$ h before harvesting. Oxygen uptake was measured with an oxygen electrode using 0.5 mM substrates. Results are corrected by subtraction of the endogenous respiration.

inducer	substrate	Rate of oxygen uptake (nmol O_2/min/mg dry wt)	
		strain 41Z	strain D40G
none	D-mandelate	0	0
	L-mandelate	0	0
5mM-	D-mandelate	61	0
L-mandelate	L-mandelate	163	0
5mM-	D-mandelate	125	152
D-mandelate	L-mandelate	242	0

were obtained when 0.05 mM-phenylglyoxylate was added to the agar. Phenylglyoxylate is the inducer of the RI enzymes and was added at low concentration in the hope that it would induce newly introduced D-mandelate utilizing enzyme. Out of 306 transformants obtained, 36 were D-mdh$^+$, L-mdh$^-$ and three of these were retained for further study (D40E, F and G). The other 270 were D-mdh$^+$, L-mdh$^+$, two of these were also retained (41Y and 41Z). The L- and D-mandelate dehydrogenase activities of both these classes, when induced with L- or D-mandelate, were measured by Mr I.C. Matson of this laboratory (Matson, unpublished results) using an oxygen electrode (Table 14). Neither 41Z or D40G metabolised D- nor L-mandelate without previous induction. When induced with L-mandelate 41Z utilized both D- and L-mandelate, the activity of its D-mandelate dehydrogenase being less than that of its L-mandelate dehydrogenase. D40G utilized neither substrate when induced with L-mandelate. When induced with D-mandelate 41Z utilized both substrates, its L-dehydrogenase activity again being more than its D-dehydrogenase activity. D40G utilized only D-mandelate when induced with D-mandelate, the activity of its D-dehydrogenase being similar to that of 41Z.

4.iii. Isolation of a constitutive L-mandelate dehydrogenase mutant

In previous searches for constitutive L-mandelate dehydrogenase mutants using the alternate-culture technique (Cohen-Bazire and Jolit, 1953) only mesoconstitutive mutants

were obtained (Livingstone, 1970). Therefore, it was thought that continuous-culture might be a better tool for isolating constitutive L-mandelate dehydrogenase mutants of this organism. A series of samples was removed from a continuous-culture run (operated by Dr C.A. Fewson) of wild-type bacteria growing on limiting L-mandelate. The samples were plated on nutrient agar and individual colonies examined for constitutive L-mandelate dehydrogenase activity by the DCIP tube-test. Those strains which appeared to have constitutive activity by this test were examined spectrophotometrically, by the method of Beggs (1974), for their induced and uninduced L-mandelate dehydrogenase activities. One mutant was obtained which, though not a magnoconstitutive, had greater mesoconstitutive activity than Livingstone's mesoconstitutive strain. The average L-mandelate dehydrogenase activities (in nmol/mg protein/min) of this strain (S25F) were: induced, 196 (± S.E.M. 12.1, 4 expts.); uninduced, 34 (± S.E.M. 4.3, 4 expts.). For the wild-type these activities were: uninduced, 3.3 (± S.E.M. 1.1, 4 expts.); induced, 172 (± S.E.M. 18.0, 4 expts.).

4.iv. Attempts to select aromatic catabolism mutants with toxic analogues

Analogues of metabolisable substrates can be found which restrict the growth of bacteria. Mutants which are unable to utilize a toxic analogue and its analogous substrate will not have their growth restricted in the presence of the analogue. Therefore, such mutants can be

positively selected from a population of growth-restricted bacteria by a method similar to the selection of antibiotic-resistant mutants. The analogue is added, at growth-restricting concentration, to medium containing a growth-supporting substrate; the bacteria are added and eventually the mutants overgrow the restricted bacteria. By this method. Clarke and Tata (1973) used fluoroacetamide to isolate amidase-negative mutants of Pseudomonas aeruginosa from a population of constitutive amidase mutants. Fewson et al. (1968) found that A. calcoaceticus NCIB 8250 would metabolize monofluorobenzoates to some extent but never as the sole source of carbon and energy. p-fluorobenzoate was found to restrict growth in the presence of other carbon sources while being metabolised itself. The possibility was considered that if an RI or RII mutant could not utilize the corresponding p-fluoro compound then these compounds might be useful as selective agents. Two experiments were done to examine the feasibility of using p-fluoro compounds as selective agents.

