THE EFFECT OF PHENETHYL ALCOHOL, AND OTHER ANTIMETABOLITES, ON GROWTH AND EXTRACELLULAR PROTEIN PRODUCTION IN *Staphylococcus aureus*

BY

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Sir, old man,
Will you read my book,
It took me years to write,
Will you take a look.

Lennon and McCartney

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OBJECT OF THE RESEARCH

Staphylococcus aureus produces at least thirty-one different proteins which can be detected extracellularly, and many of these are involved, either directly or indirectly in its pathogenicity. Most research into these proteins has been concerned with their nature and mode of action and little is known about their synthesis. The best known extracellular protein is the alpha-haemolysin which appears to be a truly extra-cellular protein. Some proteins appear to be cell-associated and one of these may be the acid phosphatase. The use of phosphatases by the cell is a metabolic function rather than a pathogenic one, so a comparison of the production of the alpha-haemolysin and the acid phosphatase under normal or inhibited conditions was instituted. There were two major objectives :

(1) To throw some light on the conditions required for synthesis of these proteins

(2) To study the effect of phenethyl alcohol to help elucidate its mode of action.

To assist in the work, two minor objectives were to determine something of the nature and location of the phosphatase activity and to produce and compare extracellular protein mutants.

ABBREVIATIONS

BS	Bernheimer-Schwartz Medium
c.f.u.	colony forming unit
H.U.	Haemolytic Unit
I.E.F.	Iso-electric Focussing
MNNG	N-Methyl-N'Nitro-N-Nitroso Guanidine
PBS	Phosphate Buffered Saline
PBSA	PBS + 0.1% Bovine Serum Albumin
p.f.u.	plaque forming unit
Kd	Kilo daltons

SYMBOLS

G	Diffusion rate
i	Inhibitor concentration
K_i	Inhibitor constant
K_s	Substrate constant
μ	Specific growth rate $= \frac{1}{x} \cdot \frac{dx}{dt}$
μ_m	Maximum specific growth rate
p	Product concentration
q_p	Metabolic quotient for product formation $= \frac{1}{x} \cdot \frac{dp}{dt}$
S	substrate concentration
x	Biomass concentration
Y	Growth yield
$Y_{p/x}$	Product yield

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

SUMMARY

Staphylococcus aureus can excrete at least 31 different proteins which are either wholly extracellular or cell-associated to some degree. Conditions required for production of the two classes are likely to be different since the former are produced late in the growth cycle whereas the latter are probably constitutive. Two proteins were chosen to represent each class and the effect of various antimetabolites on their production was investigated. Particular attention was paid to the effect of phenethyl alcohol since it was hoped to clarify its mode of action.

Acid phosphatase was chosen as the example of a cell-associated protein. Since its degree of association was uncertain this was investigated in two ways. A culture which had been grown overnight was separated by centrifugation and the phosphatase activity in the supernatant and in the cells was determined. Activity associated with the cells was fractionated by washing to remove loosely bound enzyme, protoplasting to determine activity in the wall and periplasm, and lysis to determine activity in the membranes and cytoplasm. It was found that 40% of the total activity was extracellular, 35% was associated with the outer layers of the cell and the remainder was in the protoplast. Electron microscopy revealed considerable activity in the cytoplasm, membranes and walls much of which could be easily washed out. The phosphatase activity appeared to have three pH optima at 6.1, 6.8 and 7.8. These results confirmed that the acid phosphatase was largely cell-associated.

Alpha-haemolysin was the other protein chosen, since it was widely accepted to be a wholly extracellular enzyme. This was confirmed.

Production of the two proteins under different conditions was assessed. In rich media, such as the glucose-casamino acids-yeast extract diffusate medium most commonly used in these experiments, alpha-haemolysin activity was first detected at a population density of 0.68 ± 0.11 measured by extinction at E_{600nm}. There was no evidence of growth linking; indeed production of the haemolysin increased most markedly while the culture was slowing down at the end of log phase. In poorer media with lower nitrogen content or with alternative carbon sources production of alpha-haemolysin began much later and yield was greatly reduced. Some other extracellular proteins were assessed in a few experiments and seemed to parallel the appearance of the alpha-haemolysin. Acid phosphatase activity was always detected and its production appeared to be proportional to the population density, although it was not directly growth-linked.

The effect of phenethyl alcohol on growth and extracellular protein production was examined by varying concentration of inhibitor and by varying either the time of addition or the cell number. Concentrations of phenethyl alcohol above about 25mM were totally inhibitory to growth due to damage to membrane. Analysis of the results indicated that the effect on growth was competitive up to 25mM and non-competitive above this concentration. Production of alpha-haemolysin was delayed until an E_{600nm} of 1.25 ± 0.2 and the yield was reduced by 80%, when treated with phenethyl alcohol at 15mM. Above this concentration it was not detected. Acid phosphatase production was inhibited in line with growth inhibition. Inhibition of both growth and extracellular protein production was independent of growth rate and population density, although if inhibitor was added when the culture was nearing stationary phase and maximum protein level this was not always easy to detect.

Certain amino acids had been suggested to be involved in alpha-haemolysin production. Phenethyl alcohol did not directly mimic the effect of phenylalanine analogues but addition of excess histidine to complex media did partially reverse the inhibition of alpha-haemolysin production. A comparison of the effect of phenethyl alcohol analogues indicated that the inhibitory effects increased with decreased polarity of the side chain, suggesting that membrane penetration might be involved.

A number of structurally unrelated inhibitors were also tested. Mitomycin C caused lysis which released excess acid phosphatase from the cells, but alpha-haemolysin was not produced. Comparison of cultures treated with either chloramphenicol or actinomycin D indicated that alpha-haemolysin was produced by a more durable form of mRNA than those for growth related proteins. Since membrane or membrane associated functions seemed to be involved in alpha-haemolysin production the effect of inhibitors which affected membranes was investigated. The effect of sodium nitrite was similar to that of phenethyl alcohol, although the mechanism was probably different. Potassium ferricyanide, sodium azide, potassium cyanide and 2,4 dinitrophenol were different in their action. In particular KCN at 1mM appeared to inhibit growth but had less effect on alpha-haemolysin synthesis which suggested that a reduction in respiration rate was not significant. Dinitrophenol was inhibitory in a non-competitive fashion and this appeared to be similar to but not identical to the effect of phenethyl alcohol; the effect of 1mM dinitrophenol being apparently identical to that of 15mM phenethyl alcohol.

Since there appeared to be evidence that phenethyl alcohol was interfering with the uptake of amino acids its effect on the uptake of radio-labelled amino acids was investigated, and compared with that produced by dinitrophenol. The results indicated that 1mM dinitrophenol reduced the uptake of the amino acids whereas 15mM phenethyl alcohol caused efflux of label from the cell.

Some aspects of the genetics of extracellular protein production were also investigated. The alpha-haemolysin activity could not be permanently eliminated by treatments intended to eliminate plasmids. About 77% of mutations produced by various treatments were pleiotropic. On the basis of the pattern of mutation five groups were suggested, possibly related to five genes or gene groups. These were the structural gene for the alpha-haemolysin;some part of the excretion mechanism; some aspect of transcriptional control; an alpha-haemolysin activator; and the genes involved in 'dwarfing'.

The general conclusion was that alpha-haemolysin and acid phosphatase were produced by different types of genetic organisation and that production of the former was probably controlled by available intermediates, particularly amino acids. The latter was probably constitutive and due to at least three enzymes which were distributed uniformly throughout the cell. Phenethyl alcohol produced its inhibitory effects by interfering with membrane transport, particularly of aminoacids.

INTRODUCTION

INTRODUCTION

A. *Staphylococcus aureus* ROSENBACH

Staphylococcus aureus is a successful and versatile pathogen whose pathogenicity has generally been accepted as being due, directly or indirectly, to its production of extracellular proteins (Elek, 1959; Blair, 1962). The production of these proteins, and the mechanisms which control their synthesis and excretion, are not well understood so this investigation was undertaken in an attempt to discover some of the factors involved. The presence of these proteins, particularly haemolysin, coagulase, nuclease and phosphatase is often used as a classification character. However, since part of the work reported here involved the isolation of mutants lacking extracellular proteins it was useful to define the species in standard terms. Except as otherwise stated, the description given here was adapted from Baird Parker (1974).

Staphylococci are non-motile, gram-positive cocci (0.7 - 1.0 μm in diameter) occurring in pairs or clusters. They can be distinguished from members of the genus *Micrococcus* by their possession of a fermentative metabolism. At a more fundamental level, staphylococci have a DNA base ratio of 30-39% G+C compared to 63-73% for micrococci. *Staph. aureus* is a catalase-positive, facultative anaerobe; mesophilic (with a growth optimum between 35-40^{o}C) and halotolerant (up to 15% w/v of sodium chloride). Acid is produced anaerobically from glucose and mannitol and frequently from lactose, maltose, sucrose and trehalose. Colonies are usually smooth, low-convex, glistening, butyrous, entire. Pigmentation ranges from white to golden orange and it may vary with the growth medium (O'Connor, Willis and Smith, 1966), or be dependent on the presence of a plasmid-like entity (Grinstead and Lacey, 1973). Antibiotic resistant strains are frequently yellow (Willis, Jacobs and Goodburn

1964). Extracellular slime layers (Yoshida and Ekstedt, 1968) or distinct capsules (Scott, 1969) are produced by some strains.

The problem of speciation in the genus Staphylococcus has attracted much attention with some workers, notably Kloos (1980), identifying as many as twenty different species in addition to Staph. aureus However, only Staph. epidermidis and Staph. saprophyticus are likely to be encountered as contaminants which might be confused with mutant isolates. Using the criteria proposed by the ICSB Subcommittee on the Taxonomy of Staphylococci and Micrococci (Baird-Parker et al., 1976), the ability to ferment mannitol and sensitivity to Novobiocin could be used as confirmatory tests for Staph. aureus.

B. EXTRA-CELLULAR PROTEINS PRODUCED BY Staph. aureus

As stated above, the production of extracellular proteins by Staph. aureus is an important feature of the genus and was considered to be the principal reason for its pathogenicity. In most instances the term "extracellular" means that the activity of the protein can be detected by zones of reaction around colonies of Staph. aureus growing on suitable solid media. However, as pointed out by Pollock (1962), the presence of such a zone is not, in itself, sufficient grounds for considering that a truly extracellular product is causing the reaction. The detection of protein in the culture medium is not sufficient grounds either since the protein may get there by leakage from a periplasmic or cell wall locus, or as a result of autolysis. For a protein to be regarded as truly extracellular, therefore, it should be demonstrated that the majority of the activity is detectable in the culture medium preferably during log phase and that there is little or no evidence of autolysis. Raynaud and Alouf (1970) echoed this view in their discussion of the extracellular nature of bacterial toxins. A low cystine content has often been accepted as a feature of extracellular proteins (Glenn, 1976), an idea first proposed by Pollock and Richmond (1962) who suggested that a lack of di-sulphide bridges might facilitate transmembrane passage.

This section describes the variety of extracellular proteins which have been detected in Staph. aureus culture filtrates and then briefly considers the evidence that these proteins are truly extracellular.

B.1. The Variety of Extracellular Proteins Produced by Staph. aureus

Several workers have examined filtrates from broth cultures of Staph. aureus in an attempt to relate the total number of extracellular proteins to the degree of pathogenicity. In these studies, potentially pathogenic strains (normally strains isolated from cases of infection), were compared with probable non-pathogens (strains not shown to have been involved in infections).

Bernheimer and Schwartz (1961) (using analytical starch gel electrophoresis) detected between twelve and fourteen different protein bands in filtrates of potentially pathogenic strains, compared to less than half this number from probably non-pathogens, and Wadström (1973a)((using Isoelectric Focusing (I.E.F.) in gels)) detected 25-30 different bands in potential pathogens compared with 4-10 in probably non-pathogens. The same author, and co-workers (Wadström, Thelestam and Möllby, 1974), demonstrated 18-20 different immunoprecipitates using cross-immunoelectrophoresis against commercial antiserum. The identity of each of the bands was not determined. Variation in the number of proteins found was probably due to differences both in technique and in the selection of producing strains.

At least thirty-one extracellular proteins have been demonstrated in culture filtrates of Staph. aureus isolated from clinical materials whereas presumable non-pathogens usually produced only a few conventional enzymes (proteases, lipases, etc.). (Todd 1968; Jeljaszewicz, 1972). Many workers have attempted to relate pathogenicity to the production of one or more of these proteins but, with the exception of the enterotoxins and exfoliative toxins, which cause highly specific pathogenic conditions, these studies have not been very conclusive.

The nature and mode of action of most of these proteins have been extensively studied over the last twenty-five years. However, the development of new or improved analytical techniques has led to the view that many reports were based on studies using incompletely purified materials. Many early conclusions should therefore be reassessed. A particular problem has been variation in molecular weight and charge properties which has been attributed to microheterogenicity caused by deamidation, proteolysis or charge alteration of ligands (Wadström, 1978).

B.2. Membrane-Damaging Toxins

McCartney & Arbuthnott (1978) have proposed that "membrane-damaging" is more appropriate than "cytolytic" as a general designation for those toxic proteins which exert their primary effect through an interaction with the cytoplasmic membrane of susceptible cells.

B.2.1. Haemolysins

Staph. aureus can produce four distinct, haemolytic proteins, designated Alpha, Beta, Gamma and Delta, which may be distinguished on a number of grounds, the simplest being their haemolytic spectrum. Table 1 is a composite table showing the haemolytic spectrum of purified samples of each haemolysin against rabbit, sheep, horse and human erythrocytes and although the assay systems used were not identical, the pattern shown by each haemolysin can be used taxonomically since similar patterns have been obtained by other workers. Only one reference has been used in each case since the intention is to show the haemolytic spectrum of the four products rather than to make a definitive statement about degrees of activity.

B.2.1.a. Alpha-haemolysin

The alpha-haemolysin is the classical haemolytic toxin produced by Staph. aureus and has been extensively studied by many workers. It was early recognised as being haemolytic, dermonecrotic and lethal (Burnet, 1929) and these three functions are still generally considered to be manifestations of alpha-haemolysin (Arbuthnott, 1970; Jeljaszewicz, 1972).

Molecular Nature

Alpha-haemolysin was extensively purified by many workers in the early and mid 1960's (e.g. Bernheimer & Schwartz, 1963; Lominski, Arbuthnott & Spence, 1963; Coulter, 1966) and it was generally considered to be a single protein although Bernheimer (1968) suggested that multiple forms of alpha-haemolysin might exist, with molecular weights ranging from 21 to 50 kilo-daltons (Kd).

Two major sedimentation species were found by Bernheimer and Schwartz (1963), an active 3S form and an inactive 12S form. The latter was thought to be due to polymerisation of the former (Bernheimer, 1965) and Arbuthnott, Freer and Bernheimer (1967) found that the 12S could be dissociated by 8M Urea with the recovery of active 3S. Electron microscopy revealed that the 12S material was in rings 10nm in diameter composed of 2-2.5 nm subunits and this ring formation remained after disc-gel electrophoresis (Freer and Arbuthnott, 1972). Bernheimer et al. (1968) have suggested that the polymerisation reaction took place through

TABLE 1

†Relative Sensitivity of Erythrocytes from Rabbit, Sheep, Horse and Man to Individual *Staph. aureus* Haemolysins

Erythrocyte Species	Haemolysin Alpha[1]	Beta[2]	Gamma[3]	Delta[4]
Rabbit	250	16	512	1.0
Sheep	10	526	128	0.2
Horse	1	1	1	1.0
Human	2	66	8	0.5

References : (1) Bernheimer (1965)

(2) Wiseman (1965a)

(3) Möllby and Wadström (1971)

(4) Kreger *et al.* (1971)

† Calculated by taking lysis of horse erythrocytes as equal to one Sensitivity Unit for the haemolysin concerned.

sites on the molecule which were also responsible for the toxic activity since toxoiding with formaldehyde removed the capacity for polymerisation as well as the toxicity. However, toxoiding might alter many parts of the molecule, so this would not be the only possibility. Wadström (1968), using I.E.F., isolated four components in purified preparations of alpha-haemolysin and similar results were obtained by McNiven, Owen and Arbuthnott (1972) and by Dalen (1975). The major component was at a pI of about 8.6 and this was the only component present in 6M Urea (Soderholm, Alleston and Wadström, 1972; McNiven et al., 1972).

Six and Harshman (1973a,b) reported the isolation of two stable proteins, A and B, which both appeared to be alpha-haemolysin. The two proteins had a sedimentation coefficient of about 3S with identical specificities and specific activities in haemolytic assays and were immunochemically identical. They considered the two forms to be "charge isomers". Molecular weight estimations were of the order of 34 Kd based on amino acid analyses (Watanabe and Kato, 1974) and this agreed with other estimations, eg. Bernheimer (1970) (33 Kd), and McNiven et al. (1972) and Watanabe and Kato (1974) (36 Kd), although Dalen (1975) reported a value of 39 Kd. The sources of variability in pI and molecular weight determinations have been reviewed by Dalen (1976).

Most of these detailed studies employed the Wood 46 strain (NCTC 7121) (Burnet 1929), which has a tendency to lose its haemolytic ability. Kjems (1963) isolated two different strains from NCTC 7121 subcultures, a coagulase positive strain yielding large quantities of alpha-haemolysin and a coagulase negative strain which produced low levels of alpha-haemolysin and deposited these with the NCTC (NCTC 10344 and NCTC 10345 respectively). Goode and Baldwin (1974) found that alpha-haemolysin was identical when produced by different strains and therefore results of studies employing only one strain might be validly applied to other strains.

Cytolytic Activity : Freer, Arbuthnott and Bernheimer (1968) demonstrated ring-like structures on the outer lamellae of artificial liposomes whose integrity had been disturbed by treatment with 3S alpha-haemolysin. This suggested that the membrane damaging effect might involve a hydrophobic interaction followed by a change in membrane orientation which activated enzymic capability. This primary enzymic event could cause membrane damage leading to lysis as suggested by Cooper and co-workers (Cooper, Madoff and Weinstein, 1964a,b; Madoff, Cooper and Weinstein, 1964). A similar reaction sequence was noted by Lominski and Arbuthnott (1962), Marucci (1963a,b) and Robson-Wright (1969).

The initial hydrophobic interaction could involve the production of the 12S form from the 3S monomer since Arbuthnott et al. (1967) have shown that cell membranes and phospholipids facilitated such polymerisation and Arbuthnott, Freer and Billcliffe (1973) found that the ability to induce polymerisation varied between different lipids. Hydrophobic interactions were also noted by Buckelew and Colacicco (1971) and by Freer, Arbuthnott and Billcliffe (1973). Evidence for an enzymic step came from the observation by Robson-Wright, Arbuthnott and Lominski (1968) that the alpha-haemolysin activity was inhibited by suramin, a known inhibitor of enzymic activity.

In contrast to the results quoted above, which involved membrane lipid in the haemolytic activity, Wiseman and Caird (1970, 1972) postulated membrane proteins as the active agents involved. They related the level of natural protease in the erythrocyte membrane of different species to their susceptibility to alpha-haemolysin and concluded that a direct relationship existed in which α-haemolysin was secreted as an inactive protease which was activated by erythrocyte membrane protease. More recently, Wiseman, Caird and Fackrell (1975) demonstrated that this activation could be achieved by pretreatment of the alpha-haemolysin with trypsin. In these experiments, the alpha-haemolysin was assayed as a protease, which must introduce some doubt into the interpretation of the results.

Freer et al. (1973) found no alteration in membrane polypeptides or in sedimentable protein following alpha-haemolysin treatment and freeze-fractured ghosts did not show changes which could be attributed to protease action. They therefore concluded that alpha-haemolysin did not behave as a protease, a conclusion which was also reached by Watanabe and Kato (1974). The brief, but extremely detailed, review of this topic by Freer and Arbuthnott (1976) also concluded that membrane lipids were more likely to be substrates than membrane proteins. However, the studies of Cassidy and Harshman (1976) on the binding of the haemolysin to rabbit R.B.C.'s indicated that membrane proteins might be involved. Using ^{125}I labelled haemolysin, they detected about 5.10^3 binding sites on the membrane surface spaced about one per 30 μm^2 and showed that initial incubation with low concentrations of haemolysin would prevent subsequent binding of excess. Production of 12S rings required about 10^7 molecules of haemolysin per cell which is 10^3 - 10^4 times the number of binding sites, suggesting that ring formation might be non-specific. Binding could be reduced by pre-treatment of the R.B.C.'s with pronase or trypsin, which implicated membrane proteins.

A model which reconciles all of these observations is difficult to conceive. However, the evidence suggests that the initial event may involve binding to specifically orientated lipids with proteins serving to maintain the orientation.

Other Activities : Activities other than haemolysis have often been reported and these are briefly reviewed here. Toxic effects involving cytopathic changes or cytolysis have been observed on a variety of other cell types (Thal and Enger, 1961; Paradisi and D'Aniello, 1969; Bernheimer and Schwartz, 1965a,b) although Thelestam, Möllby and Wadström (1973) suggested that much of this activity might be due to contaminating delta-haemolysin. Toxic changes have been observed on leucocytes (Gladstone, 1966) and mast cells (Kwareki et al. 1968) and cytopathic effects have been observed on both smooth (Thal and Enger, 1961) and skeletal muscle (Lominski et al. 1962). Protease digestion has been shown to produce fragments of the alpha-haemolysin in which different parts show cytolytic, dermonecrotic or toxic effects (Watanabe and Kato, 1976).

Lethality for small mammals was an accepted manifestation of alpha-haemolysin (Elek, 1959; Gladston, 1966) and it was also found to be lethal for a variety of cold-blooded animals (Lominski, Arbuthnott and Gemmell, 1965). An action on the thalamus in rabbits, leading to abnormal EEG patterns has been suggested to be the main cause of death (Edelwenjn et al., 1968; Edelwenjn, Jeljaszewicz and Zak, 1970). Serotonin and histamine are released, probably from mast cells, in rabbits challenged i.v. but this may not be related to the lethal effects (Jeljaszewicz Szmigielski and Hryriewicz, 1978) since treatment with antihistamines and antiserotonins did not prevent death.

Kapral, Keogh and Taubler (1965) found that production of alpha-haemolysin occurred during in vivo growth. Rahal, MacMahon and Weinstein (1968) have suggested that alpha-haemolysin may cause extensive tissue damage in terminal septicaemia in man, and Plant (1969) has detected activity resembling that of alpha-haemolysin in serum of a patient suffering from staphylococcal septicaemia. Antibody to alpha-haemolysin was found in both healthy and infected humans (Johnson and Svantesson, 1975) which implied that alpha-haemolysin was produced during infection.

B.2.1.b. Beta-haemolysin Glenny and Stevens (1935) detected a new haemolysin in culture filtrates of *Staph. aureus* and reported it to be a hot-cold lysin because of the enhanced haemolysis which was observed when treated erythrocytes were transferred from 37°C to a lower temperature. Low and Freer (1976), using highly purified material reported a sedimentation coefficient (S_{20w}) of 3.1, a pI of 9.3 and a molecular weight of 32.5 Kd.

As shown in Table one, sheep erythrocytes were the most sensitive and this activity was greatly enhanced by divalent cations (Wiseman, 1965a,b). Magnusson, Doery and Gulasekharen (1972) and Doery *et al.* (1963) noted phospholipase activity in beta-haemolysin preparations and Wiseman and Caird (1967) showed that the haemolysin hydrolysed sphingomyelin to phosphorylcholine and N-acylsphingosine. It therefore seemed likely that beta-haemolysin was a sphingomyelinase C and this has been confirmed by subsequent studies (eg. Wadström and Möllby, 1971a,b; Low and Freer, 1976). The stability of a membrane is dependent on its ability to retain its integrity while being subjected to changes in fluidity. Temperature shifts cause alterations in fluidity but untreated sheep erythrocytes are stable during changes from 37°C to 10°C. Membranes in which sphingomyelin has been hydrolized to phosphoryl choline and N-acyl sphingosine have altered ratios of hydrophilic to lipophilic components and this results in a change in membrane fluidity.

When the temperature is lowered, the altered membrane loses its integrity and the cell lyses. A similar effect can be induced by adding chelating agents rather than lowering the temperature (Smyth, Möllby and Wadström, 1975), which suggests that divalent cations might be involved in stabilising the damaged membrane.

Several workers have reported cytotoxic effects of beta-toxin (Wiseman, 1968; Wadström and Möllby, 1971b) but Wadström et al. (1974) and Thelestam et al. (1973) later reported that beta-haemolysin was not cytotoxic. Enlargement of cells in tissue culture, with release of lysosomal markers and alteration of organelle structure has been noted (Jeljaszewicz et al. 1978). Wadström et al. (1974) reported lethality for rabbits, mice, guinea-pigs and chick embryos but Low and Freer (1976) found their purified enzyme to be non-toxic in doses up to 7.5 mg/kg.

B.2.1.c Gamma-haemolysin. Smith and Price (1938) were the first to report a third haemolysin in culture filtrates of Staph. aureus, distinguished by its haemolytic activity on rabbit, human and sheep erythrocytes. Marks (1951) confirmed this difference and Smith (1956) isolated a rough strain, designated 5R, which produced gamma-haemolysin almost exclusively. Guyonnet and Plommet (1970) isolated two components which acted synergistically from the Smith 4R strain. Möllby and Wadström (1971) (using I.E.F.) also identified two haemolytic components in culture filtrates of strain 5R, but considered one of the two components to be alpha-haemolysin. The other component, with a pI of 9.5 they considered to be gamma-haemolysin. It is possible that there are two components which cannot be separated by IEF (pI 9.8 - 9.9) and with molecular weights of 26 - 29 Kd, as summarised by McCartney and Arbuthnott (1978) and Wadström (1978). The toxin was thermolabile (one component more than the other, (Guyonnet and Plommet, 1970)) and its activity was inhibited by agar. It was antigenically distinct and active on rabbit, human and sheep erythrocytes. Möllby and Wadström (1971) detected haemolysis of horse erythrocytes at about 0.2% of the activity on rabbit erythrocytes. It is toxic for cells in tissue culture (Szmigielski et al. 1976).

Fackrell and Wiseman (1976a) have recently produced a highly purified toxin which they have found to be a single protein with a pI of 6.0 (Fackrell and Wiseman, 1976b). Since this pI does not agree with that detected by any other workers it is difficult to believe that they were studying gamma-haemolysin. However, the only possible candidates (Wadström, 1978) for a pI of 6.0 are coagulase or lymphocyte mitogen which are not known to have any haemolytic capability. It seems likely that Fackrell and Wiseman (1976a,b) were either dealing with a new haemolysin or with some form of charge isomerisation.

B.2.1.d. Delta-haemolysin Delta-haemolysin was first identified as a separate haemolysin by Williams and Harper (1947) and extensively studied by Marks and Vaughn (1950), who reported its distinction from the others on the basis of its ability to lyse horse and human erythrocytes. They also noted that its activity was inhibited by normal serum and this inhibitory effect was found to reside in the phospholipid portion of β-lipoprotein (Whitelaw and Birkbeck, 1976).

According to Kreger et al. (1971) delta-haemolysin contains a high percentage of hydrophobic amino acids which might account for its solubility in some organic liquids. These authors found two components by I.E.F., both of which behaved like delta-haemolysin. One was a basic protein (pI 9.5) and the other was an acidic protein (pI 5.0), but these differences in pI might be due to "charge masking" (Bernheimer and Fishman, 1974). Controversy exists over the molecular weight of this compound which has been variously estimated between 5 and 100 Kd. The smallest estimate may be that of a monomer (Wadström, 1978; McCartney and Arbuthnott, 1978).

Heatley (1971) reported only 250-300 HU/mg of protein on human erythrocytes, but specific activity was found to be higher if erythrocytes from marine fish, especially cod, were used (Birkbeck, Chao and Arbuthnott, 1974). Wiseman and Caird (1968) reported phospholipase activity, but this was not confirmed by other workers (Kreger et al. 1971; Rahal, 1972). It has been suggested that the delta-haemolysin might be acting in a non-enzymic manner, perhaps as a surface active polypeptide (Bernheimer, 1970; Kreger et al. 1971; Bernheimer, 1974) like the strongly surface active protein melittin. This view was supported by Rahal (1972) who reported that the lytic activity resembled that of Triton X-100 or deoxycholate. Protoplasts and spheroplasts were lysed by purified delta-haemolysin (Kreger et al. 1971; Kreger and Bernheimer, 1971) and cytotoxic effects on a variety of cell types, due to surface activity, were noted by Thelestam et al. (1973). Antigenicity was reported by Elek (1959) and confirmed by Kayser and Raynaud (1965) and Wiseman and Caird (1968). Other workers (Bernheimer, 1970; Kreger et al, 1971) considered it likely that serum inhibition and precipitin lines in gels were a non-specific reaction between the delta-haemolysin and serum phospholipids, but Birkbeck and Whitelaw (1976) have raised highly specific antiserum in rabbits by subcutaneous injection of delta-haemolysin with Freunds Complete Adjuvant.

B.2.1.e. Other Haemolysins An epsilon-haemolysin produced by coagulase negative strains has been described (Elek and Levy, 1950; McLeod, 1963). Ali and Haque (1974) reported seven different haemolysins α, α_1, β^+ and β^-, ϵ, δ and δ_1 but their relationship to the classical α, β, γ and δ was not clear.

B.2.2. Leucocidin Panton and Valentine (1932) distinguished between the leucocidic and haemolytic activity. Leucocidin consisted of two proteins which could be separated by their speed of elution from CM-cellulose columns and were designated F (fast) and S (slow) on this basis (Woodin, 1959, 1960). The two components acted synergistically (Woodin, 1961) and it was suggested that a hydrophilic reaction, due to the S component, and a hydrophobic reaction, due to the F component, resulted in changes in membrane permeability. This reaction required triphosphotidyl inositol (Woodin and Wienke, 1964, 1967). A novel leucocidin isolated from bovine strains has been studied by Blobel, Schaeg and Hasche (1976) who reported a similar pattern of F and S components functioning synergistically. Activity was mainly on granulocytes of bovine origin in contrast to the traditional leucocidin which is most active on human cells. Heterologous leucocidins produced by mixing the F component of one variety with S component of the other displayed no synergism.

McKay and Arbuthnott (1974) observed that a non-toxinogenic, non-proteolytic, leucocidin-producing strain was as virulent as a toxinogenic strain, in the neo-natal mouse model which they had developed and a similar observation was made by Van der Vijver, Van Es-Boon and Michel (1975b). Jeljaszewicz et al. (1978) reported that purified leucocidin was not lethal for whole animals although granulocytes were destroyed and promyelocytes and myelocytes were apparently stimulated to increased proliferation.

B.2.3. Epidermolytic Toxin (Exfoliatin) Toxic Epidermal Necrolysis (the scalded skin syndrome) was shown to be caused by phage group II staphylococci (Melish and Glasgow, 1970; Arbuthnott et al., 1971; Kapral and Miller, 1971). The agent responsible for this effect was produced extracellularly and was named exfoliatin by Kapral and Miller (1971). Estimations of molecular weight range from 24-33 Kd and of pI range from 6.5-7.3 (Melish et al, 1976). Arbuthnott, Noble and Billcliffe (1974) found two main components by I.E.F. and reported that purified exfoliatin produced blistering in human volunteers. The toxin induces a split at the stratum granulosum by altering cell adhesion without affecting viability. The mechanism is uncertain. There may be a direct action, perhaps altering the membrane of the keratinocytes or hydrolysing the intracellular material, or the action may be indirect, stimulating a natural enzyme which performs such functions (McCartney and Arbuthnott, 1978).

B.2.4. Succinic Oxidase Factor Lominski et al. (1964) noted that culture filtrates from Staph. aureus could impair the succinic-oxidase activity of mitochondrial suspensions and called the entity producing this effect, succinic oxidase factor. Later work (Lominski, Gemmell and Arbuthnott, 1968) revealed two activities, a more heat resistant protein which impaired electron transfer in the succinate-cytochrome C reductase region and a more heat sensitive protein which impaired electron transfer in the cytochrome C oxidase region. Addition of coenzyme Q and cytochrome C restored function. The two components had pI's of 3.4 and 4.5 respectively (Gemmell, 1978). The organ from which the mitochondria were isolated affected the degree of activity, those from liver and kidney being most affected, those from muscle and brain less so and those from heart unaffected. Mitochondrial damage appeared to be due to changes produced in the organelle membranes which disrupted the sequential process of oxidative phosphorylation.

B.2.5. Lymphocyte Mitogen. Using I.E.F., Kreger, Cuppari and Taranta (1972) isolated two extracellular proteins which were capable of "transforming" lymphocytes, in vitro, into large, blast-like cells capable of mitosis. One component had a pI of 8.6-9.0 and a molecular weight of 21 Kd and the other had a pI of 5.5-7.0 and a molecular weight of 14 Kd. The mode of action of these components is unknown, but McCartney and Arbuthnott (1978) suggested that the effect might be exerted at the membrane. Mitogenic activity has also been detected in enterotoxin and protein A preparations and it is possible that these materials might be the lymphocyte mitogens although the molecular weights do not agree.