Wild-type bacteria were grown to an E500 of 0.4 in 5 mM-benzyl alcohol, 10 mM-D,L-mandelate, 10 mM-succinate-salts medium and nutrient broth. The mutants NA37 (Badh II^-, Bdh^-) and NF1408 (L-mdh^-) were grown in nutrient broth. All cultures were washed and resuspended in basal medium then the following mixtures were plated on 2 mM-succinate-salts agar + 5 mM-p-fluoro-benzyl alcohol:

(a) 10^8 succinate-grown wild-type;

(b) 10^8 benzyl alcohol-grown wild-type;

(c) 10^8 nutrient broth-grown wild-type;

(d) 10^8 nutrient broth-grown NA37;

(e) 10^8 succinate-grown wild-type + 10^2 NA37;

(f) 10^8 benzyl alcohol-grown wild-type + 10^2 NA37;

(g) 10^8 nutrient broth-grown wild-type + 10^2 NA37.

Similarly, wild-type and NF1408 were plated on 2 mM-succinate-salts agar + 10 mM-p-fluoro-D,L-mandelate, D,L-mandelate-grown wild-type being used instead of benzyl alcohol-grown wild-type.

In a second experiment nutrient broth-grown NA37 and NF1408 were similarly plated with succinate or nutrient broth-grown wild-type on the following media:

(a) nutrient agar + 0.2 mM p-fluoro compound;

(b) nutrient agar + 2.0 mM p-fluoro compound;

(c) 2 mM-succinate-salts agar + 0.2 mM p-fluoro compound;

(d) 2 mM-succinate-salts agar + 2.0 mM p-fluoro compound.

5 mM and 10 mM-fluoro compounds were found to prevent the growth of the mutants but not the wild-type and different kinds of preinduction had little effect on the growth of the wild-type. Reducing the concentration of the fluoro compounds to 0.2 and 2.0 mM allowed the mutants to grow but not better than the wild-type, therefore, no selection was possible using this technique.

DISCUSSION

1. Mutagenesis

The four mutagens studied were chosen partly because of their convenience in use and partly because of their successful use in other organisms. Two methods can be considered for assaying the effectiveness of a mutagen; determination of the number of auxotrophs induced by the mutagen (e.g. Nečásek et al., 1967; Townsend et al., 1971), and measurement of the induced reversion rate of an auxotrophic gene by the mutagen (e.g. Delić et al., 1970). For a comparison of different mutagens the disadvantage of the second method is that some mutagens may not be capable of reverting a particular mutant site, even though they can cause mutation at other sites in the same genome. However, by measuring the number of auxotrophs produced by each mutagen one is measuring their effect on a very large number of potentially mutable sites rather than on just one mutant site. Therefore, the number of auxotrophs produced is a more useful general assay with which to compare different mutagens and this was the method used in this work. If a mutagen is known to revert a particular mutant site then reversion of that mutation is a suitable method for studying the effects of different physical, biological and chemical conditions on that mutagen (as in Delić et al., 1970).

Bacterial cells usually contain more than one genome under normal cultural conditions, therefore the formation of pure mutant clones from bacteria plated directly after

mutagenesis may not occur. To obtain pure clones the bacteria are generally grown in broth for a few generations after mutagenesis. However, Hopwood (1970) noted that plating directly after mutagenesis would probably produce pure mutant clones if mutagenesis caused appreciable killing. In such a case a "viable" but mutant genome is unlikely to find itself in the same bacterium as a "viable" but non-mutant genome. Since all four mutagenic treatments did produce high levels of inactivation it was considered that plating mutated bacteria directly on solid media would be an acceptable method for the assay of auxotrophs.

Of the mutagens studied, EMS is the most convenient to handle and also gave the greatest number of auxotrophs (Table 6). The 15 min EMS treatments devised by Loveless and Howarth (1959) for Escherichia coli and Salmonella typhimurium were not effective in A. calcoaceticus and therefore longer treatments were tested. Nečásek et al. (1967) had obtained 15% auxotrophs, at a surviving fraction of 0.013%, from Corynebacterium VÚA9366 after 18 h treatment with 50 mM-EMS. A similar effect of EMS was observed for A. calcoaceticus although the rate of inactivation (Fig. 7) and mutagenesis was slower.

NTG is also an effective mutagen in this organism but again the fraction of auxotrophs obtained was lower than is obtained in other organisms. Under conditions where NTG induced about 4% auxotrophs in A. calcoaceticus (Table 5) it produced approximately 50% in Escherichia coli

(Adelberg et al., 1965). This smaller amount of mutation is useful since, presumably, it will not give rise to such a high proportion of multiple mutants as originally feared (Introduction, section 2).

Much lower levels of mutagenesis were obtained with u.v. and near u.v. with 8-methoxypsoralen (Tables 3 and 7). In both cases auxotrophs appear for short periods then rapidly become undetectable. This may be due to the rate of inactivation overtaking the rate of induction of new mutants. Under the same conditions of 8-methoxypsoralen mutagenesis Townsend et al. (1971) obtained 1 to 4% auxotrophs from Streptomyces species. However, the success of the auxotroph selection from u.v. irradiated bacteria (Tables 10 and 11) shows that even at such low levels of mutagenesis u.v. is a useful mutagen for A. calcoaceticus. In view of the similarity between u.v. and 8-methoxypsoralen mutation levels, 8-methoxypsoralen could probably also be used successfully. The failure of Livingstone (1970) to obtain u.v. induced mutants of aromatic catabolism from A. calcoaceticus, can probably be attributed to the low incidence of u.v. induced mutants and to the lack of an effective selection system. The fact that u.v. inactivated A. calcoaceticus is photoreactivable (Table 4) suggests that it possesses the same kind of enzymic repair system that operates in other photoreactivable organisms. Because of the relatively low incidence of u.v. induced mutants it is important to shield u.v. irradiated bacteria from photoreactivating light (between 300 and 500 nm;

Kelner, 1951) immediately after mutagenesis.

The results of EMS, NTG and 8-methoxypsoralen mutation in A. calcoaceticus again demonstrate that one cannot rely on mutagenic treatments developed for one organism, having the same effect on another organism and stresses the need for a systematic study for each new organism. Further work on mutagenesis in this organism could begin with a study of some of the other mutagens mentioned in the Introduction (section 2). A study might also be made of the effects of temperature and pH on some of the mutagens already examined. A start has been made on investigating the effect of pH on NTG mutagenesis in A. calcoaceticus (E.F. Ahlquist, unpublished work).

2. Isolation of Mutants by Antibiotic Selection

2.i. The effect of the antibiotics

The results of the antibiotic-disc tests (Table 8) showed that few of the antibiotics were suitable for a selection system. Four of the five penicillins (cloxacillin, ampicillin, methicillin and penicillin) had no effect, though Gram-negative bacteria are often sensitive to ampicillin (Gale et al., 1972). Carbenicillin, which has the broadest spectrum of all penicillins, seemed the most promising for a selection system. The other wall-synthesis inhibitors, vancomycin and bacitracin, were possibles for this purpose. Neomycin and gentamycin were also considered as possible selective agents since the related antibiotic kanamycin had been successfully used in a selection system

(Evans, 1966). The final choice of antibiotics was made on the basis of their effect on A. calcoaceticus in liquid medium (Table 9).

The two antibiotics chosen for use in the selection system, vancomycin and penicillin V, both exert their effect on the synthesis of the bacterial wall polymer, peptidoglycan. During the formation of peptidoglycan the basic units, disaccharide-pentapeptides, are assembled at the inner-membrane and transported through it on an isoprenoid-alcohol carrier molecule (Higashi et al., 1967). The units are then attached to the growing points of the polymer by the enzyme peptidoglycan synthase. To complete the structure, adjacent polysaccharide chains are cross-linked via their pentapeptide chains, the reaction being mediated by a transpeptidase enzyme (Matsuhashi et al., 1967).

Vancomycin has been shown to block the cycle of reactions, involving the isoprenoid-alcohol carrier, at the stage in which the disaccharide-pentapeptide is transferred to the growing point of the peptidoglycan (Struve and Neuhous, 1965; Struve et al., 1966). It has a high affinity for free terminal sequences of pentapeptide chains, particularly acyl-D-ala-D-ala as found in Staphylococcus aureus peptidoglycan (Perkins, 1969). It seems likely that the bulky vancomycin-molecule (MW 1600 to 1800), in its interaction with the terminal pentapeptide sequence, sterically inhibits the action of peptidoglycan synthase (Gale et al., 1972). It also seems likely that

vancomycin inhibits transpeptidase activity since it probably binds to unlinked pentapeptides already in the peptidoglycan polymer. The penicillins are known to inhibit the action of the transpeptidase enzyme. There is good evidence that their action depends on the steric resemblance of part of the penicillin molecule to part of the pentapeptide chain (Tipper and Strominger, 1968).