B.2.6. Enterotoxins. Certain strains of Staph. aureus produce a relatively mild form of food poisoning lasting a few hours to a day at most (Bergdoll, 1972) although the proportion of such strains was in doubt, since Casman et al. (1967) reported an average of 33% enterotoxin producers amongst Staph. aureus strains isolated from various sources whereas Bergdoll (1972) suggested that the frequency was at least 50% and probably more. Victor et al. (1969) reported a relationship between enterotoxin production and the presence of coagulase and DNase. Six serologically distinct toxic proteins were identified and designated

A, B, C_1, C_2, D, E with molecular weights of 26-30 Kd (Taylor, 1976) and a seventh species, type F has been studied (Holbrook and Baird-Parker, 1976). The precise mode of action has not been elucidated, but vomiting appears to be a direct response to inflammation of the abdominal viscera whereas the diarrheagenic mechanism may involve cyclic AMP which is known to be involved in the enterotoxicity of Vibrio cholerae and Escherichia coli (Taylor, 1976). It seems probable that these effects are mediated through the membrane of the affected cells as has been shown for the cholera toxins (Taylor, 1976).

Comparison of the amino acid sequences of A, B, and to a lesser extent, C, indicated regions of common structure which might be implicated in the activity of these proteins (Bergdoll, 1976).

B.3. Coagulase and Staphylokinase

The two enzymes in this section are linked here because they both work by by-passing normal physiological processes.

B.3.1. Coagulase. Clotting of plasma in the presence of anti-coagulant is used as a diagnostic test for Staph. aureus and Duthie (1954a,b) distinguished two reactions, "free" coagulase causing clotting and "bound" coagulase causing cells to be clumped by plasma.

Free coagulase, an enzyme detectable in the culture filtrate of most strains of Staph. aureus, was found to be a single protein (Zolli and San Clemente, 1963). It has a molecular weight of 18 Kd and a pI of 5.9-6.1 (Wadström, 1978). It was found to activate prothrombin to a "pseudothrombin", sometimes called staphylothrombin, of molecular weight 89 Kd compared to 68 Kd for natural thrombin. Clot associated serum factors V, VIII and XIII are not activated so that the clot remains uncross-linked (Zajdel et al.(1976).

Bound coagulase was antigenically similar in serologically diverse strains and was not related to free coagulase (Rother and Kelly, 1966). It was not detected in L-forms (Elkins, Hyde and Kelly, 1970), could be removed by trypsinization (Duthie, 1954a,b) or sonication, (Gorrill, Klyhn and McNeill, 1966) and might therefore be a cell wall component, apparently, a basic protein (Bruckler, Schaeg and Blobel, 1974). Clumping involves precipitation of fibrin monomers onto the "clumping factor" which is preferentially located on old wall material, rather than on newly synthesised walls (Umeda, Ikebuchi and Amako, 1980).

B.3.2. Staphylokinase. Gerheim (1948) and Lack (1948) demonstrated that Staph. aureus produced an enzyme which converted the serum protein plasminogen into the proteolytic enzyme plasmin but did not hydrolyse other protein substrates (Hutchinson, 1962). Vesterberg and Vesterberg (1972) using I.E.F., found purified staphylokinase to be a mixture of three proteins, but Fujimura, Makino and Hayashi (1974) found that the enzyme was produced as a monomer of 15 Kd early in the growth cycle. Later a complex of molecular weight 320 Kd was found, which appeared to arise spontaneously and was through to contain a high-molecular weight substance whose nature could not be determined. The pI of the 15 Kd molecule was reported to vary from 5.3-6.7 (Wadström, 1978). The mode of action is still uncertain, but evidence suggested (Eriksson, 1976) that it was not enzymic. Instead, a novel process was proposed in which one molecule of staphylokinase bound to one molecule of plasminogen to produce a dimer and this dimerisation led to the unmasking of the plasmin active site. Although this is unproven, the experiments of Jeffries and Buckley (1980) lend some support to this. These authors tested seven strains of Staph. aureus which were fibrinolytic and found that no direct proteolysis took place, but plasminogen was activated in all cases.

B.4. Conventional Enzymes

Hydrolysis of many substrates is a feature of Staph. aureus and these reactions involve extracellular enzymes.

B.4.1. Proteases. Hydrolysis of casein is a common feature of Staph. aureus isolates and three different proteases with this activity have been isolated (Arvidson, Holme and Wadström, 1971; Arvidson, Holme and Lindholm, 1972; Arvidson, 1973a,b,c). Protease I, which was studied in more detail by Houmard and Drapeau (1972) and Ryden, Ryden and Philipson (1974), hydrolysed only glutamoyl-peptide bonds but proteases II and III had wider substrate specificities (Arvidson, 1972a,b) and the latter required Ca^{++} ions for stability (Arvidson, 1973c). Their molecular weights were recorded as 21 Kd, 12.5 Kd and 28 Kd and their pI's as 4.0, 9.4 and 5.0 respectively (Wadström, 1978). Hydrolysis of gelatin might be due to a distinct protease since caseinase and gelatinase activities were not always shown by the same strain. Todd (1968) for example, found 48% of Staph. aureus isolates possessed caseinase activity while 81% possessed gelatinase activity and Ali and Haque (1974) produced similar results showing 46% and 100% respectively. Tirunarayanan and Lunblad (1966) purified two proteases from Staph. aureus strain Walker; a caseinase with a pH optimum at 9.5 and a gelatinase with an optimum of pH 8.5.

B.4.2. Urease. This enzyme hydrolyses urea to ammonia and carbon dioxide. Although it has been used as a classification character (eg. Shaw, Stitt and Cowan, 1951; Mossel, 1962) the enzyme does not appear to have been characterised.

B.4.3. Hyaluronate Lyase. Duran-Reynals (1933) demonstrated the presence of a "spreading factor" which was found (Meyer and Chaffee, 1940) to hydrolyse hyaluronic acid. Reports from various workers indicated that the enzymic activity was due to a mixture of different enzymes, although the exact number was disputed (Vesterberg *et al.*, 1967; Abramson, 1968). A pI of 7.9 and a molecular weight of 82 Kd has been noted by Wadström (1978).

B.4.4. Phosphatases. Elek (1959) considered that *Staph. aureus* did not produce an extracellular phosphatase. Pan and Blumenthal (1961) on the other hand concluded that the acid phosphatase, which they detected in coagulase positive isolates of *Staph. aureus*, was an extracellular enzyme. Malvaux and San Clemente (1969a,b) carried out a detailed study on a loosely bound acid phosphatase. The enzyme had a pH optimum at 5.2 to 5.3 and a molecular weight of 58 Kd. Activity was inhibited by Iodoacetate and EDTA and stimulated by Cu(II) ions. Free phosphatase would appear to be the same enzyme (Arvidson, 1976). Shah and Blobel (1967) reported an alkaline phosphatase which was repressed by free phosphate and consequently was not detectable in normal growth media. In view of the variety of phosphatase enzymes produced by other species, e.g. three types in *Salmonella typhimurium* (Kier, Weppelman and Ames, 1977), five types in *Bacillus subtilis* (Yamane and Muro, 1978b) and three types in *Escherichia coli* (Brockman and Heppel, 1968) it seems strange that *Staph. aureus* should only produce one in normal growth media. However, Wheller and White (1979) have reported the possibility of three, on the basis of at least three pH optima at 6.1, 6.8 and 7.8 which they detected in the culture supernates of NCTC 10344.

B.4.5. Nuclease. A thermostable Ca^{++} activated DNase, which differed from similar enzymes from other sources (eg. pancreas, snake venom) in being a 5'-phosphodiesterase rather than a 3'-phosphodiesterase, was demonstrated by Cunningham, Catlin and Privat-De Garille (1956) in culture filtrates from Staph. aureus. Cunningham (1959) showed DNase and RNase activities had similar pH optima, thermostabilities, divalent cation requirement and 5'-phosphodiesterase activity and suggested that the two activities were due to the same enzyme, a view which was supported by other workers (eg . Cuatrecasas, Fuchs and Anfinsen, 1967). Taniuchi, Anfinsen and Sodja (1967) showed it to be a single polypeptide of 149 amino acids, with a molecular weight of 16,807 daltons. The pH optimum was about 10.

B.4.6. Bacteriolytic Enzymes. The existence of bacteriolytic enzymes in Staph. aureus culture filtrates was first noted by Richmond (1959) and Kashiba et al. (1959), and assumed to be due to a lysozyme, ie. an endo-β-N-acetylmuraminidase until Tipper (1969) produced evidence for two different enzymes, neither of which was a lysozyme. Wadström and Hisatsune (1970a,b) partially purified this "lysozyme" and found it to be an endo-β-N-acetyl-glucosaminidase contaminated with other bacteriolytic enzymes which Wadström (1970) found to be an N-acetylmuramyl-L-alanineaminidase and a staphylolytic peptidase, probably a glycyl-glycyl endopeptidase. The same three activities make up "lysostaphin" the commercial staphylolytic enzyme preparation.

B.4.7. Lipolytic Enzymes. Several different enzymic activities have been reported for lipid substrates, but little work has been done on their characterization. Hydrolysis of triglycerides, with a preference for oleic linkages, has been reported to be due to a specific lipase

(Alford, Pierce and Suggs, 1964). Stewart (1965) demonstrated that the hydrolysis occurred at the aqueous interface and Mates (1973) found that the enzyme was only synthesised late in the growth cycle. There was, however, no clear evidence that these workers were dealing with the same enzyme. Stewart (1965) also observed esterase activity on water-soluble aromatic esters which appeared to differ from the lipase. In addition to beta-haemolysin (p.1[illegible]) other phospholipase activities have been detected. Magnusson _et al_. (1962) reported a phospholipase A hydrolysing phosphatidyl choline and Doery _et al_. (1965) found another phospholipase which hydrolysed phosphatidyl inositol. The latter seems to be a phospholipase C with molecular weight between 20 and 33 Kd. Its activity was inhibited by divalent cations and by isomolar NaCl or KCl and it has been reported to cause release of membrane bound enzymes from mammalian cells (Möllby, 1978).

Gillespie and Alder (1952) reported opacification of egg yolk agar by _Staph. aureus_ which was found to be caused by free fatty acids enzymically released from lipoprotein (O'Leary and Weld, 1964). Tirunarayanan and Lundbeck (1967, 1968a,b) described a reaction sequence in which initially lipoprotein lipase released free fatty acids which precipitated as the calcium or magnesium salts giving opacification of the medium. Solubilisation of lipoprotein giving a clear zone near the colony then occurred.

B.5. _Protein A_. Protein A was shown to be a constituent of the cell wall of pathogenic staphylococci (Yoshida, Mudd and Lenhart, 1963), which might play a part in pathogenicity by making the cells more resistant to phagocytosis (Dosset _et al_., 1969). Although 90% of protein A was bound to the cell wall, the remaining 10% could be detected extracellularly (Forsgren, 1969). A study by Movitz (1974) indicated that Protein A was excreted within one minute of being synthesised and was immediately linked to the peptidoglycan of the cell wall, so extracellular protein A might be due to the production of excess protein which cannot be incorporated. This rapid incorporation after synthesis probably explains why Umeda et al.(1980) found protein A distributed uniformly across old and new cell wall areas.

B.6. Evidence for Extracellularity. Paradoxically, proving that a protein is truly extracellular is more difficult than proving it is not. If the majority of a protein can be shown to be cell-associated then clearly it is not an extracellular protein, but the converse is not necessarily true, since absence of cell-associated activity may reflect only a failure to detect it. Subject to this condition, the following results may be considered.

B.6.1. Alpha-haemolysin. Raynaud et al. (1955) using a variety of techniques, failed to demonstrate cell-associated alpha-haemolysin, but Hendricks and Altenbern (1968) did detect low levels of alpha-haemolysin in lysostaphin lysates. They reported that haemolysin was synthesised continuously throughout the logarithmic phase and immediately excreted, reaching a maximum at mid-logphase. McNiven and Arbuthnott, (1972) also demonstrated a cell-associated haemolysin which appeared to be identical with alpha-haemolysin. They found about 1% of total activity to be cell-associated, but unlike Hendricks and Altenbern (1968) found the peak extracellular concentration at the onset of stationary phase. It seems likely that this is a true extracellular protein.

B.6.2. Acid Phosphatase. Sawashige (1967) demonstrated that acid phosphatase activity was located on, or near, the membrane of the cell, but did not determine how much of the total activity was in this form. Nugent et al. (1974) and Okabayashi and Mizumo (1974) looked at enzyme levels in cell fractions and concluded that phosphatase activity was mainly located in the cytoplasm or the membrane. Wheller and White (1979) have reported that about 60% of total acid phosphatase is cell associated. One of the functions, perhaps the main function of the enzyme, would be to dephosphorylate substrates prior to transport (Arvidson, 1976) and a location on the outer surface of the cell would therefore be appropriate. Phosphatases have been located at the cell surface in a variety of other bacteria including Gram-negatives such as Escherichia coli (eg. Chang et al. (1980) and Pseudomonas aeruginosa (eg. Chang, Ingram and Costerlon, 1970) and Gram-positives such as Bacillus subtilis (eg. Yamane and Muruo, 1978) and B. licheniformis (eg. Hulett, Schaffel and Campbell, 1976). A similar situation exists in Saccharomyces cerevisiae (Linnemans, Boer and Elbers, 1977). It seems likely that this is a cell associated enzyme.

B.6.3. Nuclease. Conflicting results have been reported for this enzyme since Nugent et al. (1974) stated that only 0.3% of activity was cell associated whereas Okabayashi and Mizumo (1974) considered it to be a periplasmic enzyme. The position is therefore in doubt.

B.6.4. Bound Coagulase and Protein A. As reported above (p.18 and P.22) these two proteins are located on the surface of the Staph. aureus cell wall. They would appear to be wall components and their presence in the growth medium would be the result of overproduction or non-specific detachment. This is more likely to happen with protein A which is rapidly and continuously synthesised rather than with bound coagulase (Umeda et al. 1980).

B.6.5. Other Proteins. Detailed studies on the location of the other proteins do not appear to have been carried out although half-cystines appear to be present as one or less in those molecules which have been examined.

There seems little doubt that the enterotoxins and exfoliative toxins are truly extracellular, since they cause their pathogenic effect at a distance from the producing cell. It is also likely that the majority, perhaps all, of the degradative enzymes are extracellular because their purpose is to solubilise and degrade potential substrate molecules. Similar arguments apply to free coagulase, staphylokinase and the remaining cytotoxins.

Therefore, only acid phosphatase, bound coagulase and protein A are known to be cell associated. In view of the facility with which the former can be detected, it would be the best choice for comparative studies between cell-associated and cell-free activities.

C. ROLES OF MEMBRANES IN EXTRACELLULAR PROTEIN PRODUCTION

The cytoplasmic membrane, or plasmalemma, provides a selective barrier between the cytoplasm and the external environment. The structure is believed to conform to the fluid-mosaic model of Singer and Nicholson (1972) in which proteins, both structural and functional, float in or traverse a hydrophobic phospholipid bilayer. This structure will be involved, intimately, with the excretion of the extracellular proteins but might also be involved in a more general way through its central role in ATP synthesis and the concommitant control of the uptake of substrates.

C.1. Export of Proteins. Prior to 1970, the problem of how proteins were exported through membranes had received little attention, due largely to a lack of knowledge of membrane structure. At that time Sargent and Lampen (1970) proposed a mechanism based on studies on penicillinase release in which the molecule would assume a hydrophobic configuration to enable it to cross the cytoplasmic membrane at special sites. Once out of the cell it would assume a hydrophilic configuration.

However, evidence was beginning to accumulate for a different mechanism, based on a "translation extrusion" system, ie. the polypeptide as it was synthesised would be extruded through the membrane, assuming its active configuration outside the membrane.

Much of this work was carried out by Palade and co-workers and has been summarised in his Nobel Prize acceptance lecture (Palade, 1975). The observations were made in mammalian systems, notably the pancreatic exocrine cell, and indicated that proteins designed for export were synthesised by ribosomes (actually polysomes) attached to membrane. Evidence suggested that membranes of this type were permeable to molecules of 1 nm diameter which would permit unfolded polypeptides to pass through but would block the return of folded, ie. tertiary, polypeptides (diameter > 2 nm). The nascent polypeptide would thus assume its active configuration after secretion into the cisternal space of the endoplasmic reticulum. Other findings supported this view since structural modifications, such as the formation of disulphide bridges, occurred after excretion. Two subclasses of attached polysomes appeared to exist; one which synthesised proteins for export and the other which synthesised membrane proteins and inserted them into the membrane.

Studies in bacterial systems presented essentially the same model. May and Elliot (1972) who studied protease production in Bacillus subtilis favoured the view that the protease was being excreted as it was synthesised and extended this observation to B. amyloliquefaciens (Sanders and May, 1975). Similar conclusions were drawn by Cancedda and Schlesinger (1974) in their study of alkaline phosphatase synthesis by Escherichia coli, and by Boethling (1975) in his study of protease secretion by Pseudomonas maltophilia.

A primary requirement of any "translation-extrusion" model would be a special selection system to allow extracellular proteins to be synthesised on membrane bound ribosomes, while preventing cytoplasmic proteins from being similarly produced. Such a mechanism is provided by the signal-sequence hypothesis, which was developed for mammalian systems but is believed to be applicable also to bacteria (Blobel and Dobberstein, 1975; Olsen and Schwartz, 1978). In this model, the selectivity is imposed by the initial 5' sequence of codons - called the signal codons - on the messenger RNA (Figure One (a)). The signal codons are translated to provide the initial aminoacyl residues of the growing polypeptide chain, this sequence being known as the signal peptide. Signal peptides which have had their primary structure determined, appear to be 15-30 residues in length with a hydrophobic sequence in the middle (Blobel and Lingappa, 1978). This sequence attaches to the cytoplasmic membrane, possibly at a specific ribosome receptor protein, although since the leader sequence is composed of basic amino acids, the attachment might be promoted by binding of this portion to the acidic phospholipid surface. (Wikner, 1979). As translation continues, the remainder of the polypeptide is passed through the

Figure 1(a). Export of Polypeptides by a Translation-Extrusion System Involving a Signal Sequence.
Modified from Ramaley (1979)

5' ------- 3' Messenger RNA

 Signal Codons

 Signal peptide

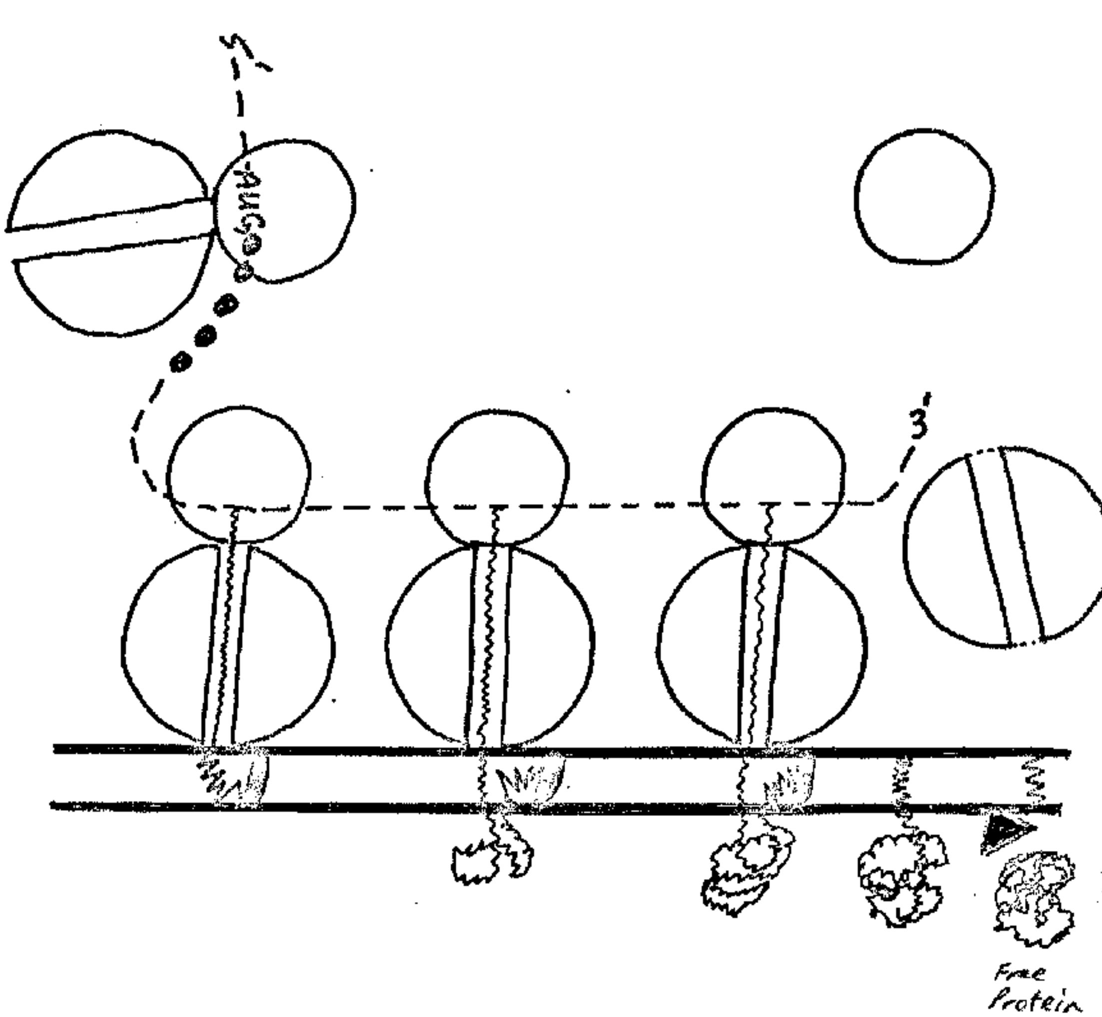
 Ribosome Receptor Protein

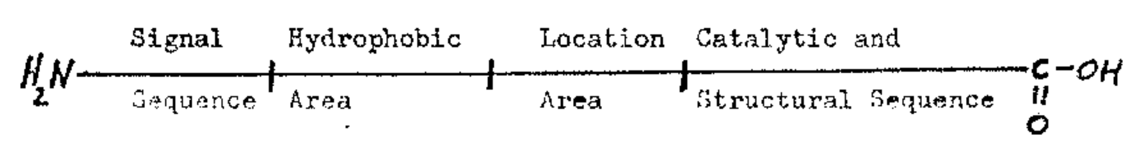
 Signal Peptidase

Recognition of the signal codons by the ribosome, or synthesis of the signal peptide, causes the ribosome to attach to a specific site on the membrane. After synthesis the exported protein is cleaved from the attachment site by a specific peptidase.

Figure 1(b). Proposed General Structure for Exported Proteins.
Modified from Ramaley(1979)

Areas not drawn to scale.


5'
AUG
3'
Free Protein


H_2N
Signal Sequence
Hydrophobic Area
Location Area
Catalytic and Structural Sequence
C−OH
O

membrane and becomes externally located. The signal sequence is finally cleaved from the remainder of the polypeptide by a membrane associated protease and the extracellular protein becomes free on the outside of the membrane.

Proteins which are located within, or on the surface of, the membrane might be exported by the same mechanism with the exception of the final cleavage. However, Wikner (1979) has suggested that since water insolubility is the main feature of membrane proteins, the passage through the membrane might trigger the folding of the polypeptide into a hydrophobic configuration which would remain membrane bound.

Having become external to the plasmalemma, the protein must then pass through the wall into the external medium, although some proteins might remain in the space between the membrane and the wall. This space is usually known as the periplasm, a term originally introduced to describe the area between the inner and outer membranes of the gram-negative cell but extended to cover the equivalent area in gram-positive cells (Ramaley, 1979). This might involve either an active or a passive transport system in the wall, but this is unclear.

Evidence for this signal hypothesis is extensive although other mechanisms are not excluded. With regard to the special mRNA, Boethling (1975) noted that synthesis of the Ps. maltophila protease continued for 30 minutes after production of messenger was inhibited, indicating that the preformed messenger was of a more durable form than normal messengers for cytoplasmic proteins. Similar observations in Staph. aureus were made by Katsuno and Kondo (1973) (p 43) and in B. amyloliquefaciens by Gould, May and Elliott (1973). The latter workers concluded that durability was due to a large messenger RNA pool for extracellular proteins (Both *et al.* 1973). which could persist for up to 60 minutes in the presence of transcription inhibitors (O'Connor, Elliott and May, 1978).

Cleavage of the signal sequence is exemplified by work on the penicillinases of B. licheniformis 749/C and 6346/C, by Lampen and co-workers. Early reports that a membrane bound 33 Kd protein was specifically converted to a free 29.5 Kd exoenzyme have proved to be impossible to reproduce, but some form of processing does take place, since an early to mid-log phase protein of 30.5 Kd has been shown to be converted to a 29.5 Kd protein by removal of 8 amino acids from the N-terminus.

Evidence for the signal sequence on the mRNA is most elegantly demonstrated by experiments in which the E. coli β-galactosidase z gene was fused to the signal sequence of the outer membrane lamB gene. From this hybrid gene, a hybrid messenger was transcribed which was then translated into a hybrid protein, with β-galactosidase function and located in the outer membrane (Beckwith et al. 1978). Ramaley (1979) has proposed a general structure for exported proteins (Figure One b). If such a generalisation is valid, it makes it likely that the difference between an extracellular protein, such as α-haemolysin and a cell-associated protein, such as acid phosphatase, resides entirely in the "location area" which probably contains the cleavage site also. However, other differences based on control of synthesis are also probable.

C.2. The Chemiosmotic Hypothesis.

The chemiosmotic hypothesis (Mitchell, 1966, 1970) proposed that during oxidative phosphorylation, the respiratory chain acts as an electrogenic proton pump which translocates protons across the plasmalemma to generate an electrochemical potential. This potential, which is known as the protonmotive force (Δp), subsequently drives a number of energy-dependent membrane reactions including ATP synthesis and substrate transport.

In bacteria the respiratory chains are organised into 2-4 sections which move protons out of the cell, each section transporting approximately 2 protons per electron pair transferred (Jones, 1979). The proton motive force generated, expressed in millivolts (mV) can be calculated from the equation : $\Delta p = \Delta\psi - z\Delta pH$ where $\Delta\psi$ is the membrane potential (inside negative) and z is a constant which converts the pH gradient (ΔpH) into mV (it equals 59 at 25°C). Calculated values for Δp in Staph. aureus at different pH's were reported to be 211mV at pH 6.5 (Collins and Hamilton, 1976), 250-270mV at pH 6.0 and 195-205mV at pH 7.0 (Kashket, 1981). These values are for aerobic respiration; anaerobic respiration was reported to yield 140-150mV (Kashket, 1981). Similar, though slightly higher values, were reported for E. coli (Jones, 1979). ATP is synthesised by a membrane-bound ATP synthetase complex composed of about seven different proteins, two of which comprise the membrane attachment site (BF_0) and the remainder form the proton translocating ATP synthetase itself (BF_1). The latter, when detached from the membrane, functions as an ATPase and it is this reverse action which is usually assayed (Jones, 1979).

C.2.1. ATP Yield and Growth Rate. Studies relating ATP production and growth rate are hampered by difficulties in determining yield coefficient for ATP (Y_{ATP}). One method which may be used is to determine oxygen consumption and use this to calculate Y_0 (gram bacteria produced per gram atom of oxygen consumed).

$$Y_O = P/O. \; Y_{ATP}$$

where P/O is the number of moles ADP phosphorylated to ATP during transport of one electron pair. Since P/O and Y_{ATP} cannot be determined independently, Y_O or Y_{O_2} (gram bacteria produced per mole oxygen consumed) may be used directly as a proportional representation of ATP production. A fall in Y_O can therefore be due either to a drop in phosphorylation efficiency or to a drop in the efficiency with which ATP is used to promote growth. The latter may reflect an increased maintenance energy requirement, the most likely cause of such an increase being turnover of cell material, which is commonly seen where uncoupled growth has been produced. Stouthamer (1979) discussed five growth conditions in which uncoupling could be observed, at least in E. coli.

C.2.1.a. Excess Energy Source. Experiments on growth in media where the energy source is in excess cannot be adequately performed in batch culture because the energy source molecule, frequently glucose, usually becomes the growth limiting factor. Continuous culture is more satisfactory. Under these conditions the cell will often employ excess ATP to accumulate storage compounds. This type of uncoupled growth would not result in an irretrievable loss of the energy potential, since if energy supply became restricted later the cell could utilise the store and therefore recover the energy.

C.2.1.b. Unfavourable temperatures. Although Stouthamer (1979) uses the term "unfavourable", uncoupling appears to be a result only of growth at elevated temperatures. The maintenance coefficient appears to be increased, but this cannot be the only reason for the decreased growth yield. One possibility which might be important is an increase in membrane fluidity due to the higher temperature, since this could easily lead to a lack of co-ordination between the components of the membrane respiratory system.

C.2.1.c. Minimal Media. Molar growth yields are highest in complex media and very high ATP yields are obtained. In minimal media growth yields would be expected to be less, due to shortage of precursors, but Stouthamer suggested that the limited capacity for assimilation in minimal medium might also be important, permitting relatively more ATP synthesis than cell growth.

C.2.1.d. Transient Periods During transition from one growth condition to another, eg. from anaerobic to aerobic, a transient drop in growth yield may be noted. Most experiments in this area have to be done in continuous culture since they involve short pulses of changed conditionspassing through a steady-state. Pulsing glucose-limited A. aerogenes with glucose or succinate, stimulated the respiration rate and succinate also stimulated the rate of ATP synthesis. When the succinate pulse was exhausted the ATP synthesis rate fell sharply and then returned to normal, suggesting that the cells possessed a rapid, fine control, of ATP levels. Switching from high aeration to low aeration increased the respiratory rate but ATP levels remained normal and growth yield fell, which probably meant that ATP turnover increased. This aspect has been reviewed by Harrison (1976).

C.2.1.e. Growth Inhibiting Compounds. A large number of compounds are directly inhibitory to growth, including antibiotics which interfere with protein or nucleic acid synthesis in addition to those antimetabolites which interfere with membrane dependent energetic processes. The former will be considered later (Section D), the latter are discussed here. Stouthamer (1979) listed a number of compounds which had been shown to reduce Y_{ATP} including Nitrite, Ferricyanide, Thiosulphate, Pentachlorophenol, 2,4 Dinitrophenol and Carbonyl-cyanide-m-chlorophenylhydrazone. The last two are known to dissipate the membrane proton gradient but the mode of action of the others is uncertain. In B. subtilis, disruption of proton translocation by these compounds has been proposed to involve a specific interaction of the uncoupler with the transport system. Interaction would take place within the membrane and be a function of the anionic form of the inhibitor (Brummett and Ordal, 1977; Nicholas and Ordal, 1978). The protein-ligand complex might be stabilised in a non-productive conformation in which short circuited protons become unavailable for the generation of proton-motive force (Konings, 1977; Jones, 1979). Maintenance respiration in dinitrophenol treated cultures appeared to increase, possibly to compensate for the loss of ATP synthesis via the membrane (Stouthamer, 1979).

Inhibition of growth of E. coli due to dissipation of the pH gradient has been shown for tributyltin chloride (Singh and Bragg, 1979) and for benzoate, sorbate and propionate (Eklund, 1980). The latter also affected B. subtilis and Ps. aeruginosa. Aliphatic diols and their esters (Akedo, Sinsky and Gomer, 1977) and alkyl esters of p-hydroxy benzoic acids (Eklund, 1980) inhibited growth of B. subtilis and the latter also inhibited E. coli and Ps. aeruginosa. Interference with transport mechanisms might be the cause in these cases and this is considered in section C.2.3. below.

As discussed by Stouthamer (1979) there is a discrepancy between ATP production and ATP utilisation in uncoupled cells and so some form of energy spilling mechanism must be employed. This might take the form of a modification to the energy yielding process itself, for example, deletion of oxidative phosphorylation sites or branching of the respiratory chain. Alternatively, there may be the establishment of futile cycles in which an essentially non-productive reaction is employed, for example, a futile ion cycle in which $NH_3 + H^+$ inside the membrane are co-transported and released on the outside as NH_4^+. This proton translocation is balanced by the inward movement of NH_4^+ which is subsequently split to NH_3 and H^+ so that a cycle becomes established $NH_4^+ \rightleftharpoons NH_3 + H^+$. ATPase activity, due to reversal of the BF_1 ATP synthetase might also be important in some organisms, particularly facultative anaerobes.

Another possibility involves the diversion of ATP energy into the production of Guanosine 3'5' Bis-diphosphate and Guanosine 3' diphosphate, 5' triphosphate, the so-called magic spot (MS) compounds. Control of RNA synthesis is central to the regulation of growth and would be particularly important when amino acids were unavailable. In "stringent" cells of E. coli , the onset of amino acid starvation led to an immediate 5-20 fold increase in the MS compounds (Cashel, 1975; Nierlich, 1978). The compounds were synthesised on the ribosomes and might be control signals acting to reduce anabolic processes since their appearance was accompanied by a 10-20 fold drop in all forms of RNA synthesis (Gallant, 1979).

C.2.2. Uptake of Substrates. Active transport of molecular species from the medium into the cell is an energy dependent phenomenon; the amount of energy involved depending, to a large extent, on the nature of the transport molecule. Three systems have been proposed: Neutral substrates are transported by a specific carrier which is termed a symport because the substrate molecule is translocated in tandem with a proton. This transport is powered by the full proton-motive force. Anions are transported by the pH gradient alone whereas cations are transported by the membrane potential alone, this being designated as a uniport (Konings, 1977).