An organism related to Acinetobacter calcoaceticus NCIB 8250, Acinetobacter MJT/F5/199A, is known to contain peptidoglycan in its wall. Thorne et al. (1973) showed that its wall consists of four layers: (i) a peptidoglycan-containing dense layer, (ii) an intermediate layer, (iii) a lipopolysaccharide-containing outer membrane and (iv) an ordered array of protein subunits. Interference with the formation of the first layer could be sufficient to inactivate the bacterium. In Gram-positive bacteria binding of vancomycin is irreversible; Staphylococcus aureus, pretreated with vancomycin, fails to grow in vancomycin-free medium and continues to accumulate peptidoglycan precursors (Reynolds, 1966). A. calcoaceticus cannot bind vancomycin irreversibly since it can grow in vancomycin-free medium after vancomycin treatment (Results, section 2.iv), this is probably due to a lower affinity of its pentapeptide for vancomycin. However, there is a difference in the growth of vancomycin-treated bacteria in nutrient broth and L-glutamate-salts medium. A possible explanation of the faster growth in nutrient broth is that peptides in the broth bind to vancomycin and help to remove it from the

bacterial walls. Penicillin V is only included in the selective medium to exert its protective effect on non-growing bacteria (Table 9). Considering the mechanisms given above for the action of penicillin and vancomycin it is difficult to imagine how such an interference could occur. Perhaps these antibiotics, particularly vancomycin, have unknown secondary effects in A. calcoaceticus; certainly the vancomycin concentration used with this organism is much greater than that required to inhibit Gram-positive bacteria; the minimum growth inhibitory concentration for Bacillus subtilis is 2 μg/mg dry weight (Best and Durham, 1965). It therefore seems possible that events other than wall-synthesis inhibition take place in A. calcoaceticus.

The antibiotic mixture can behave differently depending on the substrate in the selective medium. When bacteria are treated in L-mandelate or benzyl alcohol no turbidity increase occurs after addition of the antibiotics, however, when the substrate is succinate an increase in turbidity is observed. This effect was observed in a number of experiments though never measured quantitatively. If vancomycin acts only on the wall it is unlikely that the substrate would make a difference to its action - particularly as L-mandelate and benzyl alcohol are metabolised to succinate. The effects on vancomycin-inactivation of different substrates, substrate concentrations and live: inactivated bacteria ratio (Results, section 2.v), might be difficult to explain even if the action of the antibiotic

were completely understood. Since the effect of vancomycin on A. calcoaceticus is far from clear, no meaningful explanations can be put forward for these results. However, this did not prevent them from being used in developing the selection techniques.

2.ii. The selection techniques

To isolate a limited number of mutant classes, some kind of selection system is necessary. This was demonstrated by the attempt to isolate mutants blocked in aromatic catabolism (Results 3.ii) without prior selection. Since the attempt at positive selection of blocked aromatic mutants with p-fluoro compounds also failed (Results 4.iv), the only remaining method was antibiotic selection. Fortunately, it seems that antibiotic selection is suitable for isolating blocked aromatic mutants as well as auxotrophs.

Successful selection of auxotrophs was obtained by a 3 h vancomycin treatment in 10 mM-succinate-salts medium (Tables 10 and 11). Over each cycle of enrichment an approximately 10-fold increase in auxotroph numbers was obtained. In the reconstruction experiment (Table 12, Fig. 13) the unexpected 100-fold enrichment obtained after a 1 h vancomycin treatment could be explained as rapid removal, by nutrient broth, of vancomycin from non-growing mutants. A vancomycin-preatreated auxotroph (U4K) has been shown to grow several hours earlier than vancomycin-pretreated wild-type when both had been vancomycin treated for 2 h in 10 mM-succinate-salts medium and transferred to