A somewhat different system has been suggested by Kabach (1974). In this model, an oxidised transport protein with a high affinity for substrate, is reduced by an electron donor and undergoes a conformational change which causes it to move across the membrane and release its bound substrate molecule into the cytoplasm. The transport protein is then reoxidised and reverts to its original conformation on the outside of the membrane. Such a process would require that the transport mechanism be directly coupled to its own respiratory chain.

In Staph. aureus, group translocation of sugars appeared to be powered by substrate phosphorylation using phosphoenolpyruvate phosphotransferase whereas active transport of amino acids mainly used L-2-glycerophosphate as donor (Kaback, 1974). Although enzymic mechanisms (permeases) are widely accepted, Christensen (1979) has pointed out that non-enzymic transport is compatible with the available information, since the unenergised system appeared to operate as a facilitated diffusion. He has also proposed that uptake of a new amino acid might be balanced by counter-transport of a different one from the intracellular pool.

The transport process is usually highly specific, with a different uptake system being employed for each substrate, or for each group of closely related substrates, although the exact number of systems may vary between different organisms. Staph. aureus, for example, has been found to have twelve different transport systems for amino acids whereas E. coli and B. subtilis only have nine (Konings, 1977). The majority of detailed studies have been carried out in E. coli and, in most cases, at least two transport systems for each amino acid have been found; one with high affinity and one with low affinity eg. Anderson and Oxender, 1978). However, only one Michaelis constant for transport appears to have been published for each amino acid in Staph. aureus (Konings, 1977) and, with the exception of proline, they were all larger than 10 μM which suggests that the affinities were fairly low. This is consistent with the E.coli observations since the high-affinity processes were binding protein-mediated and gram positive organisms did not appear to possess such systems (Konings, 1977). Mutants of E. coli which lacked both uptake systems for branched chain amino acids were found to be incapable of retaining leucine (Anderson and Oxender, 1978) and similar results have been obtained for aromatic amino acids (Whipp, 1977, quoted by Anderson and Oxender, 1978), histidine (Shifrin, Ames and Ames, 1966) and arginine (Rosen, 1973). These observations suggested that specific transport systems were necessary to maintain the intracellular pools of amino acids, although the precise mechanism by which this could be controlled is doubtful. It is possible that leakage is a process which functions continuously, but which is usually masked by the transport process. This might be related to the excretory process discussed by Christensen (1979) (p.37).

C.2.3. Membrane Directed Inhibition of Uptake. As discussed above, uncoupling agents reduced or eliminated the protonmotive force and this would be expected to inhibit the rate of transport in a non-competitive manner. However, experiments by Brummett and Ordal (1977) and Nicholas and Ordal (1978) suggested that inhibition of amino acid transport in B. subtilis, by several chemically dissimilar compounds, could be explained by a mechanism other than the uncoupling of oxidative phosphorylation. They looked at transport of glycine or proline in the presence of 3,3'4'5 - tetrachlorosalicylanilide (TCSA), trifluoromethoxycarbonylcyanide-phenylhydr-azone (FCCP) or pentachlorophenol (PCP) and its analogues. FCCP competitively inhibited proline transport but inhibited glycine transport in an uncompetitive manner; TCSA also proved to be a competitive inhibitor for proline transport while being non-competitive for glycine transport; PCP was like TCSA in being non-competitive for glycine transport, but was, in contrast, uncompetitive with respect to proline transport. These conflicting results were explained by assuming that a specific protein-ligand interaction was taking place, which depended on the anionic form of the inhibitor. Studies with PCP and its analogues showed that the degree of inhibition depended on the degree of acidity of the analogue. It was concluded that the free energy of interaction which produced the protein-ligand complex resulted in the stabilisation of unproductive comformations of the specific membrane protein.

A similar explanation could be applied to the effect of N-ethylmaleimide on amino acid transport in E. coli (Janick, Grunwald and Wood, 1977), although in this case the binding reaction was known to be a covalent and non-specific linkage to accessible sulphydryl residues. Aliphatic diols produced a similar type of growth inhibition in B. subtilis which has been ascribed to a reduction in uptake of amino acids (Akedo et al. 1977). Inhibition decreased with increasing chain length and 1,2 diols were found to be more effective than 1,3 diols. Esterification of one alcohol grouping enhanced activity, whereas esterification of both caused a slight decrease. These results are consistent with a model in which the inhibitor is an amphipathic molecule which becomes partitioned into the plasmalemma and causes disruption of the normal structure, since such disorganisation could lead to dissipation of the membrane proton gradient or to configurational changes in transport proteins.

Alkyl esters of p-hydroxybenzoic acid also appeared to be growth inhibitory for B. subtilis, E. coli and Ps. aerugonisa due to an inhibition of uptake, which was suggested (Eklund, 1980) to be due to an increase in membrane permeability. NADH-oxidation and O_2 consumption were also inhibited. Weak acids such as benzoate, sorbate and propionate seemed to inhibit purely by disrupting the pH gradient (ΔpH) which produces part of the protonmotive force. Dissipation of the membrane proton gradient by tributyltin chloride was detected by Singh and Bragg (1979) who also noted leakage of amino acid from the intracellular pools. They concluded that the creation of a transmembrane OH^- -anion exchange was responsible.

From the diverse reports it is possible to conclude that membrane mediated inhibition of amino acid transport may be due to either disruption of the proton gradient or to changes in the configuration of the transport proteins. However, the observation that tributyltin chloride caused leakage (Singh and Bragg, 1979) suggests that there may be a further possibility, especially if this observation is related to the leakage observed in transport deficient mutants (Anderson and Oxender, 1979) and the excretory process discussed by Christensen (1979).

Some of the reported effects of phenethylalcohol can be explained by assuming a membrane directed effect, but other effects have been described which might require a different mode of action. Since the function of this compound is therefore uncertain, and since it plays an important part in this thesis, it will be considered separately below (p.59) after a brief review of other relevant antimetabolites.

D. RELEVANT STUDIES ON OTHER ANTIMETABOLITES

A number of workers have looked at non-membrane directed inhibition of Staph. aureus and these studies can be divided into two groups - those in which the overall effect, at least on growth yield, was determined at a fixed time, and those in which inhibition, at least of growth rate, was followed over a period of time.

D.1. Fixed Time Studies.

D.1.1. Amino Acid Analogues. Amino acid analogues are generally considered to be inhibitory because they are incorporated into proteins in place of the natural analogue, resulting in the production of inactive materials. Leboeuf-Trudeau et al. (1969) showed that certain tryptophan analogues depressed growth, DNA synthesis and alpha-haemolysin synthesis in Staph. aureus, although the total protein content was not affected. All of the effects could be reversed by L-tryptophan or by precursors such as indole or anthranilic acid. Mathieu, De Repentingy and Trudeau (1970) found that analogues of tyrosine, methionine, cysteine and leucine were apparently without effect, but that metafluorophenylalanine inhibited growth and alpha-haemolysin production, although ortho- and paraflurophenylalanine only affected growth. These effects were reversed by L-phenylalanine or phenylpyruvic acid.

The reason for these observations in unclear, since although the possibility that the analogues were incorporated in place of the correct amino-acid was not ruled out, the absence of cross-reacting material argued against this. It is possible that the aromatic amino acids were under feed-back control mechanisms operating at the level of prephenate or anthranilate, since controls of this type are known to exist in other bacterial species. (For a review of this topic, see Gibson and Pittard, 1968).

Sharma and Haque (1973) studied the effect of tryptophan analogues on beta-haemolysin production and obtained results similar to those of Leboeuf-Trudeau et al. (1969) for the alpha-haemolysin. They suggested that tryptophan limitation might preferentially repress haemolysin synthesis because haemolysin molecules had a higher content of tryptophan than other cellular proteins but, without knowing the tryptophan content of a number of different staphylococcal proteins, it is difficult to see how this conclusion could be sustained.

D.1.2. "Thymineless Death". When a thymine requiring mutant of E.coli is deprived of thymine, DNA synthesis ceases immediately, but RNA and protein synthesis continue for about 30 min, at which time the viable count decreases rapidly (Cohen and Barner, 1954). Attempted DNA synthesis in the absence of thymine was reported to be the cause of death (Maaløe and Hanawalt, 1961; Hanawalt et al., 1961). Thymineless death was shown to occur in Staph. aureus (Mathieu et al. 1968) and alpha-haemolysin was not produced during the period of deprivation which suggested that alpha-haemolysin synthesis might be more sensitive to this treatment than other more central synthesis.

D.1.3. Chloramphenicol and Actinomycin D. Both these antibiotics inhibit protein synthesis, chloramphenicol by preventing translation and actinomycin D by preventing transcription (Franklin and Snow, 1975; p.126 and p.90. Actinomycin D. also inhibits DNA replication at higher concentrations (Muller and Crothers, 1968). The use of complementary cultures treated with chloramphenicol or actinomycin D thus permits the possibility of determining the life of an mRNA molecule for a specific protein and Katsuno and Kondo (1973) examined the synthesis of enterotoxin B in this way. They found that production of extra-cellular proteins ceased immediately after the addition of chloramphenicol, while addition of actinomycin D did not stop production until about 30 minutes had elapsed. Enterotoxin B production paralleled that of total extracellular proteins but intracellular protein synthesis was halted immediately by treatment with either of the antibiotics. These results indicated that there was little or no intracellular accumulation of extracellular proteins as a whole, and enterotoxin B in particular, while the mRNA molecules which specified these proteins appeared to be relatively long lived compared to those responsible for the synthesis of intracellular proteins.

D.1.4. Mitomycin C. This antibiotic was demonstrated to be rapidly lethal to bacterial cells (Iyer and Szybalski, 1963), causing an immediate inhibition of DNA synthesis. In *Staph. aureus* it has been used to study lysogeny (eg. Hendricks and Altenbern, 1968; German, Panouse-Perrin and Ardouin, 1971) and the latter considered that the induction of lysogenic phage was due to inhibition of the production of repressor by mitomycin C. Current theory, however, has implicated the SDS-error prone repair system in phage induction in *E. coli* (Radman, 1975; Witkin, 1976). According to this hypothesis DNA damage leads to induction of the Rec A protein (protein X) which destroys or inactivates the phage repressor leading to the onset of the lytic cycle.

D.2. Periodic Monitoring Studies. To aid in the interpretation of these studies, a brief review of growth and extracellular protein production in the absence of antimetabolites will be presented.

D.2.1. Uninhibited Conditions.

D.2.1.a. Growth. Most experiments have employed a casamino acid based medium, although proprietory media, notably Lab Lemco, Nutrient broth, Trypticase - Soya broth, have been employed on many occasions. To ensure that extracellular proteins were detected rapidly, most studies have employed large inocula of between 1% and 20% of stationary values. Although it is usually impossible to be certain, these inocula contained between 10^7 and 10^9 colony forming units (c.f.u.) per cm^3. The presence of a lag phase lasting 30-90 mins was common although the results presented by McNiven and Arbuthnott (1972) do not show such a feature. Exponential phase then ensued, its length depending on the concentration of inoculum, with most studies showing a generation time of about 60 mins, although the conditions employed by Gemmell and Shibl (1976) showed a generation time of about 3 hours. However, the precise conditions of this experiment were not given and the growth rate in any case declined after one generation, to about 6 hours. Over the next 12 hours, the culture entered into stationary phase without apparently completing a further doubling of population.

In most other studies, growth rate remained exponential for three to four generations and then began to decline so that growth became non-exponential, although the population continued to increase until it reached a stationary level of about 10^9-10^{11} c.f.u. cm^{-3}, the final density being dependent on the nature of the growth medium. Holme and Arvidson (1976) noted that growth rate was not affected by switching from glucose to glycerol as the energy source (both apparently at 15g.dm^{-3}), but the growth rate was slightly reduced by switching from 40g.dm^{-3} casein hydrolysate to 20g.dm^{-3}. Growth rate fell further when casein levels were reduced to 10g.dm^{-3} (Bjorklind and Arvidson, 1976). The latter workers also looked at cultures in 20g.dm^{-3} casein supplemented with tryptic or protease I digests of casein. Although these experiments were not precisely detailed in the report, it appears that both supplements were inhibitory rather than stimulatory. Dissolved oxygen tension was shown to have an important effect on growth rate (Holme and Arvidson, 1976) with generation times being doubled by lowering the dissolved oxygen below about 40%. However, the slowing down of the culture during the transition period between exponential and stationary phases was ascribed by these authors to exhaustion of media components, since they found that addition of glycine, serine and threonine at this stage prolonged the exponential phase.

D.2.1.b. Extracellular Protein Production.

Alpha- haemolysin : Classically an atmosphere enriched with CO_2 was considered essential for optimal yields of alpha-haemolysin (Burnet, 1929) and it has been suggested by Hendricks and Altenbern (1968) that the CO_2 may have been acting as a buffering agent, preventing an elevation of pH which they demonstrated would inhibit the production of alpha-haemolysin. This was not confirmed by McNiven and Arbuthnott (1972). Kapral (in discussion to Gladstone, 1966) commented that alpha-haemolysin synthesis in Staph. aureus had an absolute requirement for CO_2: in continuous culture conditions no alpha-haemolysin was produced unless CO_2 was introduced and the prompt response to CO_2 addition suggested that it acted as an inducer of the alpha-haemolysin structural gene.

When the organism was grown in batch culture it seemed to produce enough CO_2, as a by-product of metabolism, to off-set this requirement for an exogenous source. This requirement for CO_2 in continuous culture might be related to Coulter's observation (Coulter, 1966) that the capacity to produce alpha-haemolysin was apparently lost during continuous culture. As pointed out by Arbuthnott (1970) growth is not always accompanied by production of alpha-haemolysin and this suggested that some specific growth factor was necessary for its biosynthesis. Braman and Norlin (1951) had found that yeast extract contained such a stimulatory factor, although they could not identify it and Bernheimer and Schwartz (1963) described a semi-synthetic medium containing yeast extract dialysate and casamino acids which gave optimal yields of alpha-haemolysin under normal aerobic conditions. This medium, sometimes slightly modified, has been used in many studies (eg. Six and Harshman 1973a,b; Paradisi and D'Aniello, 1969; McNiven et al., 1972). Dalen (1973a,b) concluded that the stimulatory component of yeast extract was histidine, although histidine dipeptides were more potent, apparently because they were taken up more rapidly (Dalen, 1973c). He suggested that serine, glycine and CO_2 exerted a stimulatory effect due to their role as precursors in purine biosynthesis, since histidine could be derived from ATP, and the presence of a histidase active above pH 8.0 was the reason for the depression of alpha-haemolysin synthesis above that pH noted by Hendricks and Altenbern (1968). In the completely synthetic medium which Dalen employed, growth and haemolysin synthesis proceeded at relatively low rates and he therefore described the effect of histidine as non-gratuitous, since growth was also stimulated. Keller, Hanson and Bergdoll (1978) have shown that several amino acids, including 2 mM histidine, can function as energy sources for Staph. aureus.

The rate of synthesis of alpha-haemolysin and other extra-cellular proteins remained exponential after growth rate began to decline (McNiven, 1972) and this behaviour is in accord with the model proposed by Coleman, Brown and Stormonth (1975) as noted by Abbas-Ali and Coleman (1977). These workers considered that this differential behaviour was due to competition for transcriptional precursors and related the intra-cellular concentration of nucleotides to the production rate. These observations make it clear that alpha-haemolysin **was produced at maximum** rate when the culture was slowing down. However, this leads to a certain degree of contradiction since this was the time at which glycine and serine became exhausted (Holme and Arvidson, 1976) and yet Dalen (1973c) claimed that these two amino acids were stimulatory to alpha-haemolysin synthesis.

Lipolytic enzymes As pointed out in section B.4.7. there appears to be no clear differentiation of the lipolytic enzymes but at least two enzymes, a lipase and an esterase, have been distinguished. Gemmell and Shibl (1976) using "suitable culture media and incubation conditions" found that lipase was produced exponentially from zero time and reached a maximum level before the culture entered stationary phase. Esterase, on the other hand, appeared a little later while the culture was in log phase and continued to increase as the culture gradually slowed down. However, as discussed above (p. 44), the growth rate of this culture appeared abnormal, so it might be premature to draw any general conclusions from these observations. Mates (1974a) noted that lipase production was inhibited by free aliphatic acids, which is consistent with the view that specific degradative enzyme production would be inhibited by a specific reaction product.

Phosphatases. Arvidson (1976) and Holme and Arvidson (1976) have reported on the production of acid phosphatase. Arvidson (1976) reported that only loosely bound phosphatase was produced during log phase and that free phosphatase was first detected when the culture began to slow down (4h in the casein hydrolysate-glycerol medium used). At the same time, bound phosphatase ceased increasing, which was interpreted as indicating that a number of phosphatase attachment sites (possibly teichoic acid) were synthesised during log phase, but that their formation ceased at the transition point. Phosphatase, synthesised continuously, would occupy the attachment sites (by electrostatic attraction) during log phase and thereafter de novo enzyme would be released directly into the medium. However, there appears to be no evidence that free phosphatase is exclusively composed of de novo molecules. Release of free phosphatase was found to be stimulated by reduced oxygen tension and Holme and Arvidson (1976) noted that, once stimulated, restoration of high oxygen levels did not reduce the rate, even 'though growth rate was stimulated. Since Y_{O_2} was noticeably lower in glycerol medium compared to glucose medium, Holme and Arvidson (1976) suggested that phosphatase production might be stimulated by the higher turnover rate.

Little work has been done on the alkaline phosphatase, but Shah and Blobel (1967) reported that free inorganic phosphate caused repression of production of this enzyme, a further example of product feed-back inhibition.

Protease I Holme and Arvidson (1976) also looked at the production of Protease I (Serineprotease) and further observations were made by Bjorklind and Arvidson (1976). The enzyme (in Casein Hydrolysate medium with glucose or glycerol as carbon source) was first detected during log phase and production was reduced, or delayed, by low oxygen tension. Supplementing the culture with lactalbumin hydrolysates indicated that protease I hydrolysate was stimulatory to protease I synthesis although no reason was deduced. This observation is in contrast to the product feed-back inhibition which has been considered above. However, it is worth noting that the original growth medium also contained a protein hydrolysate and that the controlling factor for protease production might well be the intracellular amino acid or total nitrogen level rather than random peptides.

Staphylokinase. Makino, Fujumura and Hagashi (1976) found no relationship between aeration and staphylokinase yield but they did note that the nature of the carbon and energy source was important with glycolytic intermediates being stimulatory, whereas acetate and citric acid cycle intermediates were either non-stimulatory or slightly inhibitory. This suggests that fermentive metabolism might be more favourable than oxidative.

Protein A. Studies by Movitz (1976) indicated that protein A resembled acid phosphatase in being preferentially attached to a cell wall component, in this case peptidoglycan, with excess being released into the medium. Synthesis of protein A was exponential until just after growth entered the transition period. Cell bound protein A production then slowed down in parallel with growth finally ceasing when the culture entered stationary phase. Cell-free protein A continued to increase over a period of about 36 hours, apparently due to autolytic release from the cell wall.

Enterotoxin B. Morse, Mah and Dobrogosz (1969) and Morse and Baldwin (1971, 1973) noted repression of enterotoxin B synthesis during growth in the presence of glucose or pyruvate, but were unable to decide if they were observing catabolite repression. Keller *et al.* (1978) found several amino acids, including histidine, increased the yield of enterotoxin B, although concentrations in excess of 10 mM seemed to be inhibitory. No clear reason for this observation was deduced.

Delta-haemolysin. Production of delta-haemolysin has been shown (Turner, 1978) to require adequate aeration and a high concentration of yeast extract diffusate, although the precise levels appeared to be strain variable.

D.2.2. Effect of Antibiotics on Growth and Extracellular Protein Production.

A number of workers have looked at the effect of low levels of antibiotic on the production of extracellular proteins. In these experiments growth rate was usually inhibited by about 20% and attention has been centred on the degree to which the production of specific extracellular proteins was reduced.

In one of the earliest studies of this kind, Hinton and Orr (1960) found that protein synthesis inhibitors of different kinds (chloramphenicol, tetracycline and oleandomycin) inhibited growth and alpha-haemolysin production to the same extent. Mates (1974b) found that tetracyclines at concentrations which did not affect growth, could nevertheless inhibit production of lipase. Later he reported that lincomycin exerted a similar effect (Mates, 1975). Gemmell and Shibl (1976) also found that both lipase and esterase were totally inhibited by lincomycin at concentrations which only reduced growth by about 20%. They reported similar results for alpha-haemolysin and coagulase. Sub-inhibitory concentrations of chloramphenicol apparently suppressed production of these four extracellular proteins to a lesser extent with coagulase, lipase and esterase, eventually attaining the same levels as untreated control and alpha-haemolysin reaching 50%. The results suggest a delay in initial expression since the rate of production appeared to be the same in controls and treated cultures once the production was under way. Unfortunately the growth rate was not reported for these cultures, although an overall reduction of not more than 20% is implied, but it seems possible that the delay in production was a consequence of the cells diverting limited resources away from non-essential synthesis. Some of the results presented in this paper appear contradictory but the effect of lincomycin and polymyxin in tandem was clear cut. Under these conditions, release of esterase and lipase was detected and the authors suggested that this was due to increased membrane permeability caused by the polymixin allowing release of intracellular pools of enzyme, although release was delayed for about 6 hours (probably about 2 generations but growth rates not given). Polymyxin alone caused apparent spontaneous release of alpha- and delta- haemolysin, DNase, lipase, esterase and Succinic Oxidase Factor from non-growing cells. Neither the exact amount of material released nor the time required for this release were given however and therefore the possibility exists that the effect of the two antibiotics might be the reverse of that proposed above, that is that lincomycin might be retarding the spontaneous release induced by polymixin.

E. PHENETHYL ALCOHOL

E.1. Bacteriostatic Effect. The bacteriostatic effect of phenethyl alcohol, first described by Lilley and Brewer (1953) was suggested by Berrah and Konetzka (1962) to be due to the reversible inhibition of DNA synthesis. These authors found that protein and RNA synthesis were inhibited to a lesser extent, which could be partially reversed by an unidentified component of yeast extract. Later work confirmed these inhibitory effects (Rosenkrantz, Carr and Rose, 1964, 1965; Prevost and Moses, 1966). Studies on E. coli by Lark and Lark (1966) indicated that the effect on DNA synthesis was due to a specific inhibition of the initiation of new replication forks, without stopping previously initiated replication cycles. Further work (Lark and Renger, 1969) indicated that the initiation of DNA replication involved three distinct steps. The first two steps appeared to involve the synthesis of proteins since they were inhibited by amino acid starvation and by chloramphenicol treatment whereas the third step did not require amino acids. Only the first step was inhibited by phenethyl alcohol, which suggested that phenethyl alcohol interfered selectively with protein synthesis. Kaneko, Kodama and Nagata (1977) noted that phenethyl alcohol substantially reduced the E. coli inner membrane content of a DNA binding protein and Craine and Rupert (1978) found that a similar protein formed part of a membrane-DNA complex at the origin of replication which might explain the specific effect on initiation but also implicates membrane as the site of action. This is considered further in the next section.

E.2. Membrane Effects. Silver and Wendt (1967) noticed an increased permeability in E. coli treated with phenethyl alcohol and King (1974) noted a similar effect in Streptococcus faecalis. In contrast, Urban and Wyss (1969) found that phenethyl alcohol treatment of Bacillus subtilis decreased the ability of this organism to take up transforming DNA. These observations indicated that phenethyl alcohol caused permeability changes, probably by acting on the cytoplasmic membrane.

Several other observations also implicated the cytoplasmic membrane as the site of phenethyl alcohol activity; for example, Nair, Pradhan and Sreenivasan (1975) suggested that changes in membrane structure were responsible for phenethyl alcohol inhibition of E. coli's ability to repair radiation induced single-strand breaks in DNA and Yura and Wada (1968) and Wada and Yura (1971, 1974) found that resistance to phenethyl alcohol involved specific changes at a membrane-bound locus. This might reflect a change in the susceptibility of the membrane protein referred to above (p. 52). However, membrane phospholipids have been implicated by Nunn (1975) who noted that synthesis of phospholipids in E. coli was inhibited by phenethyl alcohol, phosphatidyl ethanolamine synthesis being most sensitive followed by phosphatidyl glycerol. He proposed that unbalanced growth was due to this inhibition, but failed to reverse the effect by supplying preformed phospholipids. It seems probable therefore that inhibition of phospholipid synthesis was a consequence of unbalanced growth rather than the cause. Inhibition of uptake of amino acids in Neurospora crassa was reported by Lester (1965), and Jones (1979) noted a similar effect in Myxococcus xanthus. The latter reported a rapid drop in the intracellular methionine pool immediately after addition of phenethyl alcohol, but the reason for this observation is uncertain since further experiments did not indicate any general increase in membrane permeability due to phenethyl alcohol at the concentration used (17mM), although 85mM phenethyl alcohol did severely affect membrane integrity. An increase in membrane fluidity, as demonstrated by Halegoua and Inouye (1979), might be responsible (Jones, personal comm.). Halegoua and Inouye (1979) used phenethyl alcohol to study the process of translocation and assembly of outer membrane proteins in E. coli and noted a differential effect. Processing and assembly of matrix protein was inhibited by 22.5mM or more, whereas protol G was similarly inhibited at 30mM or above. Promatrix protein was accumulated, probably in the periplasmic space. At lower concentrations tol G and lipoprotein also accumulated in the periplasmic space and were inserted into the outer membrane after phenethyl alcohol was removed. During active growth, 22.5mM phenethyl alcohol appeared to completely block promatrix protein production. They found that phenethyl alcohol drastically increased membrane fluidity and suggested that this might be involved in the inhibitory process.

E.3. Effects on Staph. aureus. De Repentigny et al. (1968) and Cadieux, Côté and Mathieu (1970) found that addition of 5 $mg.cm^{-3}$ (47.5 mM) of phenethyl alcohol to six strains of Staph. aureus resulted in unbalanced growth and that the effects were greatest for the fastest growing strain and least for the slowest. The presence of a capsule did not affect the inhibitory action. Electron microscopy studies (Cadieux et al., 1970) revealed that phenethyl alcohol disturbed the formation of septa, and studies of the uptake of acridine orange, using fluorescence microscopy, indicated that the treated cells had become more permeable to the dye. Both these observations might be different manifestations of the same membrane directed effect.

De Repentigny et al. (1968) also examined the effect of phenethyl alcohol on the production of extracellular proteins. Gel diffusion of untreated culture filtrates against a "commercial antitoxin" revealed six precipitin lines, whereas filtrates from phenethyl alcohol treated cultures revealed only one precipitin line. The precise nature of the material producing these lines was not determined, but gel diffusion against purified anti-alpha-haemolysin or in rabbit blood agar, as well as mouse lethality tests, indicated that alpha-haemolysin was not produced by treated cultures (De Repentigny *et al.*, 1968). These results are interesting but limited since the study was confined to one concentration of phenethyl alcohol (47.5 mM) added at one time (the start of log phase).

Altenbern (1966, 1968, 1969, 1971a,b) utilised the ability of phenethyl alcohol to prevent initiation of DNA replication, to obtain synchronously growing populations of *Staph. aureus*, and suggested that phenethyl alcohol treatment of *Staph. aureus* inhibited an amino acid-requiring step in the initiation of DNA synthesis, similar to that which was inhibited in *E. coli* (Lark and Renger, 1969).

F. GENETICAL STUDIES

Genetical studies in *Staph. aureus* are not as well advanced as in the more classical organisms such as *E. coli* or *B. subtilis*. In this section some investigations which have relevance to this thesis will be described.

F.1. Mutational Classes

F.1.1. Dwarf Colony Mutants. These were first described by Hoffstadt and Youmans (1932). Browning and Adamson (1950) and Wise (1956) both isolated dwarf (G variant) colonies in the presence of streptomycin and noted that alpha-haemolysin was not produced by these mutants, although they obtained conflicting results on the production of coagulase. The dwarf mutants appeared to be deficient in some aspect of vitamin metabolism; Haem (Yegian, Gallo and Toll, 1958; Tien and White, 1968), Pantothenate (Sompolinsky, Gluskin and Ziv, 1969), Vitamin K (Sasarman et al., 1971).

F.1.2. Mutations in Genes Involved with Extracellular Protein Production. A number of workers have examined strains of Staph. aureus which are mutant for particular extracellular products. In every case isolates lacking one character only were selected (Table 2), but in each a number of pleiotropic mutants were isolated. Pleiotropic mutants are those in which an apparently single mutation results in the loss of more than one character. These observations are summarised in Table 3. Since primary isolations were made on a variety of media, and mutagenic treatments varied (even where the same mutagen was used, the conditions employed were different), it seemed unlikely that the production of pleiotropic mutants was the result of a freak production of multiple single mutations.

As shown in Table 3, twenty-one pleiotropic mutants were found in eight independent studies. It is difficult to draw any firm conclusions from this data at this stage and this matter will be reconsidered later (p. 186); but two points may be made now. Fourteen of the mutants were defective in alpha-haemolysin production. All but four of these (6a, 8b, 8f, 8g) were also defective in staphylokinase production. Of the ten which were defective in staphylokinase production, only one (6d) was not defective in alpha-haemolysin production. This may indicate a close relationship between these two products similar to that suggested by McClatchy and Rosenblum (1966a,b) (p.63).

Two studies (4 and 5) were concerned with increased production and the results indicated that all extracellular proteins tested were found in increased amounts in these mutants, possibly due to an improved ability to export the products through the membrane. Altenbern (1975a,b)

TABLE 2

Mutagens Used by Other Workers to Obtain Extracellular Protein Mutants

	Reference	Primary Mutation Sought	Mutagen Used
(1)	Ommen and Friedman (1970)	Nuclease	MNNG
(2)	McClatchy and Rosenblum (1966a)	Alpha-haemolysin	UV or NA
(3)	Harmon and Baldwin (1964)	Penicillinase	UV
(4)	Kondo and Katsuno (1973)	Enterotoxin B	None (Spontaneous)
(5)	Yoshikawa *et al.* (1974)	Nuclease or alpha-haemolysin	MNNG
(6)	Forsgren (1972)	Protein A	MNNG or EMS
(7)	Wadstrom (1973b)	Glucosaminidase	MNNG or EMS
(8)	Van Der Vijver, Van Es-Boon and Michel (1975a)	Coagulase or alpha-haemolysin	EMS

Key : MNNG : N-Methyl-N'-Nitro-N-Nitrosoguanidine.

UV : Ultra Violet Light

NA : Nitrous Acid

EMS : Ethylmethanesulphonate (Alkylating agent)

TABLE 3

Patterns of Pleiotropic Mutations Detected by Other Workers

Product	1	2	3	4	5	6 a	6 b	6 c	6 d	6 e	7 a	7 b	7 c	8 a	8 b	8 c	8 d	8 e	8 f	8 g	8 h
Alpha-haemolysin	+	-	-	++	++	-	-	+	+	-	-	+	+	-	-	-	1	-	1	-	-
Beta-haemolysin	-	±	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Delta-haemolysin	0	0	0	++	0	0	0	0	0	0	0	0	0	+	+	+	1	+	-	-	1
Enterotoxin B	0	0	0	++	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Leucocidin	0	0	0	0	0	0	0	0	0	0	-	+	+	-	1	1	1	+	-	1	1
Coagulase	-	+	+	0	++	+	-	-	-	-	-	-	+	-	1	+	1	+	+	+	1
Staphylokinase	0	-	-	++	0	+	-	+	-	-	0	0	0	-	+	-	1	1	+	+	1
Proteolytic activity	0	0	-	0	++	0	0	0	0	0	0	0	0	-	+	+	+	+	+	+	+
Hyaluronate lyase	0	0	0	0	0	0	0	0	0	0	-	+	+	-	+	1	+	+	+	+	+
Nuclease	-	+	+	++	++	+	+	+	-	-	-	-	-	-	1	1	1	+	+	1	1
Phosphatase	0	0	0	++	0	0	0	0	0	0	0	0	0	-	+	+	+	+	+	+	+
Glucosaminidase	0	0	0	0	0	0	0	0	0	0	+	+	-	0	0	0	0	0	0	0	0
Lipase	0	+	-	0	0	0	0	0	0	0	-	+	+	-	+	+	+	+	+	+	+
Protein A	0	0	0	0	0	-	-	-	-	-	0	0	0	0	0	0	0	0	0	0	0

Key: + Product detected; - Product not detected; ++ Product detected in increased amounts;

± Product sometimes detected; 0 Product not tested for or not produced by parent strain;

1 Product detected in reduced amounts, ie "leaky";

References: As Table Two. The letters a-h refer to different mutants described in a single report.

isolated mutants, thought to be defective in membrane function following MNNG treatment, and found that some possessed enhanced ability to produce enterotoxin B or alpha-haemolysin. Of twelve isolates examined, two showed an increase in alpha-haemolysin production but showed wild type levels of enterotoxin B and three showed increased levels of enterotoxin B but showed wild type or decreased levels of alpha-haemolysin, which indicated that more than one gene was involved.

F.2. Involvement of Plasmids in Extracellular Protein Production

Plasmids are extrachromosomal elements of genetic material which can replicate autonomously in the bacterial cytoplasm (Lederberg, 1952), and current theory uses the term to encompass all entities which can fulfil this definition, including lysogenic bacteriophage (Meynell, Meynell and Datta, 1968). However, for convenience, lysogenic bacteriophage will be considered separately here.