nutrient broth (Results 2.iv). This indicates that the auxotroph was released from the growth-inhibiting effect of vancomycin before the wild-type. The inactivated fraction of wild-type bacteria after 1 h vancomycin (500 μg/ml) treatment is in the region of 90% (Fig. 10), this could not give more than a 10-fold enrichment. It therefore seems that the "release from vancomycin" may be playing a considerable part in enrichment. This is a departure from the theory of antibiotic enrichment, in which the important factor is the fraction of antibiotic-inactivated wild-type compared with the fraction of inactivated mutants (Schlegel and Jannasch, 1967). Figure 13 shows that in the reconstruction experiment enrichment was highest where vancomycin-inactivation was lowest, the enrichments obtained subsequent to the 1 h vancomycin treatment could easily be accounted for by vancomycin-inactivation of the wild-type. This indicates that the "release effect" is overtaken by the "inactivation effect" as the length of vancomycin treatment increases. Therefore, though the two effects cannot be completely separated in any of the enrichment techniques, it is likely that the "release effect" would be of greater importance to enrichment during 3 h treatments than in 17 h treatments. Use might be made of this "release effect" to increase the efficiency of selection, however, 1 h vancomycin treatments were unsuccessful in obtaining aromatic blocked mutants (Results 3.ii).

Selection of blocked aromatic mutants was eventually successful using a 17 h vancomycin + penicillin V treatment in 10 mM-benzyl alcohol salts medium. Various mutants of the RIII regulon and the catechol pathway were obtained (E59 mutants, Table 1) but no RII regulon mutants were isolated. No isolation of RI mutants was tried in a similar system because it is known that intermediates would accumulate and allow the growth, and hence vancomycin-induced inactivation of any RI mutants. Beggs (1974) showed that when A. calcoaceticus is growing on L-mandelate, benzaldehyde accumulates because benzaldehyde dehydrogenase I is rate limiting. Benzaldehyde induces the RII regulon and the benzyl alcohol dehydrogenase reduces some of the benzaldehyde into benzyl alcohol. With the RII regulon induced, RI mutants would be able to grow on the benzaldehyde and benzyl alcohol. Because of repeated failures to isolate RII mutants it was thought that some benzoate might accumulate during growth of the wild-type on benzyl alcohol, possibly due to a lag in "benzoate oxidase" induction. This would allow RII mutants to grow and be vancomycin inactivated. Therefore, the benzoate preinduction step (Results 3.ii) was introduced to the selection procedure in an attempt to stop benzoate accumulation. However, if there was accumulation of benzoate, even after preinduction, then RII mutants would also be induced for "benzoate oxidase" and would be able to grow. This would make benzoate preinduction an actual disadvantage in RII mutant selection, but clearly, benzoate preinduction still allows the isolation of RIII and catechol pathway mutants. One

way of circumventing the accumulation of benzaldehyde and benzyl alcohol during growth on L-mandelate would be to use a benzaldehyde dehydrogenase II^- mutant as the starting culture for enrichment. In such a case benzaldehyde would still accumulate but RI mutants could not utilize it via their RII enzymes. However, if benzoate was accumulating during growth on benzyl alcohol, no blocked "benzoate oxidase" mutant could be used as the starting culture to prevent the accumulation. Such a mutant would not be able to grow on benzyl alcohol and would not be inactivated by vancomycin. The 10 mM-benzyl alcohol + vancomycin/penicillin V system worked well for the isolation of RIII and catechol pathway mutants and could probably be adapted to obtain other blocked mutants of carbon source catabolism, so long as there were no unknown problems of substrate accumulation.

How this system compares with the only other Acinetobacter selection system studied, that of Herman and Juni (1974), is not really known. These authors obtain a 99.9% inactivation of wild-type after 2 h incubation with ampicillin (2000 μg/ml) and cycloserine (400 μg/ml). This is higher than the inactivation obtained after incubating strain NCIB 8250 with vancomycin (500 μg/ml) for 2 h (Fig. 10). However, since they give no indication of what the surviving fraction of mutants might be, one can make no estimate of the level of enrichment they obtained. It is interesting to note that neither ampicillin nor

cycloserine have much effect on strain NCIB 8250 (Tables 8 and 9). There might, therefore, be some difference in the structure of the wall between NCIB 8250 and their organisms. A knowledge of the effect of the ampicillin/cycloserine mixture on NCIB 8250 might help to clarify this.