F.2.1. Extracellular Proteins Associated with Plasmids

Plasmids in Staph. aureus have been the subject of frequent reviews (eg. Richmond, 1968, 1972) but these reviews are largely restricted to plasmid borne resistance to antimetabolites, particularly penicillin. Penicillinase is an extracellular protein which is known to be specified by a plasmid. Chemical agents which eliminated this plasmid from Staph. aureus included acriflavine (Mitsuhashi et al., 1963); acridine orange (Harmon and Baldwin, 1964; Cannon and Dunican, 1970), sodium dodecyl sulphate (Sontein and Baldwin, 1972a,b), ethidium bromide (Bouanchaud, Scavizzi and Chabbert, 1968). Growth at elevated temperatures was also effective although results varied markedly between different plasmids and different host strains (Asheshov, 1966).

Dunican (1967) and Cannon and Dunican (1970) extracted DNA from Staph. aureus and subjected it to column chromatography on Poly-L-Lysine-Kieselguhr. Only two strains were examined, one obtained by acridine orange (25 $\mu g/cm^{-3}$)treatment and one by growth at 42^{o}C. Precise details of the selection procedures were not given. Three peaks were observed in material from the parent strain and they considered this to mean that two plasmids were carried by this strain, the third peak presumably being chromosomal DNA. In the strain derived by growth at 42^{o}C, only two peaks were found and in the strain derived by growth in acridine orange, only the chromosomal peak was found. A correlation between the loss of satellite peaks and the loss of various characters appeared to exist, which suggested that alpha and delta-haemolysin were specified by the acridine-sensitive temperature-insensitive plasmid, whereas beta haemolysin coagulase, nuclease, lipase and staphylokinase were specified by the plasmid which was sensitive to both treatments. However, the lack of experimental detail means that these results should be treated with caution. A request for further details and strains met with no response.

Witte (1976) treated eleven strains of Staph. aureus with SDS and demonstrated apparent elimination of alpha-haemolysin formation from five strains, with frequencies varying between 26% and 100%. Coagulase and DNase were still produced suggesting that if a plasmid was being eliminated, these two characters were not on the same plasmid as the alpha-haemolysin gene(s). The variants had the same growth rate as the wild type.

Rogolsky et al.(1974) examined the nature of the genetic determinant controlling exfoliative toxin production and found that percentage elimination was strain variable and also varied for different treatments.

Evidence that a bacteriocin, produced by some phage group II strains, could be co-ordinately lost with the exfoliatin, has been presented by Warren _et al._ (1974). This co-ordinate loss occurred at a frequency of 92% when either ethidium bromide or growth at 44°C was used. Coagulase penicillinase and beta-haemolysin were not lost in these experiments. Parisi _et al._ (1967) used elevated temperature alone or with sodium dodecyl sulphate in an attempt to eliminate coagulase production. Of twenty-six strains treated in this way, twenty-five showed no evidence of loss, but in one strain between 60 and 94% elimination was obtained by the combined treatment.

Dornbusch, Hallander and Löfquist (1969) considered that methicillin resistance and enterotoxin B production were both carried by a plasmid in strain DU 4916 but this was not confirmed by Lacey (1974). However, Shalita, Hertman and Sarid (1977) confirmed that enterotoxin B was coded for by a gene carried on a plasmid, of 0.75 Mega-daltons (Md), although linkage to the methicillin resistance gene was not found.

F.2.2. Extracellular Proteins Associated with Lysogeny

Lysogenic bacteriophage may, on occasion, specify the production of a "toxic" extracellular protein. Freeman (1951) found that non-toxinogenic strains of _Corynebacterium diphtheriae_ became toxinogenic when lysogenised by phage B and conversion has also been observed in other genera; _Clostridium perfringens_ (Paquette and Fredette, 1967) _Cl. botulinum_ (Inoue and Iida, 1970), _Streptococcus pyogenes_ (Zabriskie, 1964). A number of workers have reported on possible lysogenic conversions in _Staph. aureus_ involving extracellular proteins, but most of these observations were unsupported. Thus, Hendricks and Altenbern (1968) could not confirm the observations of Blair and Carr (1961) that alpha-haemolysin was associated with lysogeny, while Clecner and Sonea (1966) indicated that the delta-haemolysin gene was carried by a lysogenic phage, but a later report (Dobardzic and Sonea, 1971) suggested that this was not the case, although different phage conversions might have been involved. Casman (1965) found that the enterotoxin B gene was carried by a lysogenic phage; Van der Vijver, Van Es-Boon and Michel (1972) showed that leucocidin was carried by a lysogenic phage and Rosendal, Bulow and Jessen (1964) and Rosendal and Bulow (1965) reported that the capacity to produce lipase was lost following lysogenisation.

The only example of lysogenic conversion which was substantiated was the co-ordinate conversion of beta-haemolysin positive-staphylokinase negative strains to the reverse phenotype following lysogenisation with serogroup F phage (De Waart _et al_., 1963; Winkler, De Waart and Grotsen, 1965; Dobardzic _et al_., 1970; Dobardzic and Sonea, 1971; Van der Vijver _et al_., 1972). At least fifteen group F phage were shown to cause this conversion (Winkler _et al_., 1965) and these workers suggested that the phage might specify some kind of repressor which led to the phenotypic loss of beta-haemolysin. Alpha-haemolysin might behave like staphylokinase in this conversion (Dobardzic and Sonea, 1971). Staphylococci are frequently found to be lysogenic or even polylysogenic and so lysogenic conversions might be very frequent in this genus (Rountree, 1949; Smith, 1948; Blair and Carr, 1961).

Egan (1972) postulated that a plasmid locus might be a _sine qua non_ for genes specifying extrachromosomal proteins. His evidence was not strong, however, and he suggested that useful information might be gained from a comparison of the amount and location of penicillinase produced by genes which are chromosomally or plasmid located. Asheshov, Woods and Dyke (1970) had examined these features, but their paper probably appeared after Egan had completed his review. They found that the only difference between chromosomal and plasmid genes was that the plasmid gene produced about twice as much penicillinase as the chromosomal gene. While this could reflect a more efficient excretion, they took the view that the difference was due to "gene dosage", ie. that there were two copies of the plasmid for every copy of the chromosome.

The evidence for plasmid involvement in extracellular protein synthesis in Staph.aureus is therefore inconclusive. There does, however, seem to be grounds for considering that the genetic locus of many of the genes might be strain variable.

F.3. Genetic Mapping in Staph. aureus.

F.3.1. Transduction. Many workers have used transduction to map small regions of the Staph. aureus genome, particularly in studies involving amino acid biosynthesis, but only one group (McClatchy and Rosenblum, 1966b) have applied this technique to mapping of extracellular products. They found that alpha-haemolysin mutants fell into two groups : those which had lost only alpha-haemolysin and those which had also lost staphylokinase (McClatchy and Rosenblum, 1966a). Transductional analysis led them to suggest that these two groups represented two genes which were capable of being transduced independently. Their results, however, were very erratic, since control values frequently approached the transductional values which made it difficult to accept that transduction was really being observed.

F.3.2. Transformation. Until recently, transformation had not been observed in Staph. aureus and indeed, Spizizen, Reilly and Evans (1966) had suggested that transformation did not occur in staphylococci.

Riggs and Rosenblum (1969) were able to demonstrate transfection (transformation by phage DNA) after partial digestion of the cell wall by lysostaphin and Markov et al. (1973) found that pretreatment with trypsin could increase transformation frequencies by a factor of 10^3 when selecting for streptomycin resistance. Nomura et al. (1971) were the only group to report on transformation of extracellular proteins. They noted transformation of delta-haemolysin alone or co-ordinately with beta-haemolysin at a frequency of about 1:500 in the presence of penicillin, streptomycin and EDTA using 15 $\mu g.cm^{-3}$ DNA on about 10^4 c.f.u..cm^{-3} of recipient bacteria. Maximal competence was obtained between 30 and 45 min into the growth cycle.

The basic problems of transformation were overcome recently by Sjöström and co-workers (Sjöström, Lindberg and Philipson, 1972, 1973; Lindberg, Sjöström and Johansson, 1972; Lindberg and Novick, 1973; Rudin et al., 1974; Sjöström and Philipson, 1974). They found that transfection and transformation could both be attained under similar conditions. Transformation efficiency was quite low (Lindberg et al., 1972) with chromosomal markers being inherited at about $1:3.10^6$ - $1:5.10^5$ and plasmid markers at about $1:10^7$. Transfection occurred at about 5.10^5 p.f.u..μg^{-1} DNA which was only about 10% of the frequency observed for B. subtilis (Sjöström et al., 1973). A requirement for lysogeny by phage Ø11 (Sjöström et al., 1973; Rudin et al., 1974) or phage 83A (Sjöström and Philipson, 1974) for competence has been extended (Thompson and Pattee, 1977) to a number of other serogroup B phage. These phage conferred competence by functioning as "helper phage" without directly participating in the genetic process, although there might be a specific requirement for an early gene (allele 31), at least for Ø11 (Sjöström and Philipson, 1970). The low yield of transformants appeared to be due to the activity of the nuclease (Lindberg *et al.*, 1972; Sjöström *et al.*, 1973). In view of the extreme conditions and low yields which this work has shown to be required for transformation in *Staph. aureus*, it seems strange that Nomura *et al.* (1971) should have obtained such high frequencies under conditions of Ca^{++} shortage (EDTA present) and sub-optimal temperature (37°C).

F.3.3. Other Mapping Methods Pulse mutagenesis of synchronously replicating populations and marker frequency analysis have been applied to *Staph. aureus* by Altenbern (1966, 1968, 1971a,b). Although about twenty-nine markers were mapped by this worker, it has proved difficult or impossible to repeat or extend these observations (Graves *pers. comm.*) Extracellular protein genes were not located.

MATERIALS AND METHODS

MATERIAL AND METHODS

A. SELECTION AND MAINTENANCE OF ORGANISM

A.1. Choice of Organism. Wood 46 was chosen as the strain to be studied and six isolates were obtained, three from departmental stock, two variants from an ampoule of NCTC 7121, and NCTC 10344. They were tested for haemolytic and other abilities as described below (p82 - 84) On the basis of the results of these tests (p.88) NCTC 10344 was selected and all experiments were carried out using this strain.

A.2. Maintenance of Cultures. Stock cultures were grown on blood ~~agar base~~ slopes (Oxoid Ltd., London) and stored at 4°C. Subcultures were made at approximately monthly intervals on 10% sheep blood agar plates (Oxoid Ltd.) and after overnight incubation at 37°C colonies showing good haemolysis were inoculated onto 10 fresh slopes, incubated for 18h at 37°C, and retained as stock cultures.

B. ANALYSIS OF GROWTH AND EXTRACELLULAR PRODUCTS.

B.1. Media. Various liquid media were employed, as described below, but most experiments were carried out in the modified Bernheimer-Schwartz (BS) medium described by McNiven (1972).

B.1.1. BS Medium. This medium incorporates a yeast extract dialysate diffusate which is prepared separately. To minimise the risk of contamination during the 72h dialysis, the containers were rinsed with 70% ethanol prior to use. 200g yeast extract (Difco) was dissolved in 500 cm^3 distilled water and poured into a 75 cm length of 5.5 cm diameter Visking dialysis tubing (Scientific Instrument Centre, London). The dialysis sac was immersed in 1600 cm^3 of distilled water in a 5 dm^3 beaker and allowed to dialyse at 4°C with stirring for 72h. The contents of the dialysis sac were discarded and the diffusate from two dialyses were combined to yield approximately 2 dm^3. The complete medium was then prepared as follows:

Yeast Extract Dialysate Diffusate	Approx. 2 dm^3
Casamino Acids (Difco, technical)	64 g
Glucose (Analar - BDH, Poole, Dorset)	8 g
Thiamine (Aneurine Hydrochloride-B.D.H)	0.4g
Nicotinic Acid (B.D.H.)	3.7 mg
Distilled Water to 3.2 dm^3	

Adjusted to pH 7.1 with NaOH(Volucon- May and Baker), dispensed in required volumes, autoclaved at 121^{o}C (1 $kgf.cm^{-2}$) for 15 min and stored at 4^{o}C.

B.1.2. Modified BS Media. In order to investigate the role of each component in the production of extracellular proteins, BS medium was prepared with variations in the concentration of the ingredients as follows: Yeast Extract at half strength; Casamino Acids at half strength; Glucose at quarter strength, half strength or double strength; Glucose absent or replaced by Acetate or Succinate at the same molarity (14 mM); Whole medium diluted 50%, 60%, 70%, 80%, or 90%.

B.1.3. Minimal Medium. A minimal medium with the following composition was derived:

Glucose	4.0 g
K_2HPO_4	1.75g
Sodium Citrate $2H_2O$	125 mg
$MgSO_4.7H_2O$	25 mg
$(NH_4)_2\ SO_4$	500 mg
L-Cysteine	500 mg

L-Histidine	500 mg
L-Aspartic Acid	500 mg
Glycine	500 mg
Nicotinamide	10 μg
Thiamine (Aneurine Hydrochloride)	10 μg
Distilled Water to	1 dm^3

The effect of supplementing this medium by the addition of 500 $mg.dm^{-3}$ of other L-amino acids was investigated. The medium was filter-sterilised and dispensed into sterile flasks.

B.1.4. Proprietory Media. Nutrient Broth (Oxoid Ltd.), Lab Lemco (Oxoid Ltd.), Heart Infusion Broth (Difco Lab. Ltd.) and Trypticase Soya Broth (Difco Lab. Ltd.) were prepared as described by the manufacturers and sterilised by autoclaving at 121^{o}C (1 $kgf.cm^{-2}$) for 15 min.

B.2. Preparation of Initial Inocula. An isolated colony showing good haemolysis was inoculated into 100 cm^3 of BS medium and incubated at 37^{o}C on an orbital incubator (Gallenkamp, London) at 115 revolutions. min^{-1} for 16 h. The organisms were sedimented at 4^{o}C in an MSE High Speed 18 centrifuge (Measuring and Scientific Equipment Ltd., (MSE), Crawley, Sussex) at 15,000g for 10 min, washed twice in cold (4^{o}C) phosphate buffered saline (PBS) (Dulbecco 'A' - Oxoid Ltd.) and resuspended in their original volume of fresh medium, appropriate to the next stage, at 37^{o}C.

B.3 Analysis of Growth. Initial inocula were added to 100 cm^3 medium, usually to a final density of 10^8 $c.f.u..cm^{-3}$, in 250 cm^3

flanged Erlenmeyer flasks and incubated at 37°C on an orbital incubator. Aliquots (3 cm^3) were removed at intervals into sterile pre-cooled test tubes (100 x 12.5 mm) and stored on crushed ice.

Samples (0.1 cm^3) were transferred to 9.9 cm^3 of . medium, before the extinctions were read at 600 nm. Serial ten-fold dilutions were prepared in PBS and 0.1 cm^3 aliquots were spread over the surface of blood agar in three petri dishes and incubated at 37°C for 18h. After incubation, colonies were counted and the viable count expressed as **c.f.u..cm^{-3} of the original sample.**

B.4. <u>Deoxyribonucleic Acid Assay</u>. After the E_{600nm} was recorded, the samples were centrifuged in an MSE bench centrifuge at 2,000g for 20 min, and intracellular DNA was assayed by a modification of the diphenylamine reaction of Burton (1956), as follows:

Reagents: 1. Perchloric Acid ($HClO_4$) :

1 M solution in distilled water.

2. Diphenylamine Reagent :

Diphenylamine (Emanuel Ltd.) was dissolved in glacial acetic acid (Analar), containing 0.17M H_2SO_4 (conc.), to a final concentration of 0.08 M. This reagent was prepared within 24h of use and stored in the dark at 4°C. Immediately before use, 0.5 cm^3 of 0.35M aqueous acetaldehyde was added to each 100 cm^3 of reagent.

<u>Method</u>: 1. 3.0 cm^3 $HClO_4$ was added to the pellet from each 3 cm^3 aliquot to a final concentration of 0.25 M. The pellet was resuspended and then incubated at 4°C for 30 min. At the end of the incubation the sample was centrifuged as before and the supernatant discarded. The pellet was washed twice with 0.25 M $HClO_4$.

2. The deposit was stirred with 0.5 cm^3 of 0.5 M $HClO_4$ and then a

further 3.5 cm^3 of 0.5 M $HClO_4$ was added. The suspension was incubated at 70°C for 15 min with shaking. At the end of the incubation, the material was again centrifuged and the supernate decanted.

3. Step 2 was repeated and the supernates pooled.

4. 2 cm^3 of supernate were mixed with 2 cm^3 of diphenylamine reagent and incubated at 30°C for 18h during which time a blue colour developed.

5. After incubation, the Extinction was read at 600 nm in an SP600 (Pye Unicam Ltd.). The spectrophotometer was zeroed against diphenylamine reagent incubated with distilled water.

N.B. Where it was suspected that the DNA concentration would be in excess of 16.7 $\mu g.cm^{-3}$; the DNA extract was diluted with 0.5M perchloric acid before the 2 cm^3 sample was removed for step 4. Dilution at this stage was employed because it proved impossible to dilute the material after the reaction had been completed.

For each run, a standard curve was constructed by dissolving DNA (ex herring sperm, Koch-Light) in 2.0 cm^3 of 0.5 M perchloric acid at concentrations of 1,7, 3.4, 6.8, 8.3, 13.3 and 16.7 $\mu g\ cm^{-3}$. This was incubated at 70°C for 15 min and then treated as for the test samples at step 4.

B.5. Analysis of Extracellular Proteins

Products were assayed by tube or plate methods.

B.5.1. Tube Titrations. The supernates obtained above were used to prepare serial doubling dilutions in PBSA (PBS containing 0.1% Bovine Serum Albumin). After dilution, the appropriate reagent was added, the titration incubated and the end-point determined, as described individually below. Each sample was titrated in triplicate and controls without supernate were included.

For large experiments, titrations were carried out using 0.2 cm^3 volumes in bio-assay dishes (Nunc, Algade, Denmark), and dilutions were prepared using automatic pipette syringes (Baird & Tatlock, Chadwell Heath, Essex). For small experiments and for more critical estimations titrations were carried out using 0.5 cm^3 volumes in 100 x 12.5 mm test tubes. All solutions used contained 0.25 µM Thiomersalate (Analar - B.D.H.).

B.5.1.a. Haemolysin. Rabbit blood was obtained by bleeding from the marginal ear vein into 180 mM sodium citrate (Analar) as anticoagulant. Sheep blood in Alsever's solution and defibrinated horse blood were obtained from Oxoid Ltd. Citrated human blood, group 'O', was obtained from the Haematology Department, Western Infirmary, Glasgow. Erythrocytes were washed three times in PBS and standard suspensions were prepared by adding 1 cm^3 of packed cells to 99 cm^3 of PBSA.

Equal volumes of 1% erythrocyte suspensions were added to the two-fold dilution of the supernate, mixed and incubated at 37°C for 1 h. The end-point was determined, visually, as the last dilution which showed an approximate 50% haemolysis. More accurate endpoints were determined by retitrating the highest dilution to show 100% haemolysis using a series ranging from 0.1 of diluent : 0.9 of the highest dilution to 0.9 of diluent : 0.1 of the highest dilution. After incubation, the mixtures were centrifuged, the extinction of the released haemoglobin read at 540 nm and the dilution causing 50% release, determined by comparison with a standard curve prepared by osmotic lysis of the same blood.

The results were expressed as haemolytic units per cm^3 ($H.U.cm^{-3}$). One $H.U.\ cm^{-3}$ was defined as the reciprocal of the titre showing 50%

haemolysis.

B.5.1.b. Phosphatase. For routine assays, the following method, based on that used by Smith, Blasi and Dayton (1973), was used. Equal volumes of 1% (w/v) phenolphthalein phosphate (Oxoid Ltd.) in PBSA were added to the two-fold dilutions of the supernates, mixed and incubated at 37°C for 1h. The end point was determined by adding an equal volume of 1M ammonium hydroxide (Analar) and recording the last dilution to show an obvious pink colouration. More precise results were obtained by enzyme assay at pH 6.1 using the method described below (p.76).

B.5.1.c. Coagulase. Equal volumes of 20% human plasma in PBSA were added to the two-fold dilutions of the supernate, mixed and incubated at 37°C for 5h. The end point was taken as the last dilution, showing an obvious clot.

B.5.1.d. Gelatinase. Equal volumes of 5% gelatin (Oxoid Ltd.) in PBSA at 37°C were added to the two-fold dilutions of the supernate, mixed and incubated at 37°C for 5h. The end point was taken as the last dilution, showing sloppiness after storage for 2h at 4°C.

B.5.1.e. Staphylokinase. Equal volumes of 20% human plasma in PBSA clotted by purified coagulase were added to the two-fold dilutions of the supernate, mixed and incubated at 37°C for 5h. The end-point was taken as the last dilution showing dissolution of the clot.

B.5.2. Plate Assay. Some extracellular products were estimated by putting appropriate dilutions of the supernate into 0.5mm wells cut in agar plates. The plates were prepared by pipetting 20 cm^3 of the media listed below into 9 cm petri dishes. Six wells were cut in each plate which was used 18-24 h after pouring. The diameter of the zone of reaction around the wells after 18h at 37°C was used as a measure of activity. Each sample was estimated six times (two determinations on each of three plates).

B.5.2.a. Staphylokinase. Staphylokinase activity was determined on staphylokinase agar (p.83) containing 0.25 μM Thiomersalate. Clearing of the precipitated plasma around the well was used as a measure of activity.

B.5.2.b. Nuclease. Nuclease activity was determined on DNase agar (Oxoid Ltd.) contining 0.25 μM Thiomersalate. After incubation, the plates were flooded with 5M hydrochloric acid to precipitate the DNA and clear zones were produced where nuclease had degraded the DNA.

B.5.2.c. Caseinase. Caseinase activity was determined on skimmed milk agar (p.83) containing 0.25 μM Thiomersalate. Clearing of the milky area around the well was used as a measure of activity.

C. EFFECT OF ANTIMETABOLITES ON GROWTH AND EXTRA-CELLULAR PROTEIN PRODUCTION.

Various potential antimetabolites, listed on Table 4, were added to cultures growing in liquid media and the effect on growth parameters and extracellular protein production assessed. The reagents were prepared in distilled water at 100 times the concentrations listed in the table and sterilised by membrane filtration. The volume added to each flask was calculated such that the final concentration was equal to that shown in the table and control flasks received am equal volume of sterile distilled water. Controls, containing known positives pre-treated with reagent and reagent blanks, were included in all assays for extracellular products to ensure that any effects were due to changes in production rather than to post-production modification.

TABLE 4

Potential Antimetabolites Used in This Study.

Antimetabolite	Source	Final concentration
Phenethyl Alcohol	Emanuel, Wembley, Middlesex	7.5 - 45 mM
Phenethylamine	"	15 mM
Secondary-PEA	"	15 mM
Phenoxyethyl Alcohol	"	15 mM
Phenpropyl Alcohol	"	15 mM
Phenethyl Chloride	"	15 mM
Phenethyl Bromide	"	15 mM
DL-Ortho-Flurophenylalanine	Sigma, London	160 µM*
DL-meta-Flurophenylalanine	"	160 µM*
DL-para-Flurophenylalanine	"	160 µM*
Actinomycin D	"	0.8 µM - 8 µM
Mitomycin C	"	3 µM
Chloramphenicol	Parke-Davis, London	30 µM - 80 µM
Phenyl Acetic Acid	Koch-Light, Colnbrook Bucks	15 mM
Acriflavine	"	80 µM
Sodium Dodecyl Sulfate	B.D.H.	70 µM - 140 µM
Sodium Nitrite	"	10 - 30 mM
Potassium Ferricyanide	"	5 - 30 mM
Sodium Thiosulphate	"	10 - 30 mM
Sodium Azide	"	1 - 25 mM
Potassium Cyanide	"	1 - 5 mM
2,4 - Dinitrophenol	"	1 - 10 mM

* Equivalent to 80 µM of L forms

D. EFFECT OF PHENETHYL ALCOHOL AND DINTROPHENOL ON UPTAKE OF RADIOLABELLED AMINO ACIDS.

D.1. Preparation of Cell Suspensions. Cells from overnight culture in 100 cm^3 of BS or HIB medium were washed, resuspended in PBS (pH 7.0) and incubated at 37^{o}C in a water bath, to induce starved conditions.

D.2. Uptake Conditions.

D.2.1. Amino Acids. The amino acids chosen for study were obtained from the Radiochemical Centre (Amersham, Bucks). All were labelled uniformly with Carbon-14 at the stated activities : Glycine (40 $\mu Ci.mM^{-1}$), L-Alanine (171 $\mu Ci.mM^{-1}$), L-Glutamic Acid (285 $\mu Ci.mM^{-1}$), L-Lysine (342 $\mu Ci.mM^{-1}$), L-Histidine (59 $\mu Ci.mM^{-1}$).

D.2.2. Assay. Cell suspensions (20 cm^3) were presented at zero time with the amino acid at a final activity of 1 $\mu Ci.cm^{-3}$, incubated at 37^{o}C and sampled (1 cm^3) over a 20 min period. Samples were rapidly filtered through 0.45 µm Millipore filters which had been presoaked in the appropriate unlabelled amino acid (100 mM) to prevent non-specific binding. Cells on the filter were washed with 10 cm^3 of the unlabelled amino acid solution to remove exogenous label, and filters were then removed and radioactivity (counts per minute - c.p.m.) determined in a Hewlet-Packard Tri-Carb Scintillation Counter using 3 cm^3 Pico Fluor (Packard) as scintillant.

D.3. Effect of Inhibitors. Suspensions were treated as above, except that phenethyl alcohol (15 mM) or 2,4 Dinitrophenol (1 mM) was added at 6 minutes.

E. LOCALISATION OF PHOSPHATASE ACTIVITY

Cells from overnight HIB medium were analysed, as detailed in Figure 2, by the following methods. Alpha-haemolysin activity was also assessed.

E.1. Phosphatase Activity over pH range 5-9. Enzyme activity of each sample was determined by a standard assay procedure which varied only in the buffering used to obtain the different pH values.

E.1.1. Buffering. In initial experiments, buffering in the pH range 5-6 was obtained using 20mM acetate buffer and in range 6-9 using 20mM TRIS buffer. In later experiments, buffering across the whole range was obtained using 20mM Veronal Acetate buffer. No difference was detected between the two buffering systems but the latter was more convenient.

E.1.2. Substrate 4-Nitrophenylphosphate (Sigma) was prepared at 2mM in distilled water.

E.1.3. Assay Reaction tubes containing 1.0 cm^3 buffer and 0.3 cm^3 substrate were prepared and equilibrated for 10 min in a water bath at 37^oC. At zero time 0.2 cm^3 of sample were added, mixed and incubated for 30 min. A control blank composed of 1.3 cm^3 buffered substrate, plus 0.2 cm^3 fresh medium in place of sample was included in all assays. The reaction was stopped by the addition of 1.5 cm^3 of 1M Na_2CO_3, which also served to intensify the colour of the released 4-Nitrophenol anion. Colour intensity was read at 405 n.m., against the blank, in an SP600 spectrophotometer (Pye Unicam).

E.2. Preparation and Lysis of Protoplasts. Protoplasting was carried

Figure 2

Flow diagram to show fractionation stages for localization of phosphatase activity.

Fractionation Step	Fraction	Examination
Cells from 100cm^3 culture.		
11,000g x 10 min	Supernatant	pH Range of Activity
pellet	Whole cells	Electron Microscopy Diffusion from cells
11,000g x 10 min	first wash	pH Range of Activity
pellet	Whole cells	Electron Microscopy
11,000g x 10 min	second wash	pH Range of Activity
pellet	Whole cells	Electron Microscopy
Protoplasting (Lysostaphin)		
Protoplast Suspension	Whole cell material	pH Range of Activity
15,000g x 15 min	Supernatant (Wall debris and periplasm contents)	"
Protoplast pellet	Whole protoplasts	"
15,000g x 15 min	Protoplast wash	"
Protoplast pellet	Whole protoplasts	"
Lysis of protoplasts by:		
(a) Triton X-100	Lysed protoplasts	"
or (b) Osmotic Shock	"	"
15,000g x 45 min	Supernatant (Soluble fraction)	"
pellet	Insoluble fraction (mainly membrane)	"

All centrifugations were carried out on an MSE High Speed 18.

out by a method based on that described by McNiven and Arbuthnott (1972). Cells from 100 cm^3 of liquid medium were washed three times in distilled water and resuspended in 10 cm^3 of 50mM TRIS buffer (pH 7.5) containing 134 mM NaCl, 0.5M Sucrose and 200 units of Lysostaphin (Sigma) and incubated at 37°C for 60 min. After incubation the protoplasts were removed from the mixture by centrifugation at 15,000g for 15 min. When intact protoplasts were to be assayed for phosphatase activity, the pellet was resuspended into distilled water containing 0.5M Sucrose. Lysed protoplast preparations were made by resuspending in the absence of sucrose and subjecting the suspension to two 30 sec. bursts of ultra-sound (MSE, Crawley, Sussex). Alternately, the protoplast pellet was resuspended in distilled water and membranes were solubilised by the addition of 0.1 cm^3 of Triton X-100 to 100 cm^3 of suspension. Progress of protoplasting and lysis was followed by phase-contrast microscopy using a Microsystem 70 microscope (Watson, Barnet, Herts).

E.3. Electron Microscopy. Localisation of acid phosphatase activity within the cell was carried out by the following method, based on that described by Cheng *et al.* (1970). Cells from 100 cm^3 overnight culture in HIB were harvested by centrifugation and either processed directly or washed once or twice with 20 mM veronal acetate buffer (V.A.B.) before processing as follows. The cells were resuspended in 50 cm^3 of a primary reaction mixture of the following composition : Sodium β glycero-phosphate, 25 mM; Sodium Barbitol, 20 mM; $Ca(NO_3)_2$, 20 mM; Mg Cl_2, 10 mM; in 10 mM Citrate buffer, pH 6.1; and incubated at 23°C for 30 min. After incubation, the cells were removed from the mixture by centrifugation at 4°C, resuspended in 20 cm^3 of 65 mM $Pb(NO_3)_2$ and incubated at 23°C for

10 min. The purpose of these steps is for phosphatase to generate free phosphate from the glycerophosphate. This then reacts with the $Pb(NO_3)_2$ to form $Pb_2(PO_4)_3$ which is insoluble, so that it precipitates out at the site of the phosphatase. Being electron dense, the lead will be detectable by electron microscopy as black areas. After incubation, the cells were removed by centrifugation at $4^{o}C$, washed once in VAB and fixed in gluteraldehyde, 15 mM, in VAB for 2h. After fixing, the cells were removed by centrifugation at $4^{o}C$ and resuspended in 1 cm^3 2% agar in VAB at $46^{o}C$. The agar was allowed to set and then cores were made and washed 5 times with VAB for 10 min. Cores were resuspended in 30 mM osmium tetroxide containing 7 mM $MgCl_2$ and then again washed 5 times with VAB. Cores were dehydrated by 30 min passages through increasing acetone concentration. Dried cores were soaked for 2h in 1:1 solution of SPURR:Ethanol then in SPURR and finally embedded in SPURR by incubation at $60^{o}C$ for 16h. Cores were sectioned, stained with uranyl acetate and lead acetate and examined by Transmission Electron Microscopy.

E.4. Diffusion of Phosphatase Activity from Intact Cells. Cells from 100 cm^3 of an 18h culture were resuspended into the same volume of fresh medium at $4^{o}C$ and maintained at this temperature for 7h. Aliquots (1 cm^3) were removed at intervals and assayed for extracellular phosphatase activity and cell number.

F. PRODUCTION, ISOLATION AND CHARACTERISATION OF VARIANTS MUTANT IN EXTRACELLULAR PROTEIN PRODUCTION.

F.1. Mutation by U.V. Irradiation.

F.1.1. Preparation of Staph. aureus Suspensions. Heavily inoculated slope cultures were incubated at 37°C for 18 and 48h. After incubation, the growth was resuspended in 5.0 cm^3 of saline (0.85% physiological saline) by agitating on a Whirlimixer (Fisons, Loughborough, Leic.) for two min. The suspensions were standardized to a viable count of approximately 1.10^8 c.f.u. cm^{-3} by adjustment to an E_{600} of 0.4 previously calibrated against known suspensions. Cells from broth cultures were used in some experiments. These were prepared as described above (p.68), and were incubated in BS medium for 18h at 37°C on an orbital incubator. Cells were harvested and washed in physiological saline, and standardised as above.

F.1.2. Mutagenic Procedure. Aliquots (2 or 5 cm^3) of Staph. aureus suspensions were exposed, with periodic agitation, in open glass petri-dishes, to a 2537AU, model II, 30 cm, 7W, UV lamp (Hanovia, Slough, Bucks) at a distance of 60 cm. At intervals, samples were removed and the viable count was calculated. Suspensions were stores at 4°C in the dark, to prevent photo-reactivation, until the viable counts were known.

F.2. Mutation by Chemical Agents.

F.2.1. General Procedure. Cells were grown up in BS medium, harvested and washed as described above (F.1.1) and finally resuspended and treated with mutagen, as described individually below. All mutagens were sterilised by membrane filtration just before use. After treatment with mutagen, the cells were centrifuged at 2000g and washed twice in PBS. The organisms were resuspended to their original concentration in PBS and viable counts were determined. The suspensions were stored at 4°C in the dark, so that their post-treatment conditions matched those of the UV treated cells, until the viable counts were known.

F.2.2. N-Methyl-N'-Nitro-N-Nitroso Guanidine (MNNG). Cells were resuspended in 5 cm^3 physiological saline containing 20 μg cm^{-3} of MNNG (Sigma) at pH 5.5 and incubated at 37°C for 10 min.

F.2.3. 5 Bromo Uracil (BU) or 2,6 Aminopurine (AP). Cells were resuspended in 5 cm^3 Lab Lemco Broth containing 100 μg.cm^{-3} of either BU or AP (both Sigma) and incubated at 37°C for 30 min.

F.2.4. Nitrous Acid (NA). Cells were resuspended in 5 cm^3 of 5M Sodium Nitrite and the pH reduced to 4.5 by the addition of 5M Hydrochloric acid. The mixture was incubated at 37°C and the reaction was stopped after 10 min by restoring the pH to 7.0 with 10M Sodium Hydroxide.

F.2.5. Ethyl Methane Sulphonate (EMS). The method was based on that described by Van Der Vijver et al. (1975a); One cm^3 of EMS (Methane Sulfonic Acid Ethyl Ester - Sigma) was dissolved in 24cm^3 of working solution (6%v/v) of the EMS stock salts ($(NH_4)_2Fe(SO_4)_2.6H_2O$,2.8mg; $MgSO_4.7H_2O$,20.0mg; NH_4Cl,800mg; KH_2PO_4,1.8g; K_2HPO_4,3.0g:in 100cm^3 distilled water)
Cells were resuspended in 5 cm^3 of this solution, incubated at 37°C for 10 min and the reaction was then stopped by neutralising the EMS with 5 cm^3 of M Sodium Thiosulphate.

F.3. Examination of Mutant Populations.

F.3.1. Isolation of Mutant Clones. After the determination of viable counts, the mutant suspension was diluted to yield 500-1000 c.f.u. cm^{-3}. Aliquots (0.1 cm^3) were spread onto 3% sheep blood agar plates and incubated at 37°C for 18h. The concentration of 3% sheep erythrocytes was chosen to eliminate the possibility of selecting 'leaky' mutants which might have been masked by higher concentrations of erythrocytes. Spontaneous non-haemolytic mutants, isolated during other experiments, were also examined.

Non-haemolytic colonies were subcultured on fresh 3% sheep blood agar plates to check for stability of the mutation. The colonies were examined by Gram stain, growth and fermentation on Mannitol-Salt-Agar (M.S.A.-Oxoid), and by the oxidative-fermentative test of Hugh & Leifson (1953) using glucose as the carbohydrate. Isolates grown on nutrient agar slopes were checked for catalase production using 1M hydrogen peroxide (May and Baker Ltd., Dagenham, Essex). Only Gram positive, catalase positive cocci, displaying a fermentative metabolism and producing acid from mannitol on the MSA plate, were retained.

F.3.2. <u>Characterisation of Mutants.</u> Each mutant was characterised by the following procedure. An isolated colony from the MSA plate was inoculated into 5.0 cm^3 of BS medium. This was incubated for 6h at 37°C and the cells were removed, washed twice and resuspended in 10 cm^3 of sterile saline. The supernates were titrated for haemolytic activity and the suspensions were used to inoculate the media listed below:

<u>Blood Agar</u> : Sheep blood, in Alsever's solution, or defibrinated horse blood (Oxoid) was washed three times in PBS and resuspended to its original volume in physiological saline. This was used to produce blood agar by addition to molten sterile blood agar base (Oxoid) at

46^{o}C to a final concentration of 10% (v/v). Haemolysis was detected as clearing of the blood around the colony.

Staphylokinase Agar : Human plasma (Haematology Dept., Western Infirmary, Glasgow) was added to molten sterile blood agar base at 56^{o}C to a final concentration of 12% (v/v). The mixture was held at 56^{o}C for 30 min to precipitate the fibrin and then used to pour plates. Staphylokinase activity was detected as clearing of the opaque medium around the colony.

Skimmed Milk Agar : Skimmed milk powder (Oxoid) was made up to a 10% (w/v) solution in distilled water and sterilised by autoclaving for 5 min at 1 kgf.cm^{-2}. This was added to molten sterile blood agar base at 56^{o}C, to a final concentration of 10% (v/v). Casein hydrolysis was detected as clearing of the opaque medium around the colony.

Gelatin Agar : Gelatin (Oxoid) was added to molten blood agar base to a final concentration of 5% (w/v) and sterilised by autoclaving at 1 kgf.cm^{-2} for 15 min. Gelatin hydrolysis was detected, after flooding the plates with acidic mercuric chloride, as a clear zone around the colony.

Egg-Yolk Agar : Concentrated egg yolk emulsion (Oxoid), to a final concentration of 10% (v/v) and filter sterilised dextrose (Analar - B.D.H.), to a final concentration of 1% (v/v) (Gillespie and Alder, 1952), were added to molten sterile blood agar base containing 10 grams sodium chloride per litre. Lipoprotein lipase activity was detected as a zone of opalescence surrounding the colony.

Phenolphthalein Phosphate Agar : Sterile 1% solutions of phenolphthalein phosphate (Oxoid) were added to molten sterile blood agar base to give a final concentration of 0.01%. Phosphatase activity was detected by the appearance of a pink colouration following exposure of colonies to ammonia vapour.

<u>DNase Agar</u> : This was obtained from Oxoid Ltd. and prepared as directed.

All plates were incubated at 37°C for 24h. Gelatinase activity was also assessed in preliminary experiments by stab inoculation into nutrient gelatin deeps (Oxoid) followed by incubation at 22°C for 7 days. However, plate assays were found to be more reliable and sensitive.

Coagulase activity was detected by adding 5 drops of the cell suspension to 0.5 cm^3 of a 1:5 dilution of human plasma in saline. The time of clot formation at 37°C was noted. Isolates which had not clotted the plasma after 24h were regarded as negative for this character.

Carbohydrate fermentation was assessed by inoculating a loopful of the cell suspension into 2.5 cm^3 of 1% carbohydrate solution in peptone water and examined for acid and gas after 18h at 37°C. Mannitol, glucose, sucrose and lactose were tested.

Colonial morphology was examined on nutrient agar plates after 48h using a binocular plate microscope (Watson, Barnet). Cell morphology was checked by Gram stain and phase contrast microscopy and capsule staining was done by the Rose Bengal and Nigrosin method.

F.3.3. <u>Temperature Sensitivity.</u> Since the mutants were isolated at 37°C, it was thought that some of them might be temperature sensitive, so the mutants were streak inoculated onto 3% sheep blood agar plates and incubated at 30°C for 24h. After incubation, the presence or absence of haemolysis was recorded, the plates were reincubated at 37°C for a further 18h and any changes in the haemolytic reactions noted.

F.3.4. <u>Co-operative Effects.</u> Although all mutants were non-haemolytic, the possibility existed that two mutants growing together might cause haemolysis by a cooperative interaction between inactive products. This was investigated by streaking all possible combinations adjacent to each other on 10% sheep blood plates. The wild type was similarly tested against each of the mutants, to see if lack of haemolysis could be due to an extracellular inhibitor produced by the mutant.

F.3.5. Reversion Studies. Reversion to wild type by the non-haemolytic isolates was determined by platimg out 0.1 cm^3 aliquots of an overnight culture in BS medium onto a specially devised medium of the following composition :

SALTS : The following salts were dissolved, in order, in 100 cm^3 of tap water and sterilised by autoclaving at 121°C (1 $kgf.cm^2$); NH_4Cl, 2.0g; NH_4NO_3, 400 mg; K_2HPO_4, 600 mg; KH_2PO_4, 400 mg; Na_2SO_4, 900 mg; $Mg_2SO_4.7H_2O$, 40 mg.

NUTRIENTS : The following ingredients were dissolved in 5 cm^3 of tap water and sterilised by filtration: Nicotinic Acid, 20 mg; Thiamine (Aneurine Hydrochloride), 20 mg; Vitamin-free Casamino acids, 125 mg.

AGAR : Ionagar No. 2, 12.5g and NaCl, 10g were dissolved in 815 cm^3 of distilled water, sterilised by autoclaving, and cooled to 46°C.

BLOOD : Sheep erythrocytes, 80 cm^3 in Alsevers were washed four times in sterile physiological saline and resuspended to their original volume in the same diluent.

All ingredients were B.D.H. except Vitamin-free Casamino Acids (Difco) and Agar (Oxoid). The complete medium was prepared by mixing all of the solutions, and this was used to pour plates.

After inoculation, plates were incubated for 24h at 37°C. The resulting lawns of micro-colonies were examined for evidence of haemolysis and colonies from such areas were sub-cultured onto normal sheep blood agar. Subcultures were incubated as before and haemolytic colonies from these plates were sub-cultured again to confirm stability of revertants.

F.3.6. Replica Plating Studies. Aliquots (0.1 cm^3) of UV irradiated suspensions (500-1000 $c.f.u.cm^{-3}$) were spread on sheep blood agar plates and incubated for 12h at 37°C. After incubation, these "Master" plates were replicated by the following method, based on that devised by Lederberg and Lederberg (1952). An orientation mark was made on the base of the Master plate and the plate was then lined up with a similar mark on the ring which was securing a sterile velvet pad to an 8.5 cm diameter wooden block. The master plate was gently pressed onto the surface of the velvet, thereby transferring some of each colony to the velvet. Plates of the other media were similarly marked, orientated and pressed onto the velvet so they they were imprinted with the colonies. In most experiments, replicas were made on staphylokinase and sheep blood agar plates, but in some cases, egg yolk agar, gelatinase agar, skimmed milk agar and DNase agar plates were also included. Sheep blood agar was always the last plate to be "printed" as a control of transfer efficiency. The replicas were incubated at 37°C for 18h. After incubation, the colonies were scored for possession of the characters examined.

RESULTS

RESULTS

A. SELECTION OF STRAIN

Six strains of Wood 46 were obtained and tested for haemolytic ability, enzymic activities and fermentation range. Two departmental strains and one NCTC 7121 variant produced very little haemolysis on sheep blood plates and were therefore rejected. The other three strains were positive for all characters tested. Since the main aim of this work was to examine the production of alpha-haemolysin, the haemolytic spectrum of each organism was determined and this is shown in Table 5. NCTC 10344 produced alpha-haemolysin, as defined on Table 1, with little or no haemolytic activity that could be ascribed to other haemolysins so it was chosen for all subsequent work.

B. GROWTH AND EXTRACELLULAR PROTEIN PRODUCTION UNDER NON-INHIBITED CONDITIONS.

Most experiments were carried out in BS medium, but various modifications of this medium were also used as were proprietory media and defined minimal media.

B.1. Growth and Extracellular Protein Production in BS Medium.

BS medium (100 cm^3) was inoculated with 10^8 $c.f.u.cm^{-3}$ and incubated at 37^oC aerobically. Samples were taken at intervals and assayed for growth, DNA and extracellular alpha-haemolysin, acid phosphatase, staphylokinase, gelatinase and coagulase. The results are shown in Figures 3a and 3b.

Figure 3a shows growth, expressed as viable count ($c.f.u.cm^{-3}$) and E_{600}n.m., and DNA ($\mu g.cm^{-3}$). All three show a lag phase of about 30 min, followed by an exponential phase lasting about 3.5 hours

TABLE 5

Comparison of Haemolytic Spectra of Three Wood 46 Isolates

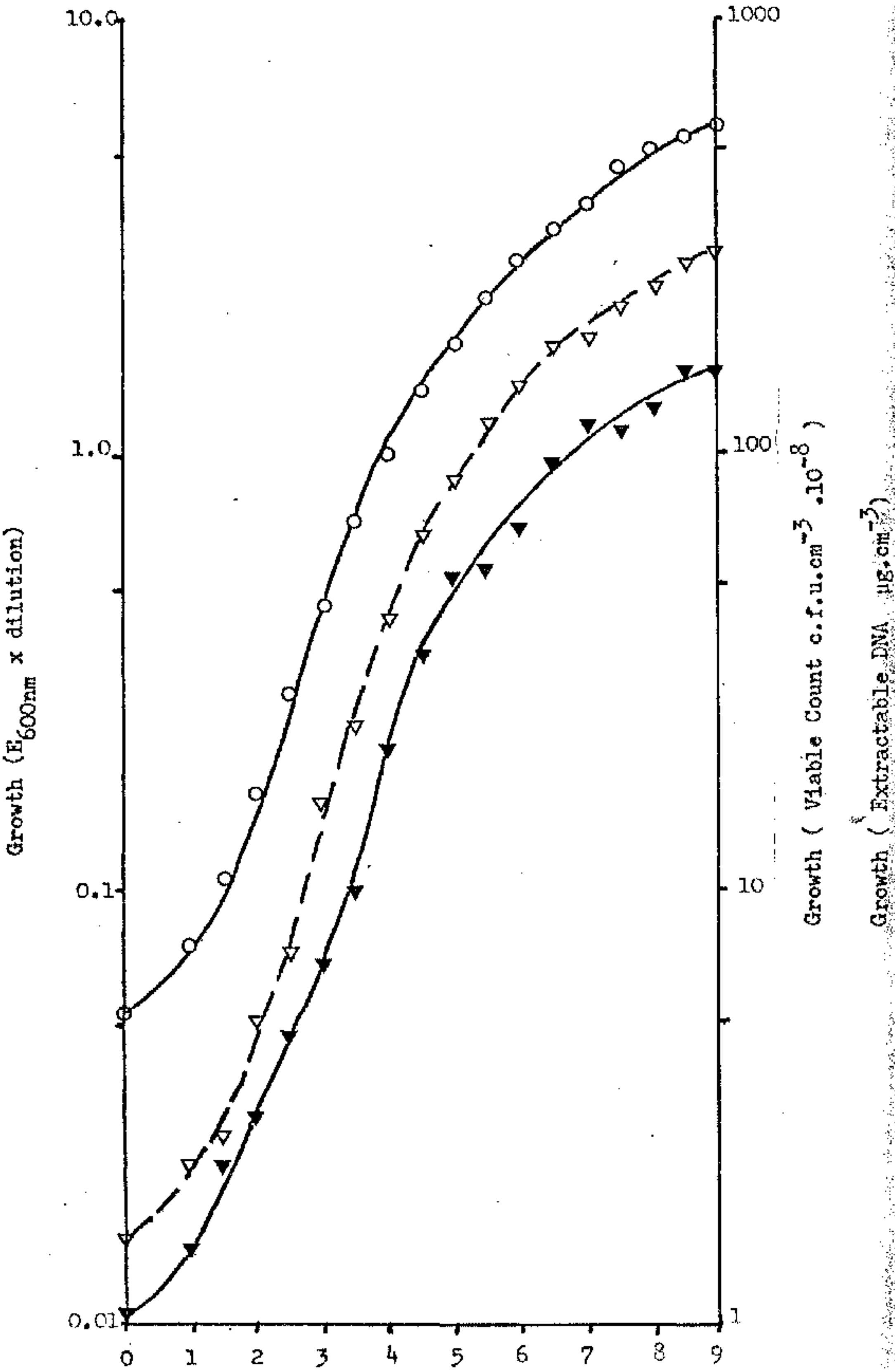
Source of Erythrocytes	NCTC 7121		NCTC 10344		Departmental Stock	
	$H.U.cm^{-3}$	Relative Sensitivity *	$H.U.cm^{-3}$	Relative Sensitivity *	$H.U.cm^{-3}$	Relative Sensitivity *
Rabbit	2048	128	2048	128	1024	64
Sheep	256	16	128	8	64	4
Horse	16	1	16	1	16	1
Human	32	2	16	1	16	1

* Calculated as for Table 1.

Figure 3a

Growth and DNA Production in BS Medium

100 cm^3 of BS medium were inoculated to give 1.10^8 $c.f.u.cm^{-3}$ and incubated at 37^oC and 115 r.p.m. Growth was measured by E_{600nm} (O) and Viable Count ($c.f.u.cm^{-3}$) (▽); DNA was measured by the Burton method (1956) (▼).

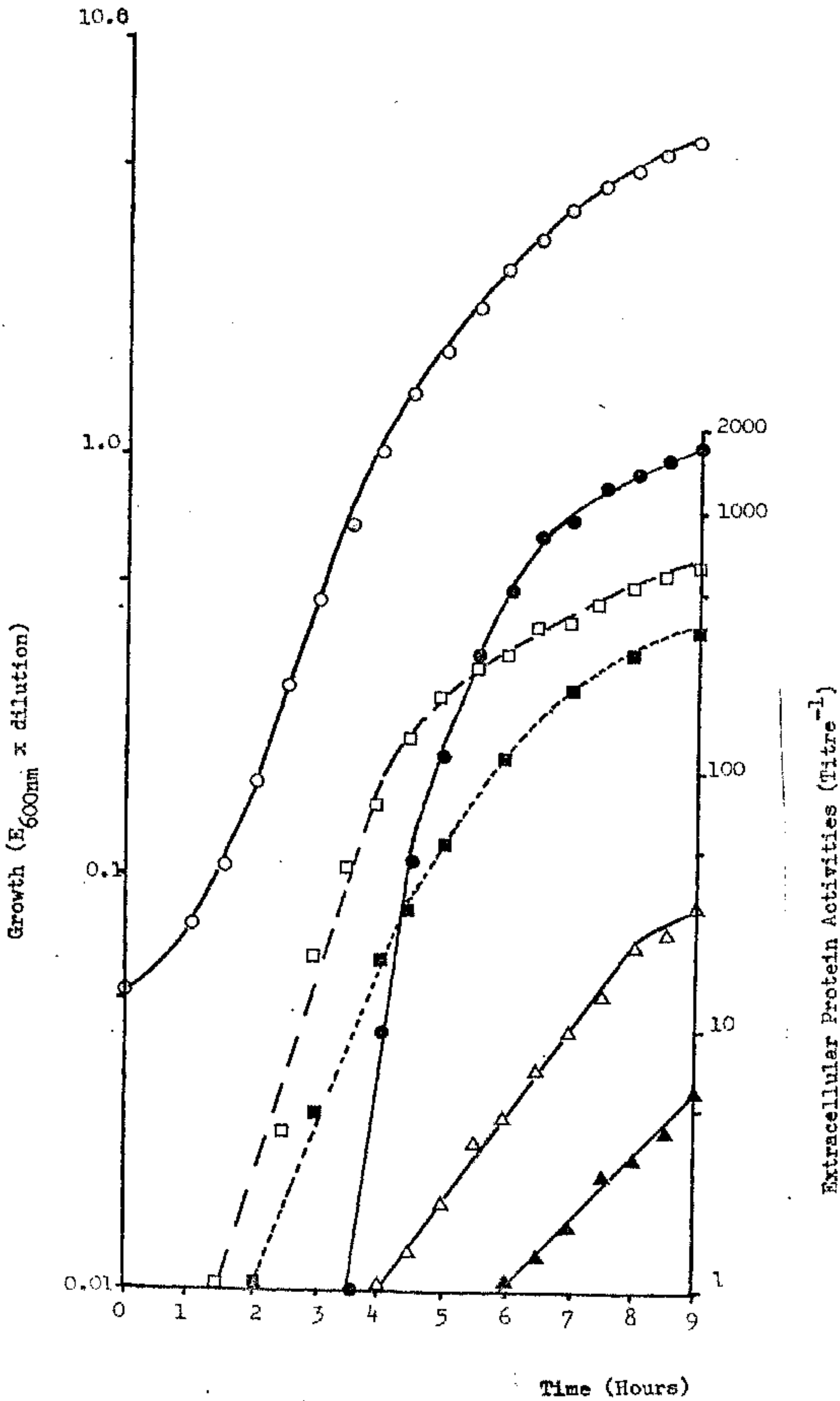


10.0
1.0
0.1
0.01
1000
100
10
1
0
1
2
3
4
5
6
7
8
9
Growth (E_{600nm} x dilution)
Growth (Viable Count c.f.u.cm^{-3} .10^{-8})
Growth (Extractable DNA µg.cm^{-3})
Time (Hours)

Fig. 3b

Production of Various Extracellular Protein Activities During Growth in BS Medium.

Conditions as for Figure 3a. Growth is expressed as E_{600nm} (○). Extracellular protein activities were determined by titration. Alpha-haemolysin (●), acid phosphatase (□), staphylokinase (■), gelatinase (△), coagulase (▲).



10.0
1.0
0.1
0.01
Growth (E_{600nm} x dilution)
2000
1000
100
10
1
Extracellular Protein Activities ($Titre^{-1}$)
0
1
2
3
4
5
6
7
8
9
Time (Hours)

followed by a gradual slowing down. Taking E_{600nm} as a standard, it can be seen that the graph of viable count is a little lower than expected during the first 90 min, 'though this may not be significant. The graph of DNA is significantly lower than E_{600nm} during the initial 90 min but the exponential phase is steeper than that displayed by the others so that all three are approximately parallel by about 4 hours.

Figure 3b compares the production of the extracellular proteins with the E_{600nm} results. None of the five protein activities were detectable in the culture medium until growth had become established. Staphylokinase, alpha-haemolysin, coagulase and gelatinase increased rapidly after the rapid exponential phase of growth was over. Acid phosphatase, however, apparently slowed down after an initial rapid increase. When cell-associated activity was assessed, (not shown) acid phosphatase activity paralleled E_{600nm} throughout the growth period, whereas the other four were essentially unchanged. Although all five seemed to appear at different times, this may reflect a failure to detect low levels of activity with the assay systems used rather than a real difference in production times.

B.2. Growth and Extracellular Protein Production in Modified BS Medium.

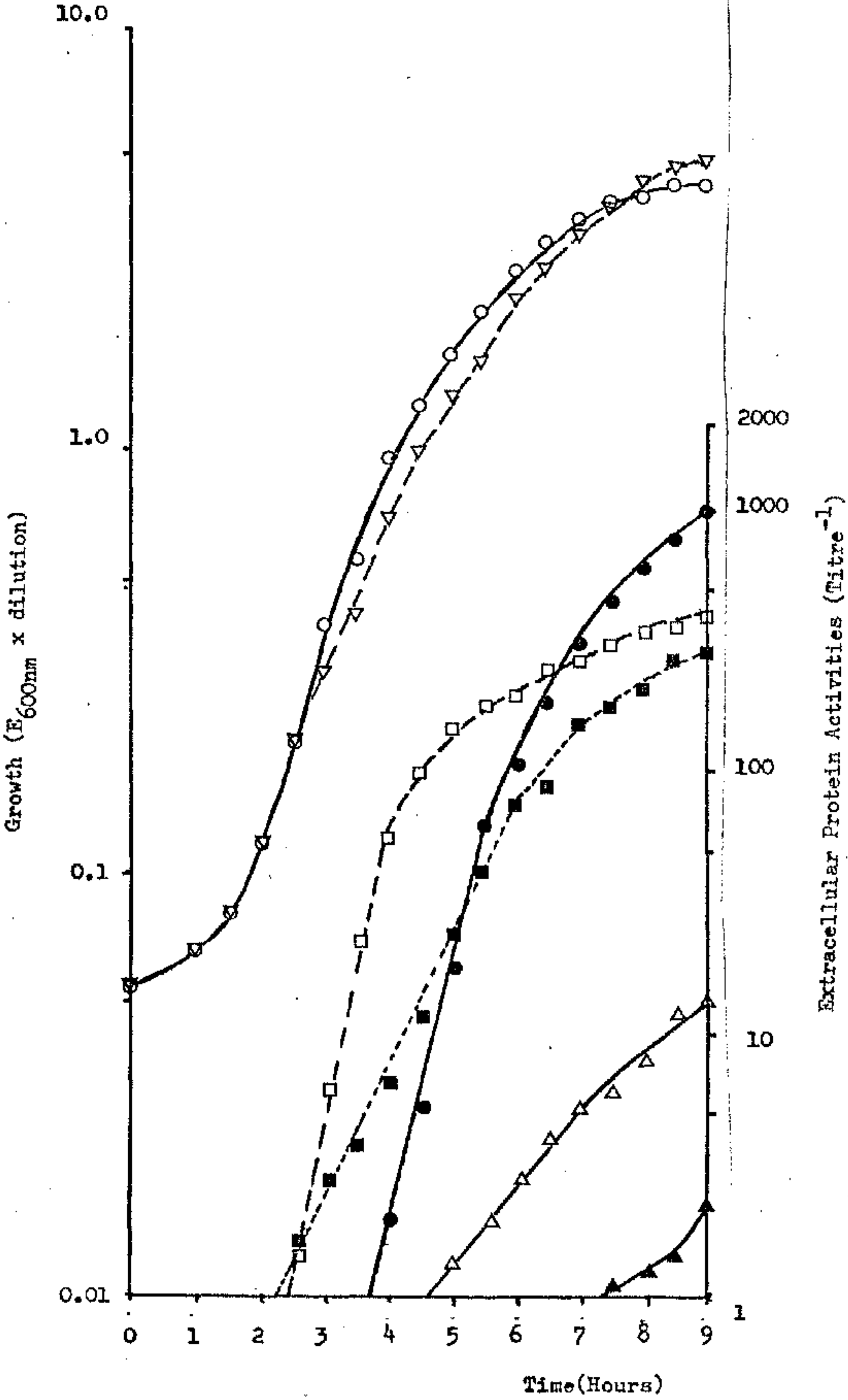
The contributions of the various medium components were investigated by varying the concentrations of the major ingredients and examining the growth rate (E_{600nm}) and assaying for alpha-haemolysin, acid phosphatase, staphylokinase, coagulase and gelatinase.

As shown in Figure 4, up to 50% reduction in either yeast extract dialysate or casamino acids increased the lag phase by about 15 min, but the exponential rate of growth was similar to the control. Entry into the period of slower growth occurred at a lower cell density than controls (E_{600nm} 1.5 rather than 2.0) and this difference was maintained

Figure 4

Growth and Production of Various Extracellular Proteins in Modified BS Medium containing 50% of Normal Yeast Extract Dialysate or 50% of Normal Casamino Acids.

Conditions were the same as those for Figure 3 except that BS medium contained only 50% yeast extract dialysate (½YE) or 50% Casamino Acids (½CAA). Growth is expressed as E_{600nm}: (○) ½ CAA, (▽) ½YE. Assays for extracellular proteins gave essentially the same results for ½ YE or ½ CAA so the mean values only are shown for alpha-haemolysin (●), acid phosphatase (□), staphylokinase (■), gelatinase (△), coagulase (▲).


10.0
1.0
0.1
0.01
Growth (E_{600nm} x dilution)
2000
1000
100
10
1
Extracellular Protein Activities ($Titre^{-1}$)
0
1
2
3
4
5
6
7
8
9
Time(Hours)

into the stationary phase where the final E_{600nm} level was only 70% of control. Activities of all the extracellular proteins were reduced in proportion to the reduction in growth.

Changes in glucose concentration, either increasing from 14mM to 28mM or decreasing to 7mM or 3.5mM, or omitting glucose entirely, had no significant effect on either growth or extracellular protein production.

Reducing the strength of the complete medium, up to 50%, by dilution with distilled water, gave similar results to reduction of yeast extract dialysate or casamino acids alone (Fig. 4). Further dilution gave the results shown in Fig. 5. Dilution up to 80% caused progressive shortening of the exponential phase and entry into the stationary phase at about 7h. Production of acid phosphatase was reduced in line with growth, but the alpha-haemolysin was only detected up to 60% and the amount was very low. The other extracellular activities were not detected above 50% dilution. At 90% dilution, a short exponential phase of no more than three generations was followed by rapid lysis. An increase in extracellular acid phosphatase activity was detected during the period of lysis.

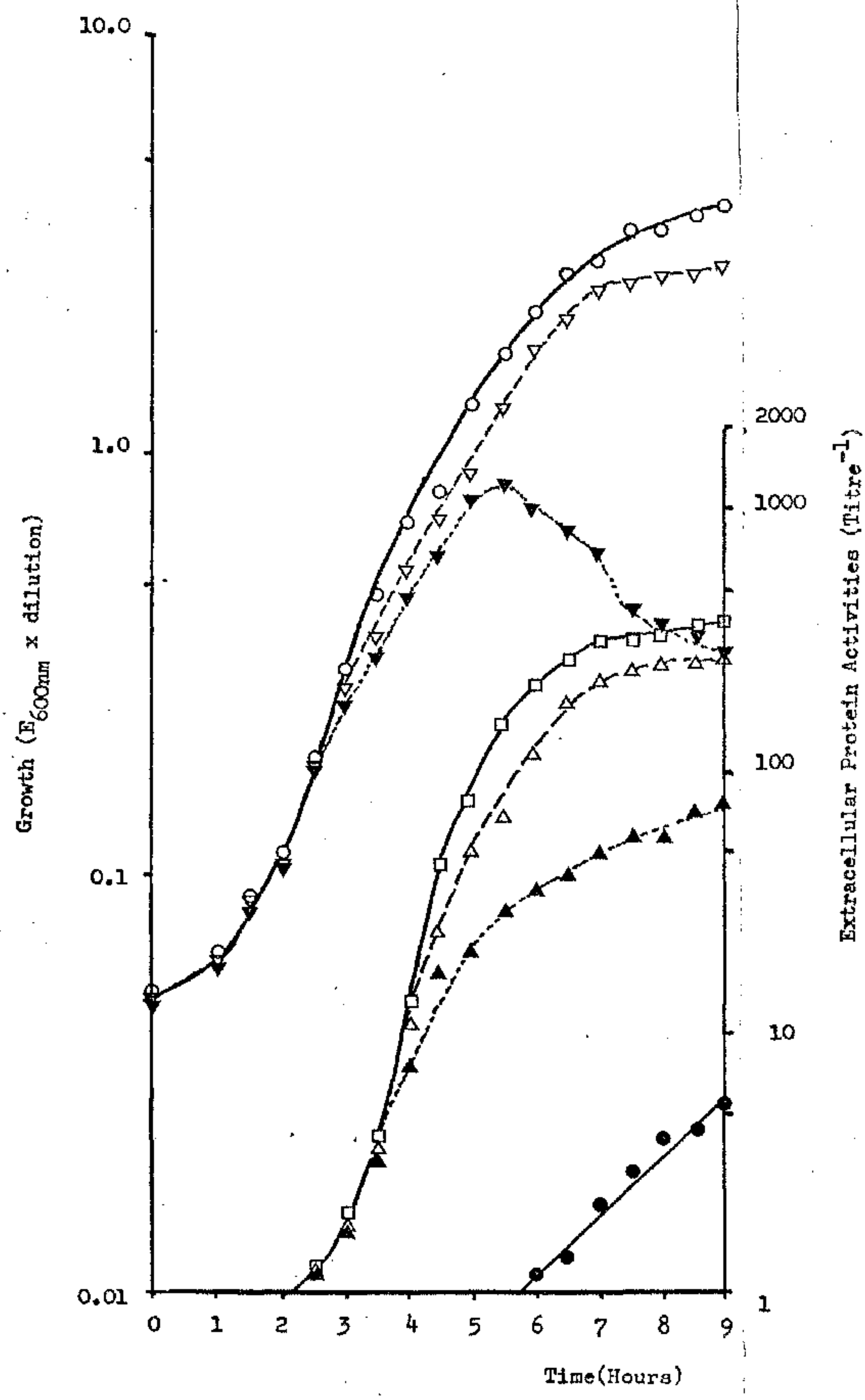
Incorporation of Acetate or Succinate (at 14mM) in place of Glucose, gave the results shown in Figure 6. For acetate grown cultures, the results for growth and extracellular acid phosphatase were very similar to those shown earlier (Fig. 4) for 50% reduction in nutrient, ie. slight extension of lag phase and about 25% reduction in final yield. Alpha-haemolysin production was, however, reduced compared to this earlier result. For succinate grown cultures, the results were more markedly reduced with lag phase being extended about one hour and the rate of growth being slower. Acid phosphatase was reduced in line

Figure 5

Growth and Production of Various Extracellular Proteins in Diluted BS Medium.

Conditions were the same as those for Figure 3 except that the concentration of the BS medium was diluted to 40%, 20% or 10% before inoculation.

Growth is expressed as E_{600nm}: (○) 40%, (▽) 20%, (▼) 10%. Assay for extracellular acid phosphatase :(□) 40%, (△) 20%, (▲) 10%. Assay for alpha-haemolysin : (●) 40%. No alpha-haemolysin was detected at higher dilutions and other protein activities were not detected.