3. Isolation of Mutants by Other Methods

3.i. Isolation of drug-resistant mutants

The method of positive selection of drug resistant mutants was shown to be successful in this organism, using streptomycin as the test antibiotic. Resistance to streptomycin in Escherichin coli is known to involve a one step mutation affecting the function of the ribosomes (Davis et al., 1968). It seems likely, from the high incidence of SmR mutants, that a one step mutation also occurs in A. calcoaceticus. No doubt the selection method could be extended to other antibiotics in cases where resistance involves a one-step mutation, it would be interesting to look for mutants resistant to some of the antibiotics to which this organism is sensitive (Table 8).

3.ii. Construction of multiple mutants by transformation

Transformation is clearly a convenient tool for the construction of multiple mutants in A. calcoaceticus (Results 4.ii). An interesting aspect of these experiments is the isolation of a D-mdh$^+$, L-mdh$^-$ strain. Lancaster (1970), who isolated the D-mandelate utilizing strain 41, suggested that the new enzyme was either a mandelate racemase as found in Pseudomonas putida (Hegeman, 1966a) or a

D-mandelate dehydrogenase. The possibility of constructing a mutant with D-mandelate utilizing ability but lacking L-mandelate dehydrogenase, allowed the hypothesis to be tested. If the D-mandelate enzyme were a racemase, the loss of L-mandelate dehydrogenase would prevent growth on D-mandelate. The construction of the L-mdh$^-$, D-mdh$^+$ strain showed that the enzyme was a D-mandelate dehydrogenase. This could have arisen in several ways, e.g. as a revertant of the D-mdh$^-$ wild-type, or as a new gene by spontaneous mutation of a duplicated L-mdh gene. A similarity in structure between the D- and L-mandelate dehydrogenase enzymes could be tested by amino acid sequencing of the two enzymes, or on a simpler level, by serological studies. The possession of a D-mdh$^+$, L-mdh$^-$ mutant will simplify further studies of the D-mandelate dehydrogenase enzyme since L-mandelate dehydrogenase activity will be missing.

3.iii. The constitutive mutant

As noted in the Introduction (section 3) the alternate-culture technique had only produced a mutant mesoconstitutive for L-mandelate dehydrogenase (Livingstone, 1970) and no non-inducing substrates or anti-inducers were available for the RI regulon of A. calcoaceticus. Therefore, continuous-culture was the only method left available for attempting the isolation of mutants magnoconstitutive for L-mandelate dehydrogenase. Using this method a mutant was obtained with mesoconstitutive L-mandelate dehydrogenase activity (Results 4.iii) but no magnoconstitutives were

obtained. In fact, no magnoconstitutive mutant has ever been obtained for an organism with an aromatic catabolic pathway (Hegeman, 1966c; Livingstone, 1970) and the reason for this is still unknown. The mutant mesoconstitutive for L-mandelate dehydrogenase isolated by Livingstone (1970) has the following properties: (i) uninduced L-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase levels only slightly above those of the uninduced wild-type (3% of fully induced wild-type); (ii) these two enzymes are uninducible above the mesoconstitutive level and (iii) benzaldehyde dehydrogenase I is hyperinducible and not constitutive. This suggested that there were 2 regulatory subgroups of the RI regulon, L-mandelate dehydrogenase + phenylglyoxylate carboxy-lyase and benzaldehyde dehydrogenase I, but both groups having a common repressor. The evidence for a common repressor was that (i) all 3 RI enzymes are co-ordinately induced and repressed by the same compounds and (ii) mutants were isolated in which all 3 enzymes were uninducible (Livingstone and Fewson, 1972).

Evidence from the construction of a PgC$^-$, L-mdh$^-$ strain (Table 13) suggests that there is no close linkage between the PgC and L-mdh genes. This means that the existence of a common operator gene for the two is unlikely, but separate operators could behave in an identical way with the same regulator protein. The tentative picture that emerges is of three separate RI genes each with its own operator but all controlled by the same regulator gene. A similar arrangement has been demonstrated for the arg

system of Escherichia coli (Jacoby and Gorini, 1969). Strain S25F could be a regulator or an operator mutant in which the regulator protein did not bind properly to the L-mandelate dehydrogenase operator but where the regulator protein + inducer could derepress normally. Further investigation of its enzyme levels and inducibility, and also genetic mapping, could help to clarify this.

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