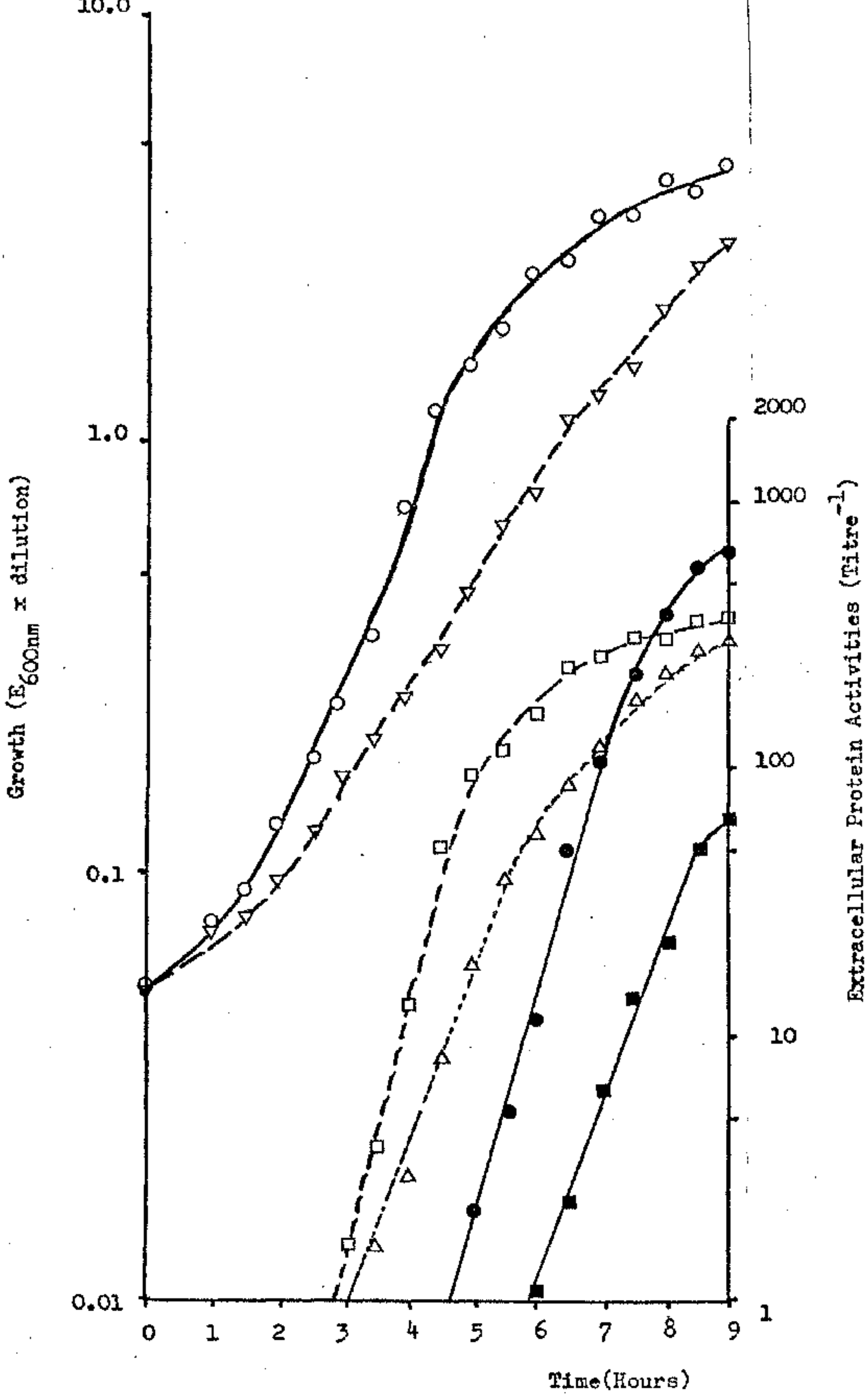
10.0
1.0
0.1
0.01
Growth (E_{600nm} x dilution)
0
1
2
3
4
5
6
7
8
9
Time(Hours)
2000
1000
100
10
1
Extracellular Protein Activities ($Titre^{-1}$)

Figure 6

Growth and Production of Acid Phosphatase and Alpha-haemolysin in BS Medium with Acetate or Succinate in Place of Glucose.

Conditions were the same as those for Figure 3 except that Glucose was replaced by either Acetate or Succinate at the same molarity (14 mM).

Growth is expressed as E_{600nm} (○) Acetate, (▽) Succinate. Assay for extracellular acid phosphatase : (□) Acetate, (△) Succinate. Assay for alpha-haemolysin : (●) Acetate, (■) Succinate.



10.0
1.0
0.1
0.01
Growth (E_{600nm} x dilution)
2000
1000
100
10
1
Extracellular Protein Activities (Titre^{-1})
0
1
2
3
4
5
6
7
8
9
Time(Hours)

with the reduction of growth but production of alpha-haemolysin was delayed by about 2½ hours. Final titre of alpha-haemolysin was about 50% down compared to about 40% reduction in growth yield. Yields of staphylokinase, gelatinase and coagulase (not shown) were reduced in proportion to the alpha-haemolysin. In subsequent experiments, only alpha-haemolysin and acid phosphatase were routinely assayed.

B.3. Growth and Extracellular Protein Production in Proprietory Media.

Proprietory media, prepared as directed by the manufacturers, were used in some experiments. In each case 100 cm^3 of medium was inoculated with 10^8 c.f.u.cm^{-3} and incubated at 37°C, as for BS medium.

B.3.1. Heart Infusion Broth. The pattern of growth and extracellular protein production for this medium was similar to that shown in BS medium (Figure 3).

B.3.2. Trypticase Soya Broth and Nutrient Broth. Growth and extracellular protein production in these media were similar to that shown in Figure 4 for BS medium with 50% reduction in yeast extract dialysate or casamino acids.

B.3.3. Lab Lemco. Growth (E_{600nm}) and extracellular protein production in this medium are shown in Figure 7. Only alpha-haemolysin and acid phosphatase were detected.

Figure 7

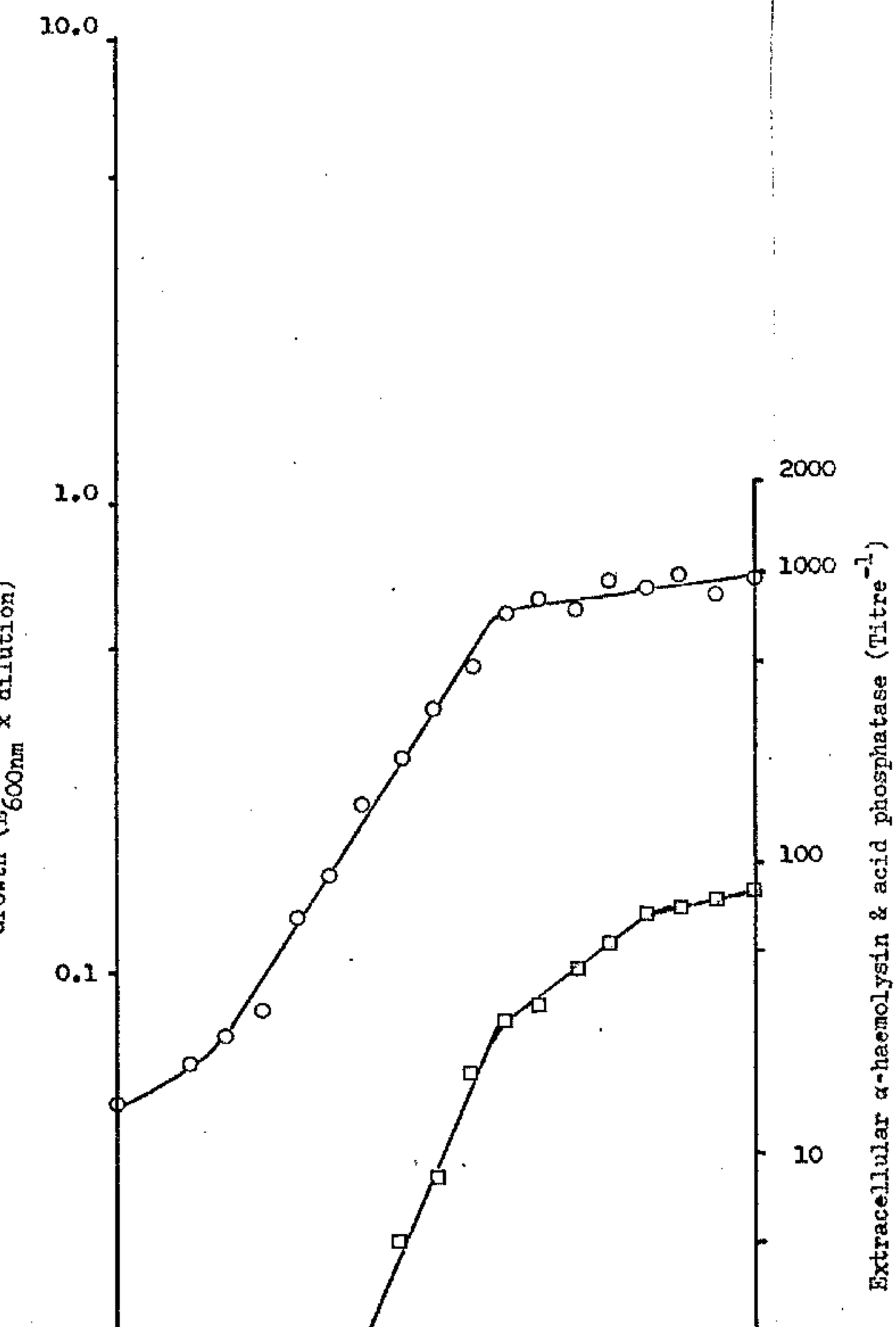
Growth and Extracellular Protein Production in Lab Lemco Broth.

Conditions were the same as those for Figure 3 except that Lab Lemco Broth was used in place of BS Medium.

Growth is expressed as E_{600nm} (○)

Extracellular Acid Phosphatase (□)

Alpha-haemolysin (●)



10.0
1.0
0.1
0.01
Growth (E_{600nm} x dilution)
2000
1000
100
10
1
Extracellular α-haemolysin & acid phosphatase (Titre^{-1})
0
1
2
3
4
5
6
7
8
9
Time(Hours)

B.4. Growth and Extracellular Protein Production in Defined Minimal Medium, With or Without Supplements.

A minimal medium containing salts, glucose, cysteine, histidine, aspartic acid, glycine, nicotinamide and thiamine was devised. This medium, supplemented by other amino acids where stated, was dispensed in 100 cm^3 volumes, inoculated with 10^7 $c.f.u.cm^{-3}$ and incubated at 37^oC aerobically. A lower inoculum was used for these experiments because, as shown on Figure 8, the maximum population density in the minimal medium was about $1.5.10^8$ $c.f.u.cm^{-3}$. In the minimal medium, only acid phosphatase was detected and this was also the case in media supplemented with any two additional amino acids. Supplementing the minimal medium with glutamic acid, glutamine, proline and lysine raised the growth rate considerably and allowed alpha-haemolysin to be produced. Phosphatase levels in the poor media were lower in total than those in the last named medium and, of course, than those in complex media. However, the amount of phosphatase produced in proportion to the amount of cell material (as measured by E_{600nm}) produced was higher for the minimal media, by a factor of ten in some cases.

C. GROWTH AND EXTRACELLULAR PROTEIN PRODUCTION IN BS MEDIUM TREATED WITH VARIOUS POTENTIAL ANTIMETABOLITES.

The effect of various potential antimetabolites on growth and extracellular protein production was investigated by adding reagent to actively growing cultures in BS medium. The majority of the experiments were carried out using phenethyl alcohol since one aim of the work was to determine the mode of action of this compound. Although coagulase, staphylokinase and gelatinase were assayed at least once for all inhibitors, the experiments concentrated on alpha-haemolysin and acid phosphatase.

Figure 8

Growth and Production of Extracellular Acid Phosphatase and Alpha-haemolysin in Defined Media.

100 cm^3 Defined Minimal medium or the same medium plus glutamic acid, glutamine, proline and lysine was inoculated with 10^6 $c.f.u.cm^{-3}$, incubated at 37^oC aerobically and assayed periodically for growth, expressed as E_{600nm}, extracellular acid phosphatase and alpha-haemolysin.

Growth (E_{600nm}) Supplemented medium (○)

Unsupplemented medium (▽)

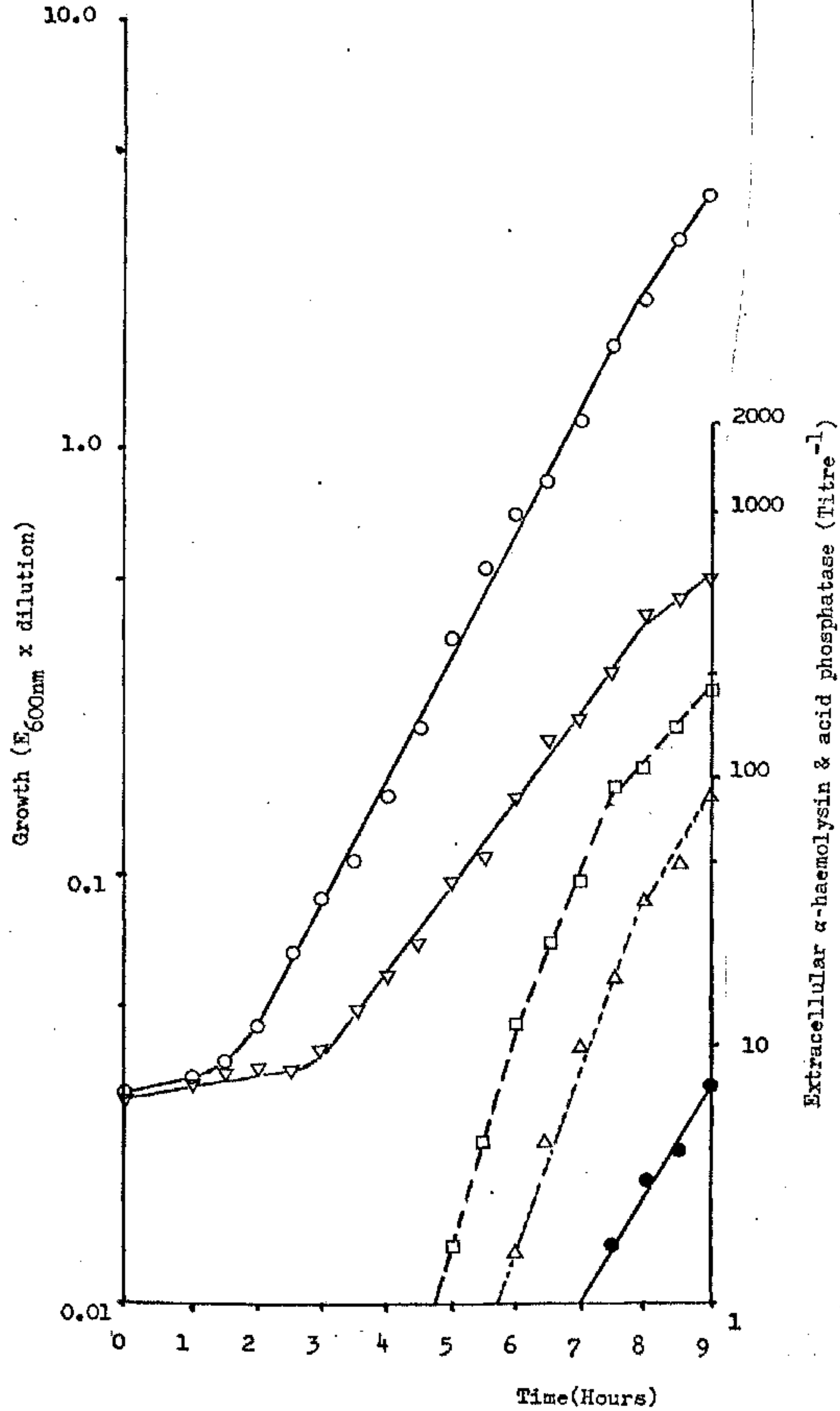
Extracellular Acid Phosphatase

Supplemented medium (□)

Unsupplemented medium (△)

Alpha-haemolysin Supplemented medium (●)

No haemolysin was detected in the minimal medium.


10.0
1.0
0.1
0.01
Growth (E_{600nm} x dilution)
2000
1000
100
10
1
Extracellular α-haemolysin & acid phosphatase (Titre^{-1})
0
1
2
3
4
5
6
7
8
9
Time(Hours)

C.1. Effect of Phenethyl alcohol.

C.1.1. Effect of Varying Phenethyl Alcohol Concentrations. Phenethyl alcohol at different concentrations was added in log phase at 2.5 hours and the effect on growth (E_{600nm}) and extractable DNA ($mg.cm^{-3}$) was assessed, as was the effect on extracellular acid phosphatase and alpha-haemolysin. The results are shown in Figure 9 a, b and c. Increasing concentrations of phenethyl alcohol, up to 30 mM were increasingly inhibitory to rate of growth, as measured by E_{600nm} and rate of increase of DNA. At higher concentrations, complete inhibition of both occurred. The E_{600nm} remained constant for cultures treated with 37.5 or 45.0 mM, but the detectable levels of extractable DNA appeared to fall in these cultures.

Long term effects were examined by either continuing incubation overnight or by spinning the cells out of the treated medium and re-suspending them in fresh medium. Cells treated with up to 22.5 mM phenethyl alcohol continued to grow and eventually reached the same stationary population as the controls. No increase in population was detected in cultures treated with 30 mM phenethyl alcohol over 12h. At higher concentrations, a slight fall of about 20% in E_{600nm} was noted and viable count fell more markedly to about 10% of pretreatment level over 12h. Removal of the cells from the treatment medium by centrifugation followed by washing and resuspension had little effect on the populations treated with up to 30 mM phenethyl alcohol, as shown in Table 6, which also shows the results of washing and resuspension on populations treated with 37.5 or 45 mM phenethyl alcohol, where losses of cellular integrity seemed to take place. Recovery was usually immediate for cultures previously treated with up to 30 mM phenethyl alcohol. At higher concentrations, recovery was considerably delayed;

Figure 9 a

Effect of Varying Concentrations of Phenethylalcohol on Growth Expressed as E_{600nm}

Conditions were the same as for Figure 3 except that varying concentrations of phenethylalcohol were added at 2½h. Control flasks received distilled water.

Control			(○)
15 mM Phenethyl alcohol			(●)
22.5mM	"	"	(◫)
30 mM	"	"	(■)
37.5 mM	"	"	(△)
45 mM	"	"	(▲)

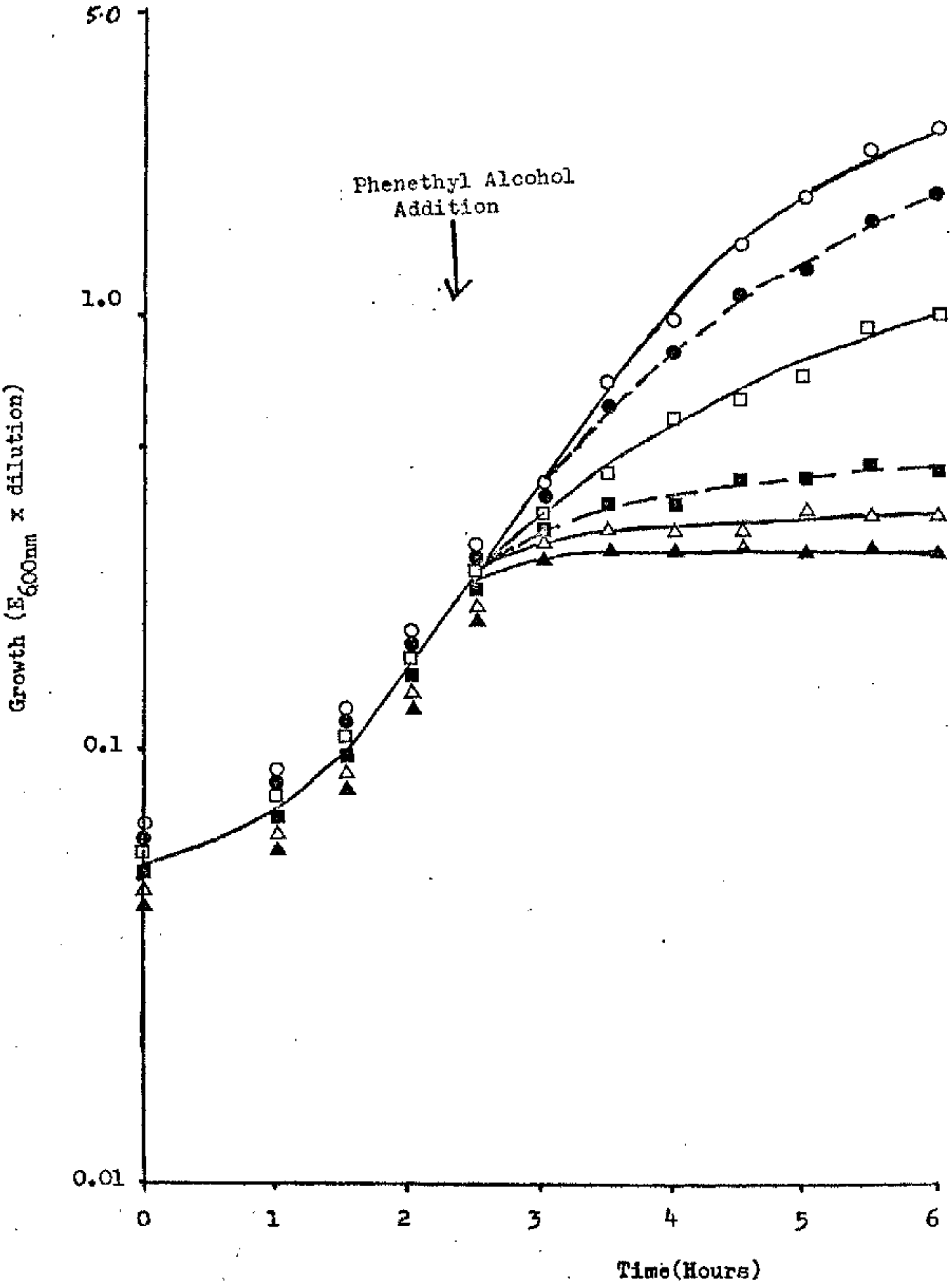
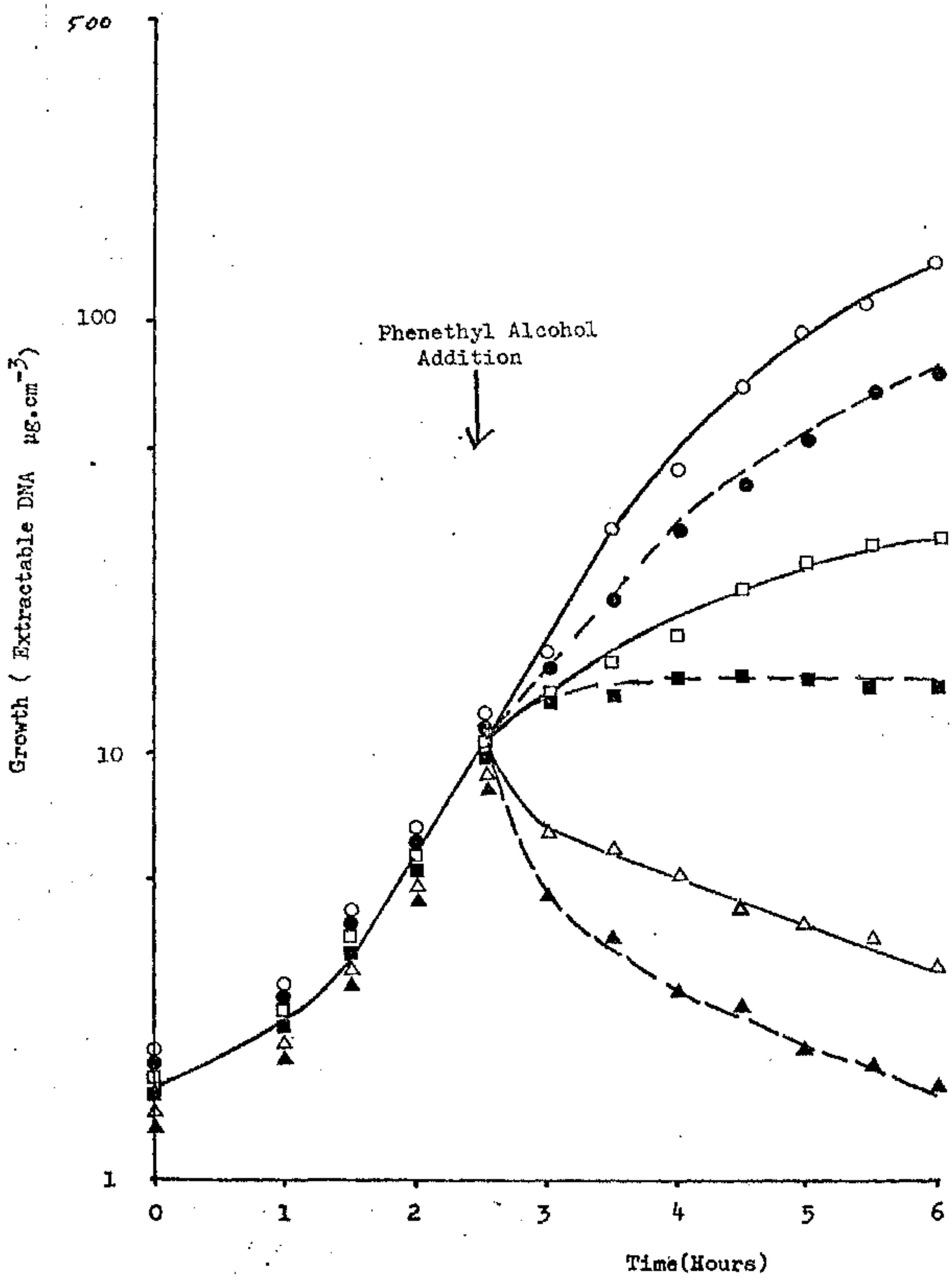

Phenethyl Alcohol
Addition
5.0
1.0
0.1
0.01
Growth (E_{600nm} x dilution)
0
1
2
3
4
5
6
Time(Hours)

Figure 9 b

Effect of Varying Concentrations of Phenethyl-alcohol on Growth Expressed as Extractable DNA ($\mu g.cm^{-3}$).

Conditions and symbols were the same as for Figure 9 a.



500
100
10
1
Phenethyl Alcohol
Addition
Growth (Extractable DNA µg.cm^{-3})
0
1
2
3
4
5
6
Time(Hours)

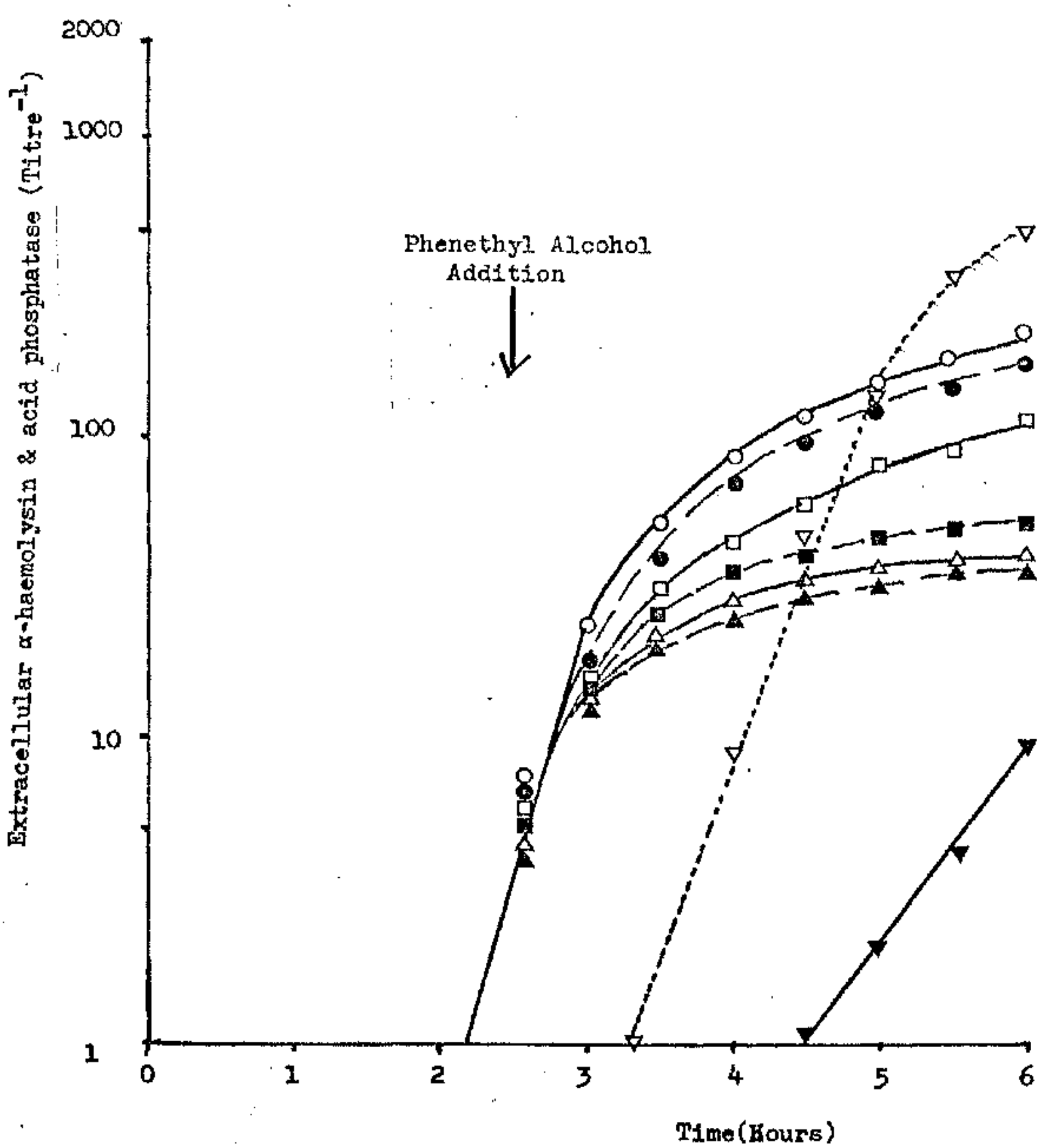
Figure 9 c

Effect of Varying Concentrations of Phenethyl-alcohol on Production of Alpha-haemolysin and Extracellular Acid Phosphatase.

Conditions were the same as for Figure 9 a

Assay for Extracellular Phosphatase	Control		(○)
	15 mM	Phenethyl Alcohol	(●)
	22.5 mM	" "	(□)
	30 mM	" "	(■)
	37.5 mM	" "	(△)
	45 mM	" "	(▲)
Assay for Alpha-haemolysin	Control		(▽)
	15 mM	Phenethyl Alcohol	(▼)

No haemolysin was detected at higher phenethyl alcohol concentrations.



Extracellular α-haemolysin & acid phosphatase (Titre^{-1})
2000
1000
100
10
1
Phenethyl Alcohol
Addition
0
1
2
3
4
5
6
Time(Hours)

Table 6

Percentage Decrease in E_{600nm} and DNA Following Washing and Resuspension of Phenethyl Alcohol Treated Cultures.

Phenethyl Alcohol Concentration (mM)	Percentage Decrease			
	E_{600nm}	SD†	DNA	SD
0	15.2	1.9	14.1	1.6
*7.5	14.6	0.9	13.2	0.9
15	14.1	1.6	15.9	2.4
22.5	13.7	1.9	16.2	3.2
30	8.6	1.1	15.8	4.1
37.5	47.6	3.2	86.0	4.8
45	54.0	2.6	93.2	4.2

*Based on only two readings - not shown on Fig. 9

†Standard Deviation.

cultures treated with 37.5 mM showed an increase after 4h and those treated with 45mM after 12h. However, all cultures finally attained the same stationary population as controls.

Extracellular acid phosphatase was detected in all cultures and the degree of inhibition resembled that of the E_{600nm} although this was less obvious in cultures treated with 30 mM or more phenethyl alcohol in that levels of extracellular enzyme continued to increase slowly after the culture had apparently ceased growing. The rate of alpha-haemolysin production was reduced at least 80% by 15 mM phenethyl alcohol and alpha-haemolysin was not detected in cultures treated with higher concentrations.

C.1.2. Effect of Population Density on Phenethyl-alcohol Inhibition.

In order to investigate how the number of staphylococcal cells affected the inhibition of growth and extracellular protein synthesis, phenethyl alcohol at 15 or 30 mM was added to different population densities at constant time.

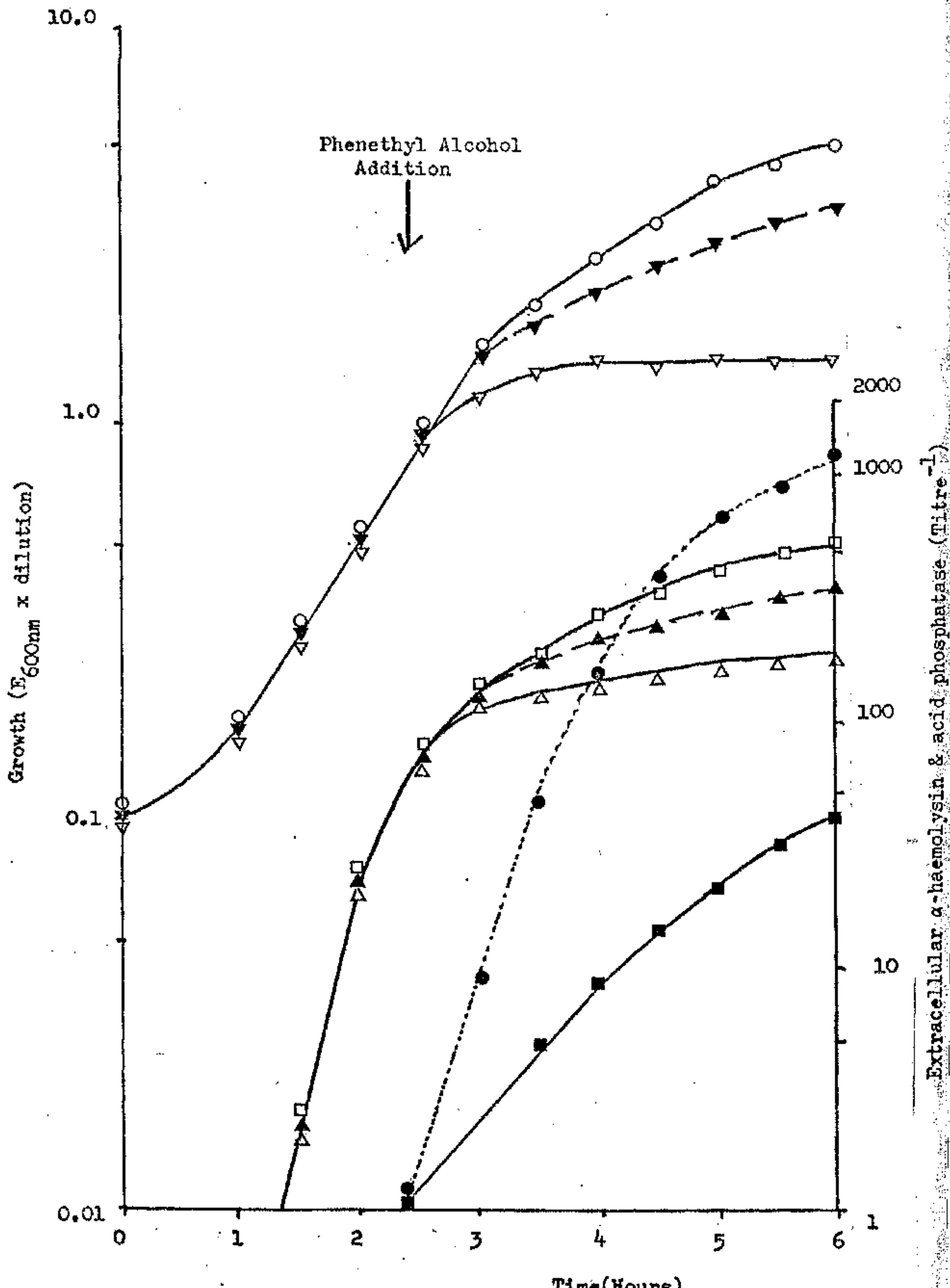
BS medium (100 cm^3) was inoculated with 2.10^8 or 3.10^8 c.f.u.cm^{-3} rather than 1.10^8 c.f.u.cm^{-3} which was previously used and incubated at 37°C aerobically. Phenethyl alcohol was added in log phase at 2.5h and E_{600nm}, extracellular acid phosphatase and alpha-haemolysin were assayed as before. The results are shown in Figure 10 a,b. Extractable DNA was assayed once and is therefore not shown, but the results were as expected, based on Fig. 9. Doubling the inoculum size to 2.10^8c.f.u. cm^{-3} (Figure 10a) produced, after an initial lag phase, an exponential phase lasting about 3h followed by a gradual slowing down. Acid phosphatase and alpha-haemolysin production were similar to that for 1.10^8 c.f.u. cm^{-3} (Fig. 9) except that their initial appearance was

Figure 10a

Effect of Phenethyl-alcohol, at 15mM or 30mM on Growth (E_{600nm}), Alpha-haemolysin and Extracellular Acid Phosphatase **Production.**

Conditions were as before, Figure 9, except that the initial inoculum was 2.10^{8} c.f.u.cm^{-3}.

Untreated	E_{600nm}	(○)
	Acid Phosphatase	(□)
	α-Haemolysin	(●)
15 mM Phenethyl Alcohol	E_{600nm}	(▼)
	Acid Phosphatase	(▲)
	α-Haemolysin	(■)
30 mM Phenethyl Alcohol	E_{600nm}	(▽)
	Acid Phosphatase	(△)
	α-Haemolysin - less than 1 H.U.cm^{-3}	



10.0
1.0
0.1
0.01
Phenethyl Alcohol
Addition
Growth (E_{600nm} x dilution)
2000
1000
100
10
1
Extracellular α-haemolysin & acid phosphatase (Titre^{-1})
0
1
2
3
4
5
6
Time(Hours)

advanced about 1h. Growth of cultures treated with 15mM phenethyl alcohol was depressed about 30% and acid phosphatase was depressed to a similar level. Alpha-haemolysin was depressed about 96%. Growth and acid phosphatase production in cultures treated with 30mM phenethyl alcohol increased about 50% in 40 min and then ceased, although phosphatase did appear to slowly increase. Alpha-haemolysin was detected in some experiments, but was never more than 2 $H.U.cm^{-3}$ and the average was less than one $H.U.cm^{-3}$ so this is not shown on Figure 10a. Trebling the inoculum size to 3.10^{8} c.f.u. cm^{-3} (Fig. 10b) produced, after an initial lag phase, an exponential phase lasting about 2.5h, followed by a period of slowing down which was more marked than that previously noted so that by 6h the growth yield was the same as that achieved by the culture which had received 2.10^{8}.c.f.u.cm^{-3}. Acid phosphatase and alpha-haemolysin productions were similar to these for lower inocula (Fig. 9 and Fig. 10a) except that their initial appearances were advanced by about 40min compared to those shown in Fig. 10a. Growth of cultures treated with 15mM phenethyl alcohol was depressed about 16% and acid phosphatase was depressed to a similar level. Alpha-haemolysin was depressed about 93%. Growth and acid phosphatase production in cultures treated with 30mM phenethyl alcohol increased about 50% in 40 min and then ceased, although phosphatase did appear to slowly increase. Alpha-haemolysin increased by no more than 17% over a 30 min period and then remained constant.

C.1.3. <u>Addition of Phenethyl-alcohol at Different Points in the Growth Cycle.</u>

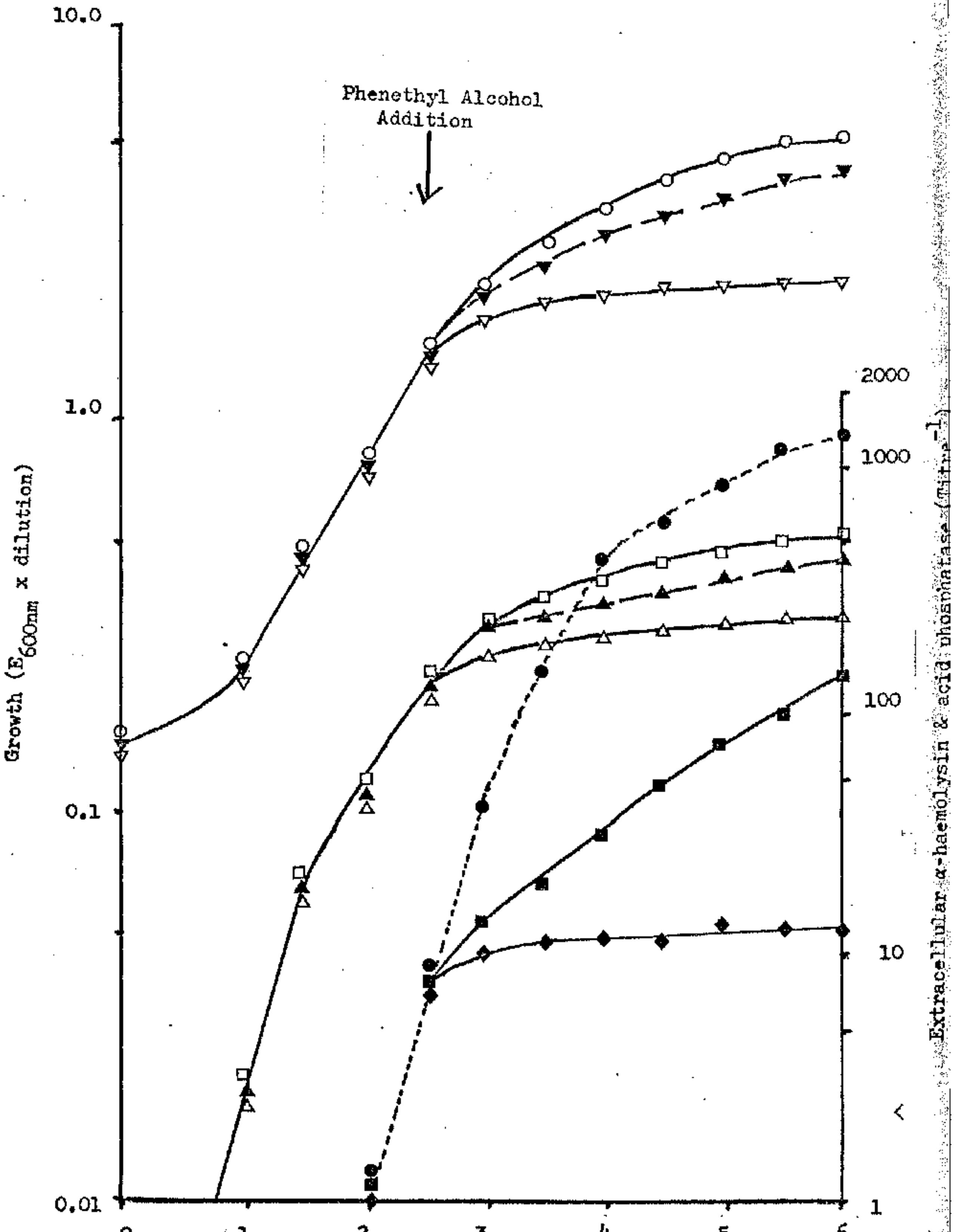
The effect of phenethyl alcohol on organisms at different stages of their growth cycle was examined using cultures in 100 cm^{3} of

Figure 10 b

Effect of Phenethyl alcohol, at 15mM or 30 mM, on Growth (E_{600nm}), Alpha-haemolysin and Extracellular Acid Phosphatase Production.

Conditions were as before, Figure 9, except that the initial inoculum was 3.10^{8} c.f.u.cm^{-3}.

Untreated	E_{600nm}	(○)
	Acid Phosphatase	(□)
	α-Haemolysin	(●)
15 mM Phenethyl Alcohol	E_{600nm}	(▼)
	Acid Phosphatase	(▲)
	α-Haemolysin	(■)
30 mM Phenethyl Alcohol	E_{600nm}	(▽)
	Acid Phosphatase	(△)
	α-Haemolysin	(◆)


Phenethyl Alcohol
Addition
10.0
1.0
0.1
0.01
Growth (E_{600nm} x dilution)
2000
1000
100
10
1
Extracellular α-haemolysin & acid phosphatase (Titre^{-1})
0
1
2
3
4
5
6
Time(Hours)

BS medium inoculated with 1.10^{8} c.f.u.cm^{-3}. The effects on growth (E_{600nm}), extracellular acid phosphatase and alpha-haemolysin are shown in Figure 11 a,b,c,d). Extractable DNA was assayed once and is therefore not shown. However, the results were consistent with those obtained before (Fig. 9). The times selected for addition of phenethyl alcohol were the onset of late lag phase, at the point where the slowing down period begins (5h), and the transition to early stationary phase(6.5h). Addition of 15mM phenethyl alcohol at 5h (Fig. 11a) caused a reduction in growth rate of 20-25% over the 90 min period post addition. However, since the untreated culture began to slow down significantly after 6.5h, the growth rate of treated and untreated cultures became very similar, although the treated culture did appear to be slightly slower until 8h, after which both rates seemed to be the same. Acid phosphatase production was reduced in line with E_{600nm}. The rate of alpha-haemolysin production was depressed about 80% until 9h. However, the untreated cultures had almost reached the final titre level by this time and so comparison of production rates was not possible beyond this point.

Addition of 15mM phenethyl alcohol at 6.5h (Fig. 11b) only caused a 10% reduction in the total rates of growth and acid phosphatase production because the untreated cultures were slowing down significantly by this stage. The rate of alpha-haemolysin production was depressed about 70% up to 9h but, due to controls achieving final titre shortly after, comparison of production rates was not possible beyond this point.

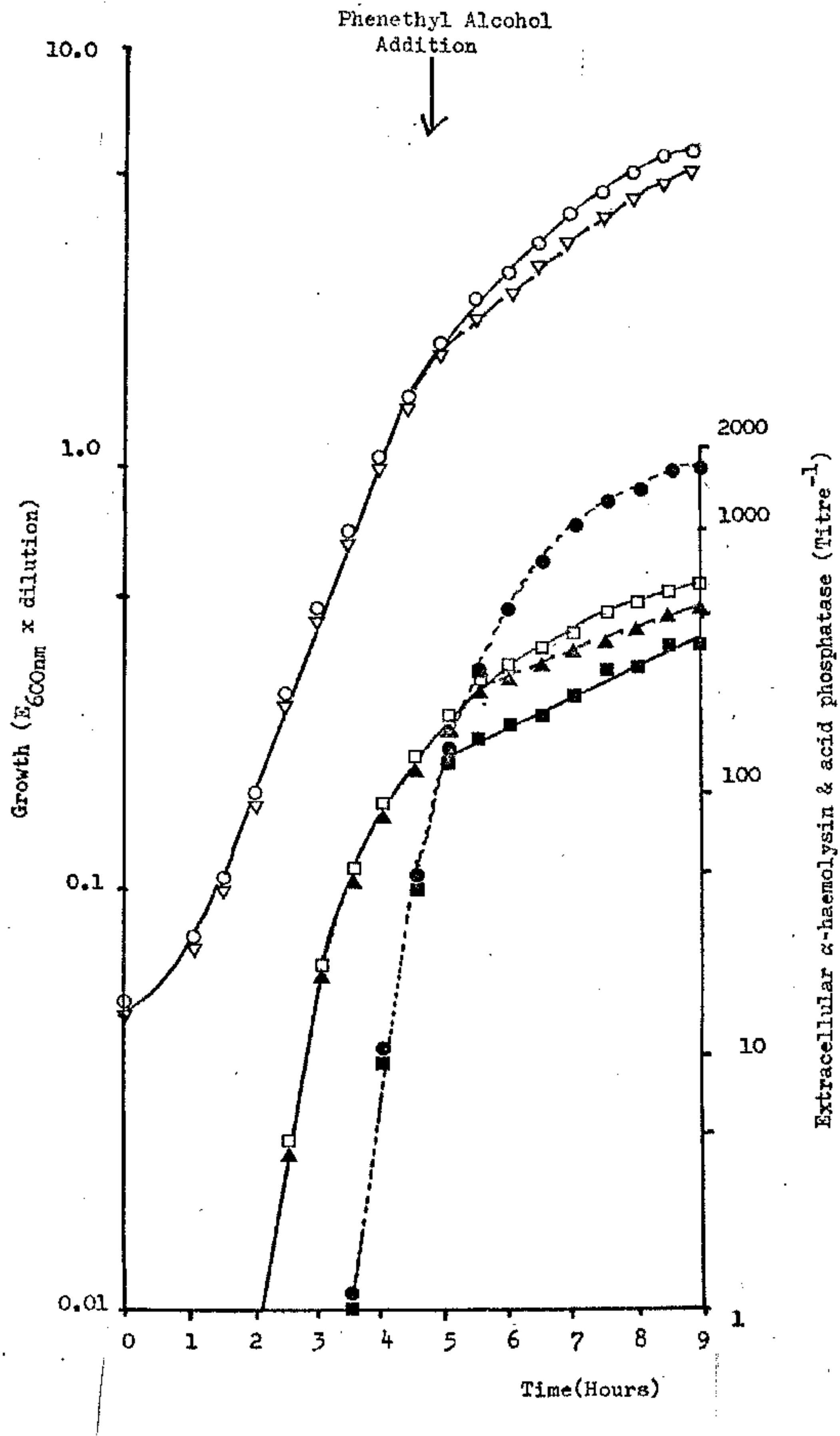
Addition of 30mM phenethyl alcohol at either 5h (Fig. 11c) or 6.5 (Fig. 11d) caused cessation of growth after about 60 min. Acid phosphatase behaved in a similar fashion although the extracellular levels did appear to increase slowly. Alpha-haemolysin production

Figure 11 a.

Effect of 15mM Phenethyl-alcohol, Added to Late Log Phase Cultures on Growth (E_{600nm}), Extracellular Acid Phosphatase and Alpha-haemolysin **Production.**

Conditions were the same as for Figure 9 except that 15 mM phenethyl alcohol was added at 5h when the culture was beginning to slow down at the end of log phase.

Growth (expressed as E_{600nm})	Control	(○)
	Treated	(▽)
Extracellular acid phosphatase	Control	(□)
	Treated	(▲)
Alpha-haemolysin	Control	(●)
	Treated	(■) or ◆

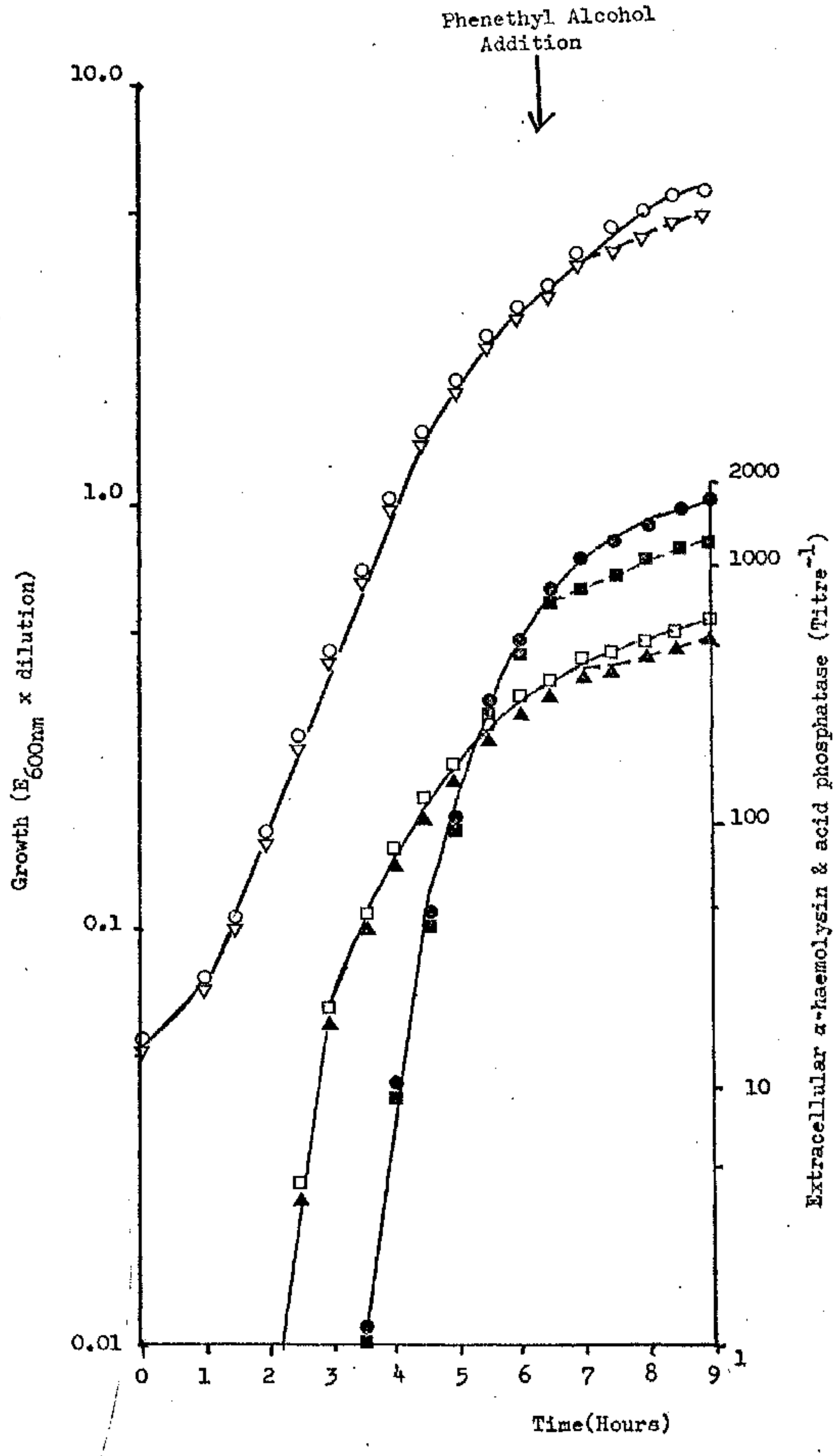


Phenethyl Alcohol
Addition
10.0
1.0
0.1
0.01
Growth (E_{600nm} x dilution)
2000
1000
100
10
1
Extracellular α-haemolysin & acid phosphatase ($Titre^{-1}$)
0
1
2
3
4
5
6
7
8
9
Time(Hours)

Figure 11 b

Effect of 15mM Phenethyl alcohol, Added to Early Stationary Phase Cultures, on Growth (E_{600nm}), Extracellular Acid Phosphatase and Alpha-haemolysin **Production.**

Conditions and symbols were as for Fig. 11 a except that 15 mM phenethyl alcohol was added at 6.5h when the culture was slowing down from late exponential phase and entering early stationary phase.

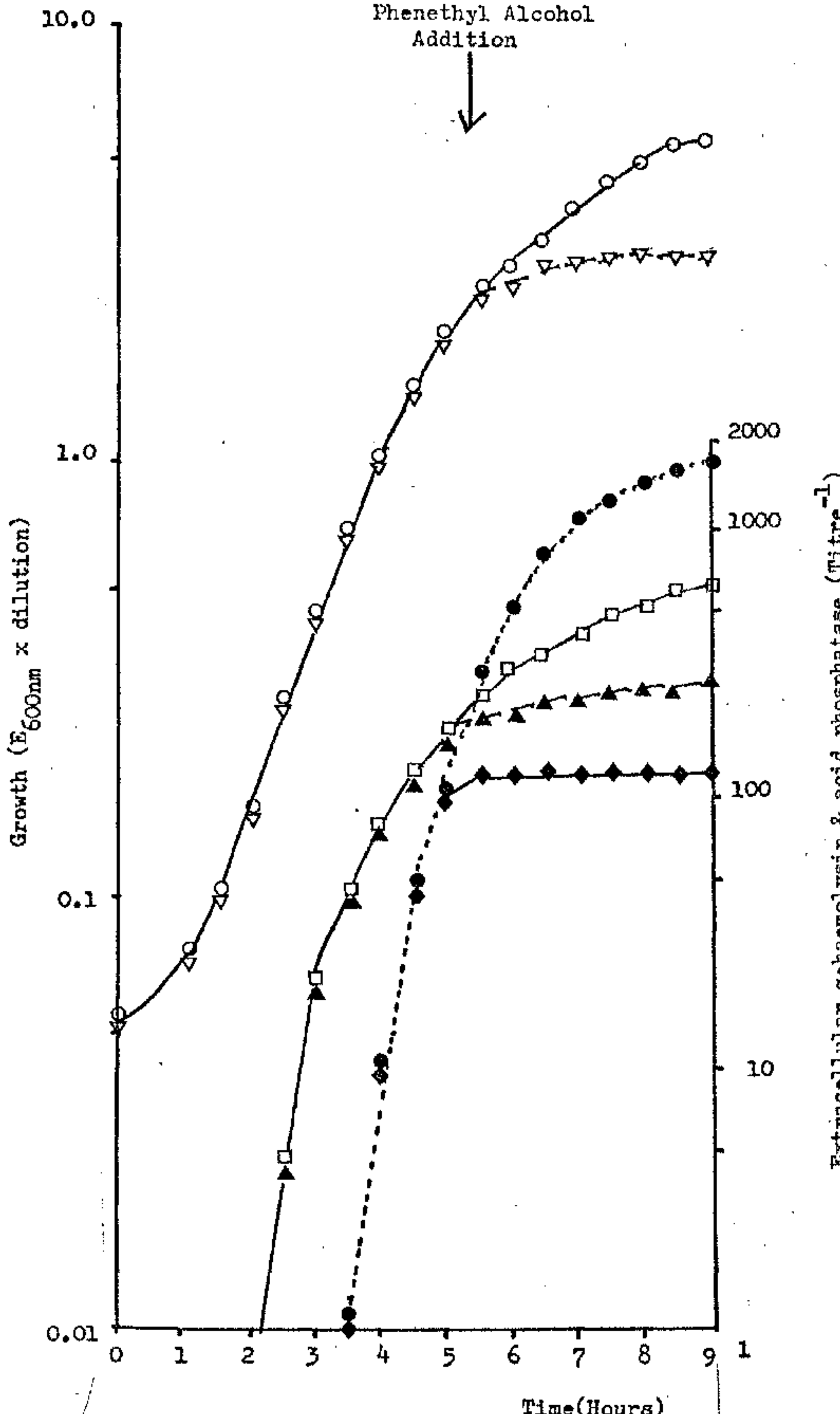


Phenethyl Alcohol
Addition
10.0
1.0
0.1
0.01
Growth (E_{600nm} x dilution)
2000
1000
100
10
1
Extracellular α-haemolysin & acid phosphatase (Titre^{-1})
0
1
2
3
4
5
6
7
8
9
Time(Hours)

Figure 11 c

Effect of 30 mM Phenethyl alcohol, Added to Late Log Phase Cultures, on Growth (^{E}600nm), Extracellular Acid Phosphatase and Alpha-haemolysin Production.

Conditions and symbols were the same as for Figure 11 a except that 30mM phenethyl alcohol was added at 5h.

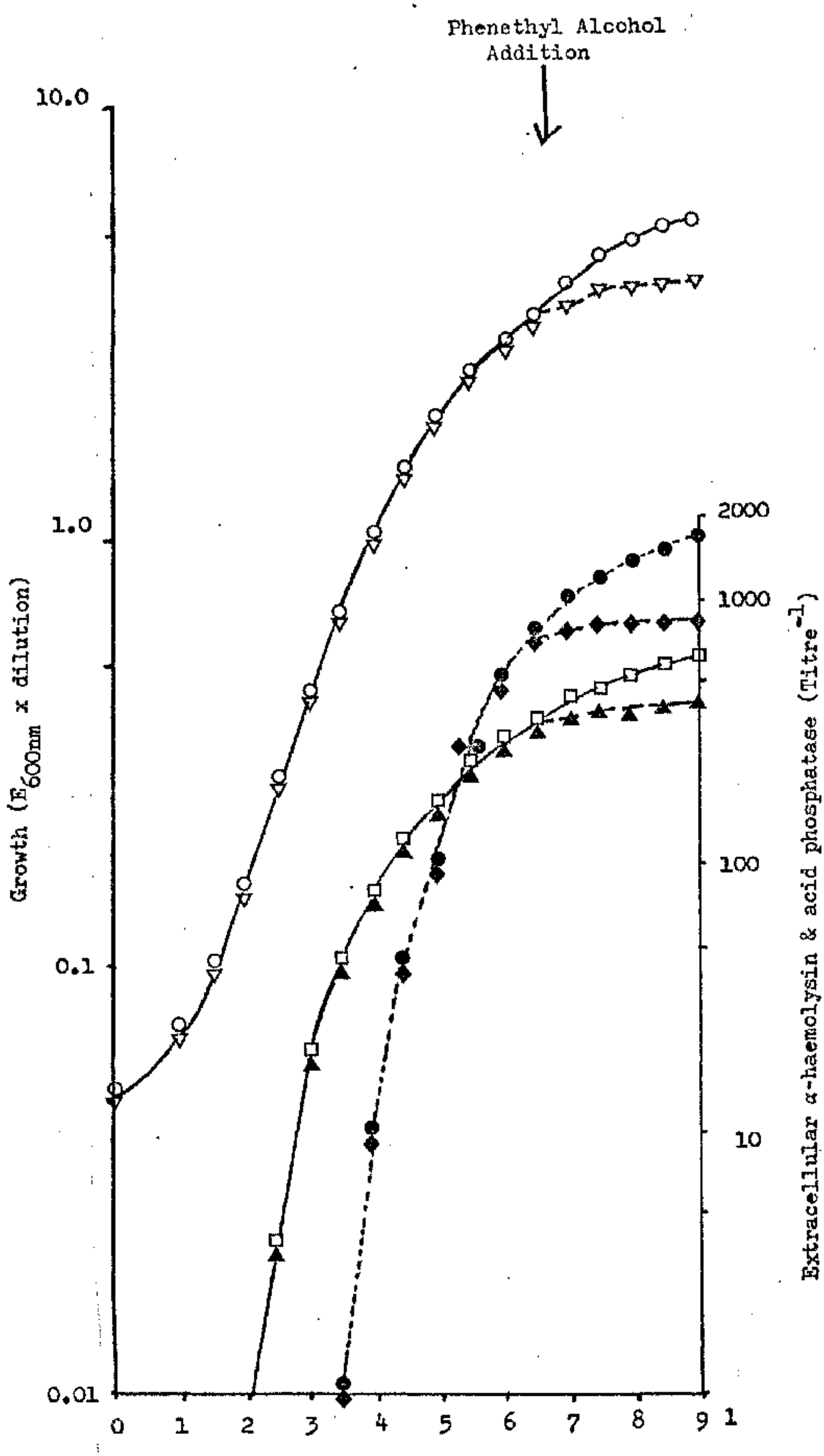


Phenethyl Alcohol Addition
10.0
1.0
0.1
0.01
Growth (E_{600nm} x dilution)
2000
1000
100
10
1
Extracellular α-haemolysin & acid phosphatase (Titre^{-1})
0
1
2
3
4
5
6
7
8
9
Time(Hours)

Figure 11 d

Effect of 30mM Phenethyl-alcohol, Added to Early Stationary Phase Cultures, on Growth ($^{E}600nm$), Extracellular Acid Phosphatase and Alpha-haemolysin Production.

Conditions and symbols were the same as for Figure 11 a except that 30mM phenethyl alcohol was added at 6.5h.



Phenethyl Alcohol
Addition
10.0
1.0
0.1
0.01
Growth (E_{600nm} x dilution)
2000
1000
100
10
1
Extracellular α-haemolysin & acid phosphatase ($Titre^{-1}$)
0
1
2
3
4
5
6
7
8
9
Time(Hours)

ceased almost immediately after addition of 30mM phenethyl alcohol and the concentration remained constant throughout the course of the experiment, indicating that there was no intracellular accumulation of this protein capable of leaking out.

C.1.4. Investigation of the Possibility that Phenethyl-alcohol Acts as a Competitive Inhibitor of Phenylalanine.

Mathieu et al. (1970) noted that phenylalanine appeared to be involved in alpha-haemolysin synthesis since meta- fluorophenylalanine could prevent its production. Phenethyl alcohol is a structural analogue of phenylalanine and its inhibitory effect could be due to competitive inhibition of a substrate molecule, so this possibility was investigated by comparing the effect of phenethyl alcohol with the effect of ortho, meta or para- fluorophenylalanine. Reversal of the inhibition by addition of phenylalanine or precursors was also attempted. Experiments were carried out in BS medium inoculated with 1.10^{8} c.f.u. cm^{-3} and incubated aerobically at 37°C. All additions were made at 2.5h and the results are summarised in Fig. 12.

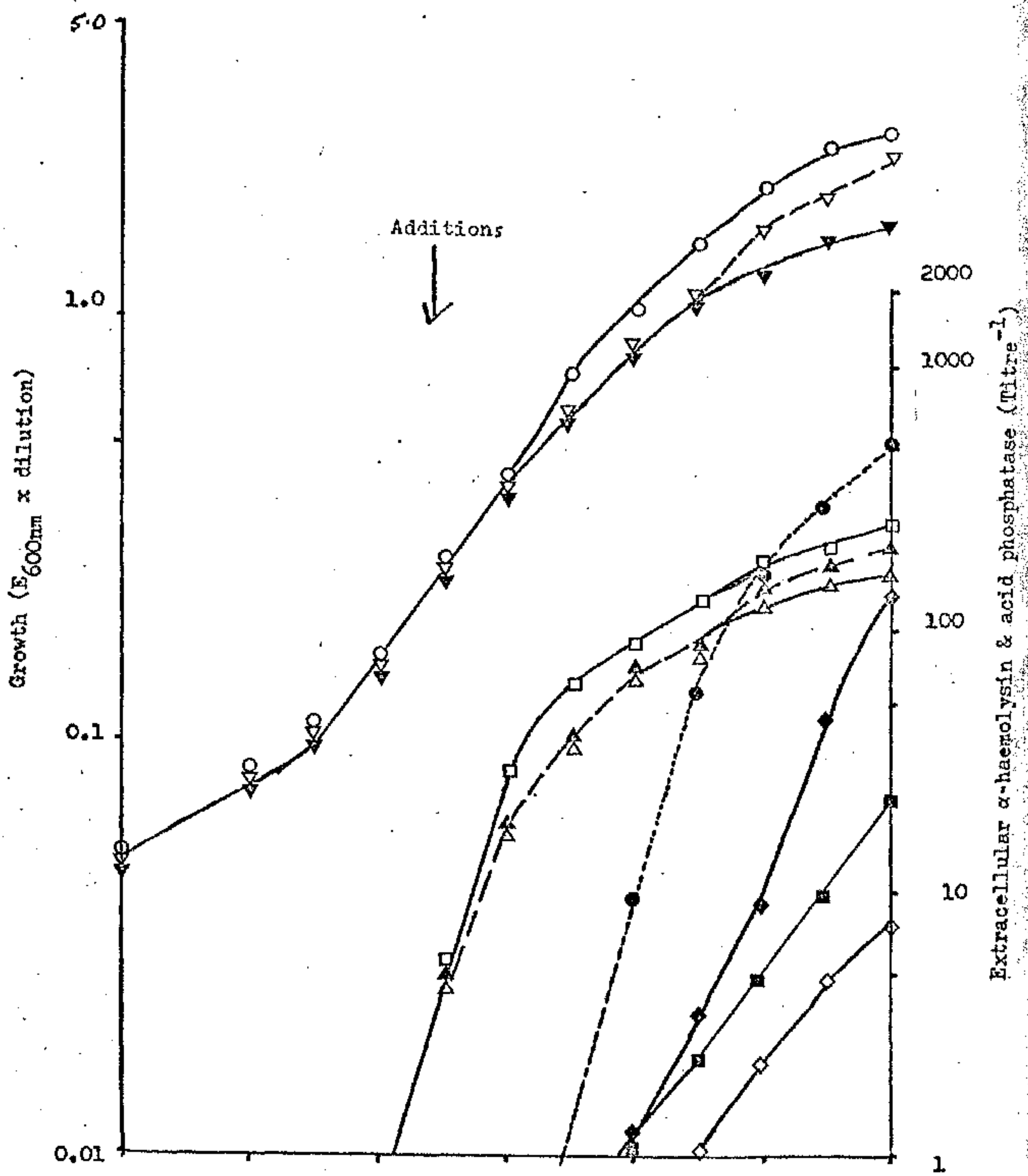
Addition of any one of the three amino acid analogues to a final concentration of 80μm of the L-form resulted in a reduction in growth ($^{E}600nm$) which closely paralleled the effect of 15mM phenethyl alcohol during the period of 2h post addition. At the end of this time, cultures treated with analogue seemed to recover, since growth rate reverted to normal. Addition of phenylalanine (200μM) with the analogue eliminated the inhibitory effect. However, phenylalanine at concentrations up to 50mM did not change the effect of phenethyl alcohol at 15 or 30mM. Combining 15mM phenethyl alcohol with any one of the analogues did not show either a synergistic effect or an antagonistic effect.

Figure 12

Effect of 80 µM ortho-, meta- or para- Fluorophenylalanine on Growth(E_{600nm}), Extracellular Acid Phosphatase and Alpha-haemolysin Production in the Presence or Absence of 15 mM Phenethyl alcohol.

Conditions were as for Figure 3 except that ortho-, meta- or para- fluorophenylalanine were added to a final concentration of 80 µM at 2.5h, either with or without 15 mM phenethyl alcohol.

Growth (E_{600nm})	Untreated (○); Treated with fluorophenylalanine (▽); Treated with phenethyl alcohol either alone or with fluorophenylalanine (▼)
Acid phosphatase	Untreated (□); Treated with fluorophenylalanine (▲); Treated with phenethyl alcohol either alone or with fluorophenylalanine (△)
Alpha haemolysin	Untreated (●); Treated with ortho-, or para- fluorophenylalanine (■); Treated with meta- fluorophenylalanine (◆); Treated with phenethyl alcohol either alone or with fluorophenylalanine (◇).



5.0
1.0
0.1
0.01
Growth (E_{600nm} x dilution)
Additions
2000
1000
100
10
1
Extracellular α-haemolysin & acid phosphatase ($Titre^{-1}$)
0
1
2
3
4
5
6
Time(Hours)

Instead the effect resembled that of phenethyl alcohol alone.

The effect of the antimetabolites on the production of alpha-haemolysin and extracellular acid phosphatase is also shown on Fig. 12. All three of the flurophenylalanines inhibited production of alpha-haemolysin. Ortho- and para-flurophenylalanine delayed the first appearance by about 30 min and reduced the rate of synthesis by 75%. Meta-flurophenylalanine also delayed the first appearance by about 30 min, but in this case the rate of synthesis was reduced by 90%. Phenylalanine (200 μM) added with analogue, abolished the inhibitory effect but had no effect on phenethyl alcohol inhibition, even when added at 50 mM. Phenethyl alcohol added with the analogue produced neither a synergistic nor an antagonistic effect. Instead the effect resembled that of phenethyl alcohol alone. Acid phosphatase was inhibited by all three analogues in line with the inhibition of growth. Phenethyl alcohol again overrode the inhibitory effect of the analogues.

C.1.5. Investigation of the Effect of L-Histidine on Inhibition of Growth and Alpha-haemolysin Synthesis by Phenethyl alcohol.

In view of Dalen's (1973a,b,c) observation that histidine was an absolute requirement for alpha-haemolysin synthesis, its effect on phenethyl alcohol inhibition was investigated. BS medium (100 cm^3) was inoculated with 3.10^8 $c.f.u.cm^{-3}$ and incubated aerobically at 37^oC. Phenethyl alcohol was added, to a final concentration of 15mM, at 2.5h and histidine was added, to a final concentration of 1mM, either at the same time or one hour later. The results showed, Figure 13, that histidine did not stimulate growth of control cultures, nor did it stimulate the production of alpha-haemolysin.

In cultures treated with phenethyl alcohol, no effect on the inhibition of growth (^E600nm) was observed when histidine was added and DNA synthesis and extracellular acid phosphatase were similarly unaffected. Alpha-haemolysin synthesis in cultures treated with phenethyl alcohol alone was only 3% of controls, but addition of histidine at the same time allowed synthesis at 25% of controls and histidine added one hour later allowed synthesis at 23% of controls.

Cultures inoculated with 1.10^8 c.f.u. cm^{-3} gave similar, but shallower, stimulation (12-15%). Lower concentrations of histidine, 200-500 µM, had little detectable stimulatory effect (5-7%) and 2mM had essentially the same effect as 1mM (21-26%).

C.2. Effect of Phenethyl alcohol Analogues.

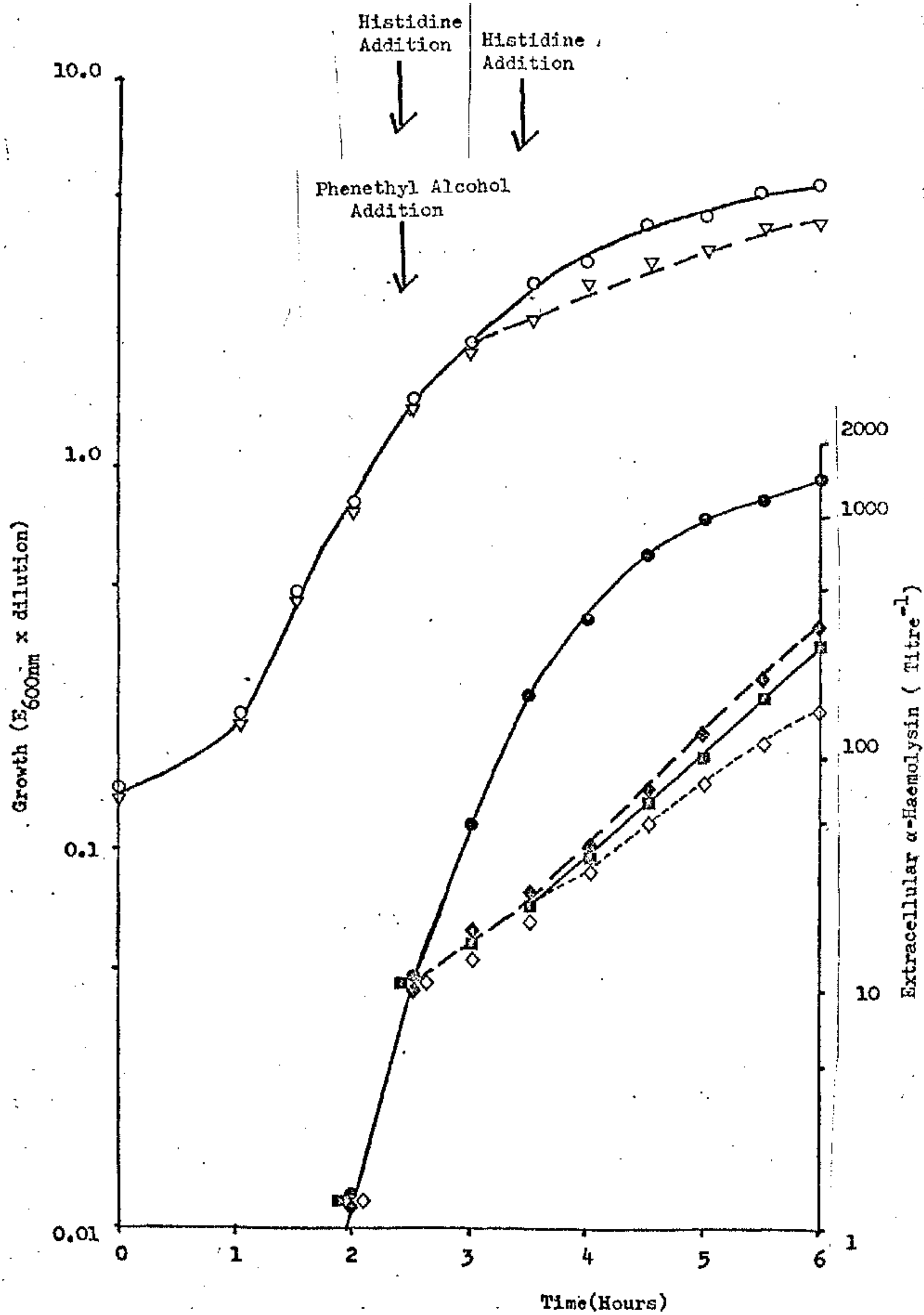
A number of compounds bearing a structural similarity to phenethyl alcohol were examined in an attempt to determine if any specific structural feature was necessary for the inhibition effects. The analogues used were phenylacetic acid, phenethylamine, phenoxyethanol,

Figure 13

Effect of L-Histidine on Inhibition of Growth and Alpha-haemolysin Synthesis by Phenethyl-alcohol.

Conditions were as for Figure 10 b except that 15mM phenethyl alcohol was added at 2.5h and 1mM L-histidine was added at 2.5 or 3.5h.

Growth (^{E}600nm)	Untreated	(○)
	Treated with phenethyl alcohol alone or with histidine	(▽)
Alpha-haemolysin	Untreated	(●)
	Treated with phenethyl alcohol alone	(◇)
	Treated with phenethyl alcohol and histidine at 2.5h	(◆)
	Treated with phenethyl alcohol and histidine at 3.5h.	(■)



Histidine Addition
Histidine Addition
Phenethyl Alcohol Addition
10.0
1.0
0.1
0.01
Growth (E_{600nm} x dilution)
2000
1000
100
10
1
Extracellular α-Haemolysin ($Titre^{-1}$)
0
1
2
3
4
5
6
Time(Hours)

1-phenylethanol, phenpropanol, phenethyl chloride and phenethyl bromide. BS medium (100 cm^3) was incubated with 10^8 $c.f.u.cm^{-3}$ and incubated aerobically at 37°C. The analogues were added at 2.5h to a final concentration of 15mM. As shown on figure 14, they produced varying effects on growth and alpha-haemolysin synthesis which could be divided into four groups. The first group, which contained only 2-phenylacetic acid produced no effect on either parameter. The second group, which comprised 2-phenyl ethylamine and 2-phenoxyethanol produced a slight increase in growth, but an inhibition of 65-70% in alpha-haemolysin synthesis. The third group, which comprised 1-phenylethanol, 2-phenylethanol and 3-phenylpropanol, produced an inhibition of 30-40% in growth and 94-98% in alpha-haemolysin. The fourth group, which comprised 2-phenylethyl chloride and 2-phenylethyl bromide, produced an inhibition of 61-62% in growth and totally inhibited alpha-haemolysin synthesis. The latter compounds appared to make the cells much more labile than any of the others, since recovery from washing and resuspension of cells treated with these compounds was only 51.6 ± 4.8% compared to 85.8 ± 3.1% for cells treated with the other compounds.

C.3. Antimetabolites not structurally related to phenethyl alcohol.

A number of other antimetabolites, not structurally related to phenethyl alcohol, were used to treat Staph. aureus under the same conditions as employed above for phenethyl alcohol. These compounds could be divided into two groups, those whose activity is directed towards nucleic acid or protein synthesis and those whose activity is directed towards the membrane or membrane based functions.

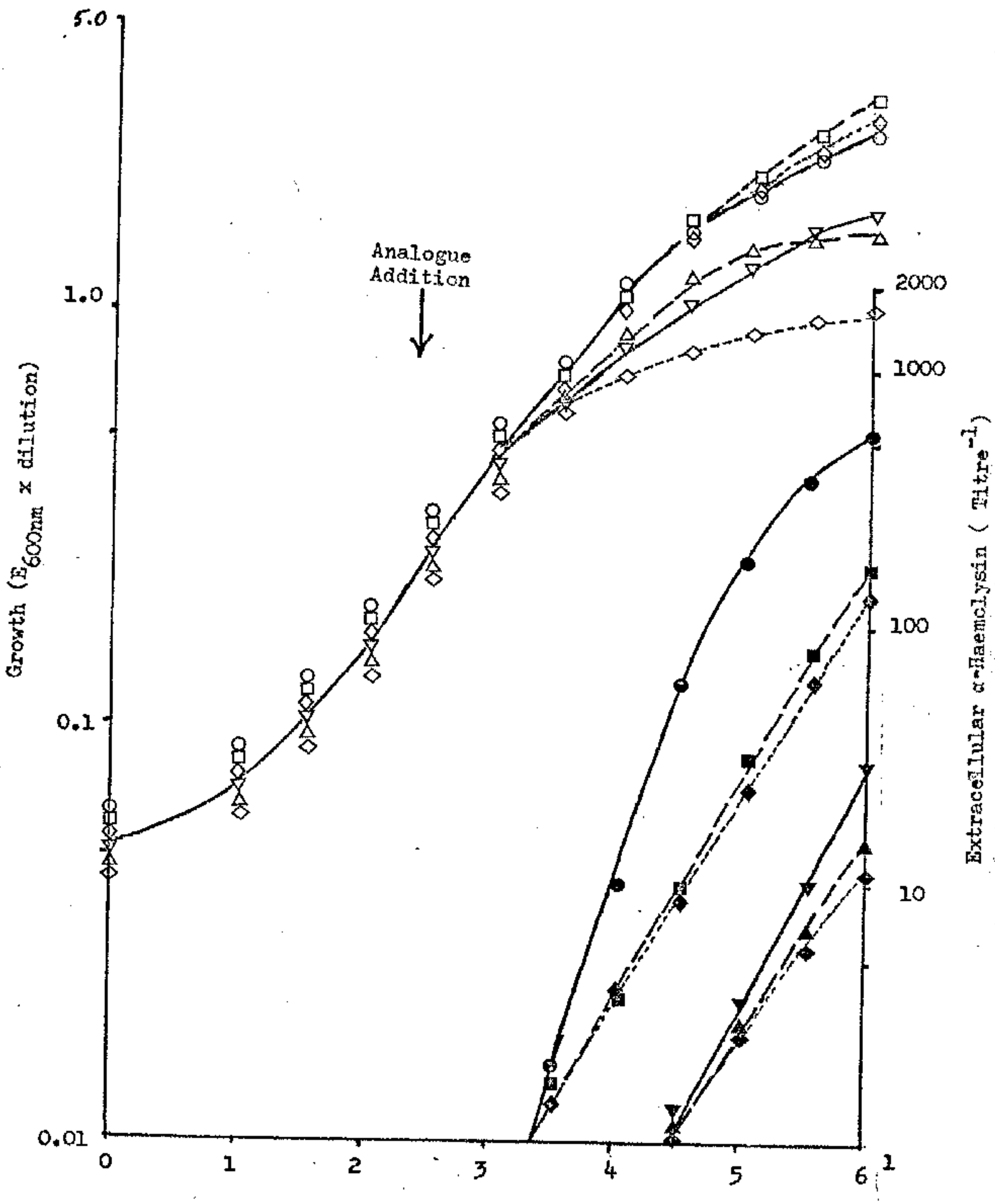
Figure 14

Effect of Various Analogues of Phenethyl-alcohol on Growth and Alpha-haemolysin Synthesis.

Conditions were as for Figure 3, except that one of the following compounds was added at 2.5h to a final concentration of 15mM.

Growth (E_{600nm})	Untreated		(○)
	Treated with	2-phenylethylamine	(◇)
	" "	2-phenoxyethanol	(□)
	" "	1-phenylethanol or 2-phenylethanol	(△)
	" "	3-phenylpropanol	(▽)
	" "	2-phenylethyl chloride or 2-phenylethyl bromide	(◇)
Alpha-haemolysin	Untreated		(●)
	Treated with	2-phenylethylamine	(◆)
	" "	2-phenoxyethanol	(■)
	" "	2-phenylethanol	(▼)
	" "	1-phenylethanol	(▲)
	" "	3-phenylpropanol	(◆)

No haemolysin was detected in cultures treated with 2-phenylethyl chloride or 2-phenylethyl bromide.



5.0
1.0
0.1
0.01
Growth (E_{600nm} x dilution)
Analogue Addition
2000
1000
100
10
1
Extracellular α-Haemolysin ($Titre^{-1}$)
0
1
2
3
4
5
6
Time(Hours)

C.3.1. Compounds Affecting Nucleic Acid or Protein Synthesis

Three compounds were used: Mitomycin C, affecting DNA and lysogeny; Actinomycin D, affecting transcription; Chloramphenicol, affecting translation. In all three cases, BS medium (100 cm^3) was inoculated with 10^8 c.f.u.cm^{-3} and incubated at 37°C aerobically. Test reagent was added in log phase at 2.5h and the effect on growth (^{E}600nm) extractable DNA (μg.cm^{-3}) and extracellular acid phosphatase and alpha-haemolysin was assessed. The results are shown on Figures 15, 16 and 17. Alpha-haemolysin was not detected in any of the treated cultures but was produced normally by the controls. To avoid confusion, this has been omitted from the figures.

C.3.1.a. Mitomycin C. As shown in Figure 15 Mitomycin C affected the growth rate within 30 min causing a reduction in increase which finally stopped after 2h post addition.

The ^{E}600nm then began to fall, indicating cell lysis. Extractable DNA appeared to decrease immediately after addition and became undetectable after 2h. Cells removed from treatment by centrifugation underwent a rapid decrease in ^{E}600nm during harvesting and viable count fell to less than 10 c.f.u.cm^{-3}. Clones of cells which survived treatment were normal in their production of extracellular proteins. Extracellular acid phosphate levels were reduced in line with reduction of ^{E}600nm up to 2h post-addition. When the ^{E}600nm began to decline, however, the phosphatase level continued to rise slowly, indicating leakage of preformed enzyme.

C.3.1.b. Chloramphenicol. Various different concentrations of Chloramphenicol were added to the cultures and the results for two of these, 30 μM and 80 μM, are shown in Figure 16. The effect of 30 μM

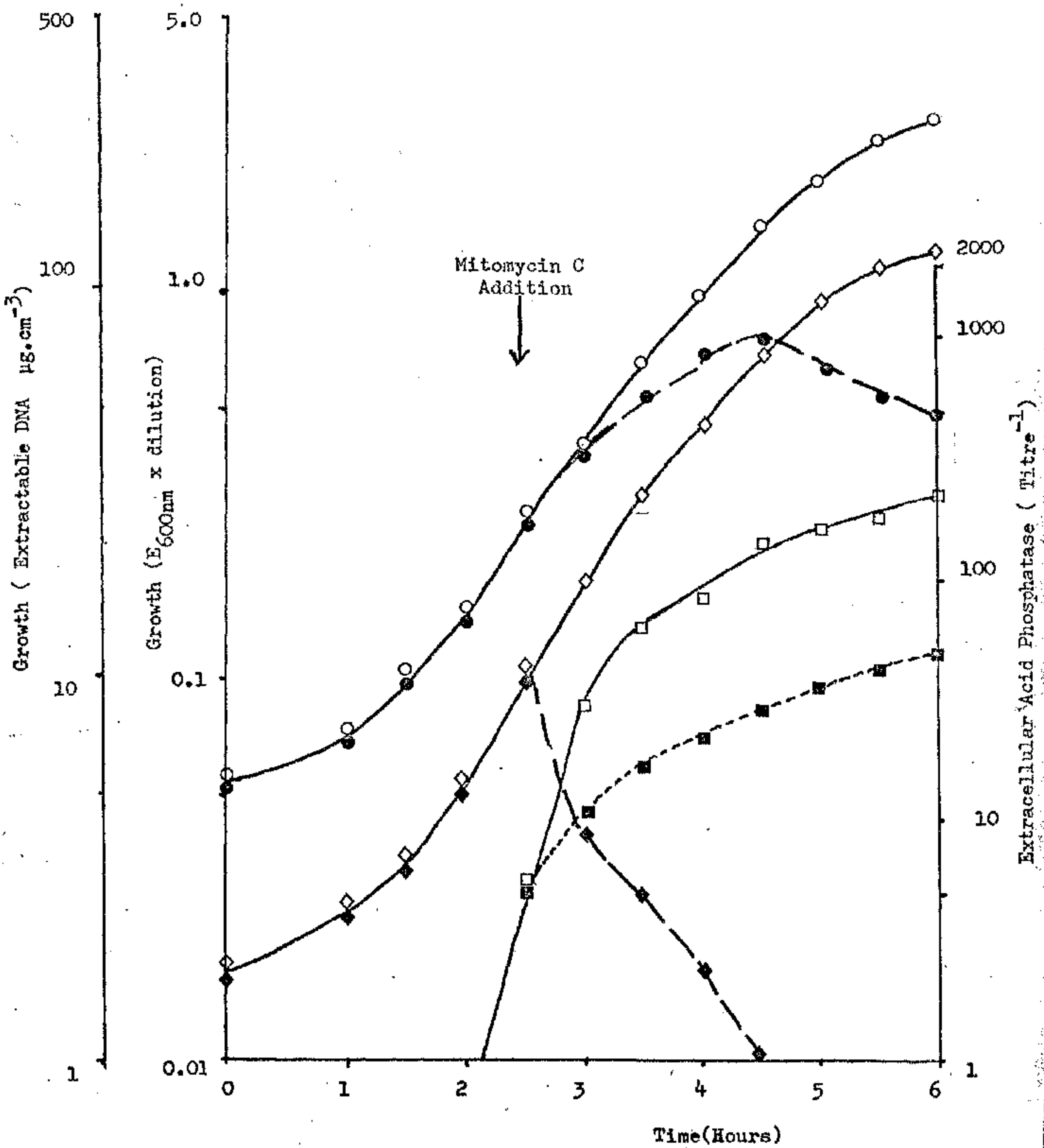
Figure 15

Effect of Mitomycin C on Growth and Acid Phosphatase Production.

Conditions were as for Figure 3, except that Mitomycin C was added at 2.5h to a final concentration of 3 μM.

Growth (E_{600nm})	Untreated	(○)
	Treated	(●)
Extractable DNA	Untreated	(◇)
	Treated	(◆)
Acid Phosphatase	Untreated	(□)
	Treated	(■)

Alpha-haemolysin was not detected in treated cultures.



500
100
10
1
5.0
1.0
0.1
0.01
Growth (Extractable DNA µg.cm-3)
Growth (E600nm x dilution)
Mitomycin C
Addition
2000
1000
100
10
1
Extracellular Acid Phosphatase (Titre-1)
0
1
2
3
4
5
6
Time(Hours)

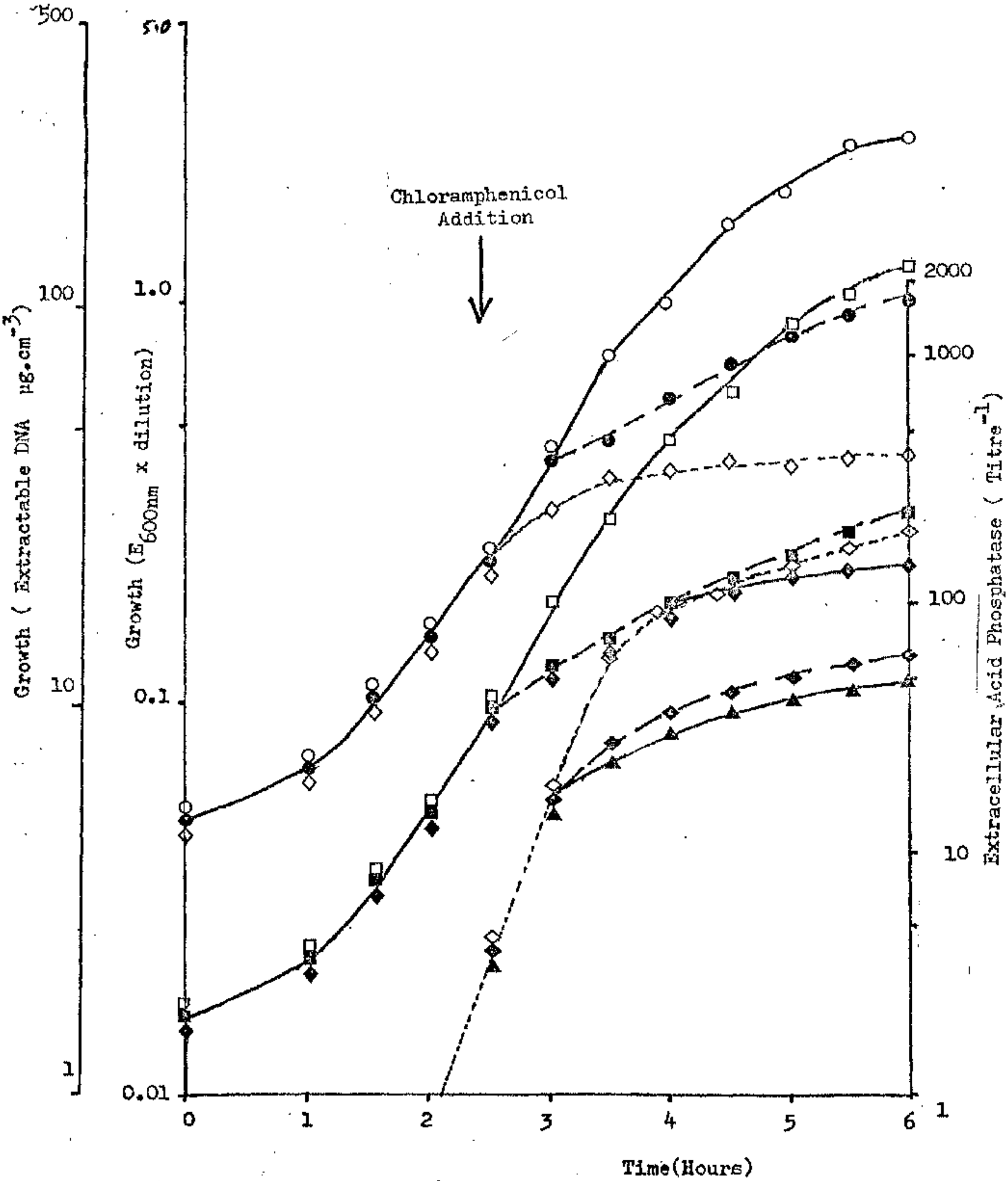
Figure 16

Effect of Chloramphenicol on Growth and Acid Phosphatase Production.

Conditions were as for Figure 3, except that Chloramphenicol was added at 2.5h to a final concentration of 30μM or 80 μM.

Growth (^{E}600nm)	Untreated	(○)
	Treated with 30 μM Chloramphenicol	(●)
	" " 80 μM Chloramphenicol	(◇)
Extractable DNA	Untreated	(□)
	Treated with 30 μM Chloramphenicol	(■)
	" " 80 μM Chloramphenicol	(◆)
Acid Phosphatase	Untreated	(◇)
	Treated with 30 μM Chloramphenicol	(◆)
	" " 80 μM Chloramphenicol	(▲)

Alpha-haemolysin was not detected in treated cultures.



Chloramphenicol Addition
Growth (Extractable DNA µg.cm^{-3})
Growth (E_{600nm} x dilution)
Extracellular Acid Phosphatase (Titre^{-1})
Time(Hours)
500
100
10
1
5.0
1.0
0.1
0.01
2000
1000
100
10
1
0
1
2
3
4
5
6

on $^{E}600nm$ and extractable DNA was very similar to that caused by 22.5mM phenethyl alcohol (Fig. 9). The effect of 80 µm chloramphenicol on $^{E}600nm$ was similar to that of 30 mM phenethyl alcohol. On extractable DNA, however, the inhibitory effect was less rapid than that caused by 30 mM phenethyl alcohol as DNA continued to increase at a reduced rate for 2h before stopping abruptly.

In the presence of 30 µM chloramphenicol, extracellular acid phosphatase continued to increase approximately in line with $^{E}600nm$. The same was true for 80 µM chloramphenicol until $^{E}600nm$ ceased increasing, but phosphatase levels then continued to rise slowly.

C.3.1.c. Actinomycin D. Various different concentrations of Actinomycin D were added to the culture and the results for two of these, 0.8 µM and 8 µM are shown in Figure 17. The effect of this compound on $^{E}600nm$ and extracellular acid phosphatase was very similar to phenethyl alcohol with 0.8 µM actinomycin being equivalent to 22.5 mM phenethyl alcohol and 8 µM actinomycin being equivalent to 30 mM phenethyl alcohol. The effect of 0.8 µM on extractable DNA was also similar to that of 22.5 mM phenethyl alcohol. However, on addition of 8µM the DNA ceased increasing almost immediately.

C.3.1.d. Comparison of Chloramphenicol and Actinomycin D on Active Alpha-haemolysin Synthesis. In the above experiments, alpha-haemolysin was not detected. In order to determine the possible durability of the alpha-haemolysin mRNA cultures were incubated until 4.5h before treatment with either 30 µM Chloramphenicol or 0.8 µM Actinomycin D, which have approximately the same effect on $^{E}600nm$. Samples were assayed every 10 min. for 30 min and thereafter at 30 min intervals. The results are shown on Figure 18.

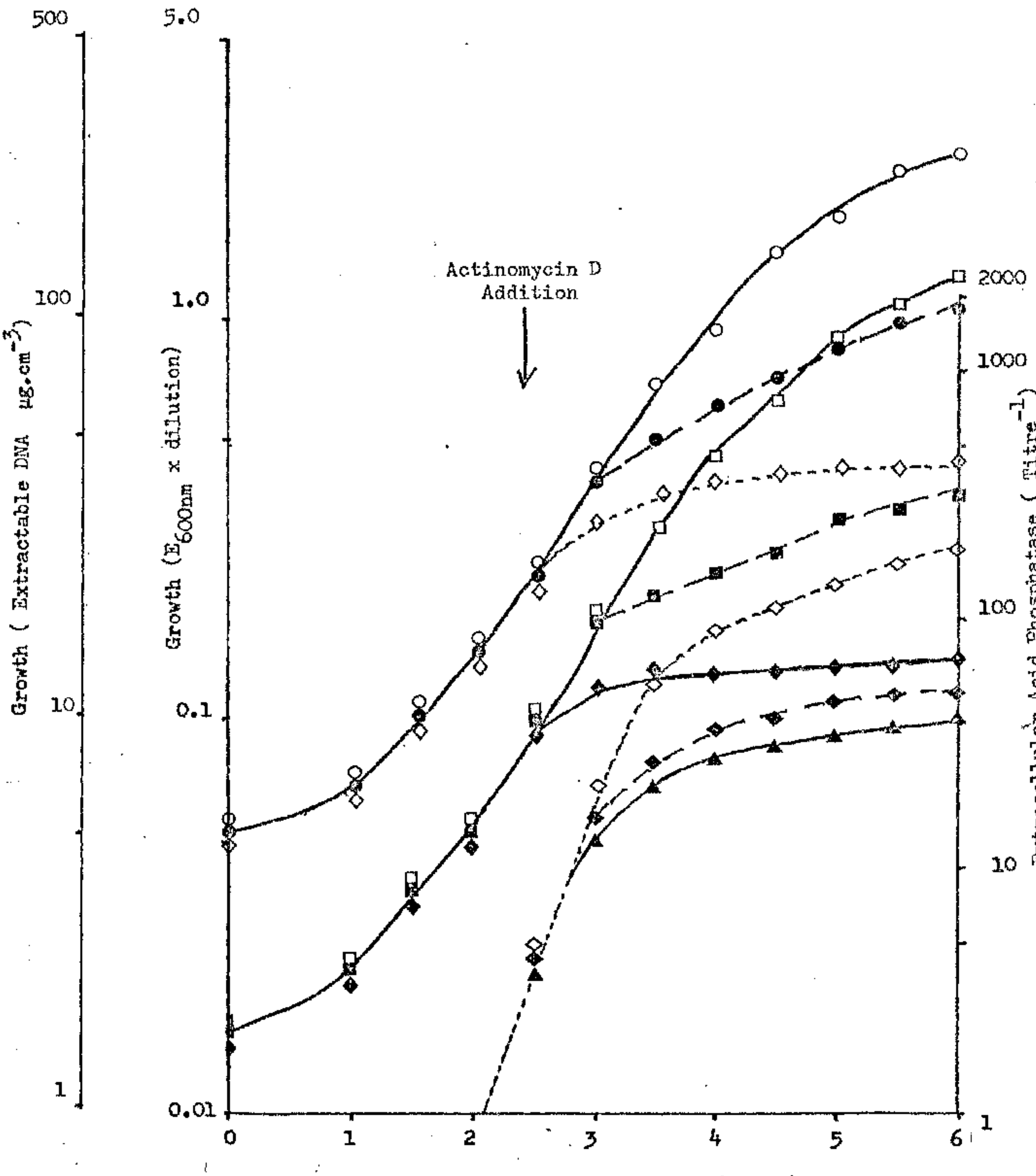
Figure 17

Effect of Actinomycin D on Growth and Acid Phosphatase production.

Conditions were as for Figure 3, except that Actinomycin D was added at 2.5h to a final concentration of 0.8 μM or 8 μM.

Growth (E_{600nm})	Untreated (○)	
	Treated with Actinomycin D at 0.8 μM	(●)
	" " Actinomycin D at 8.0 μM	(◇)
Extractable DNA	Untreated (□)	
	Treated with Actinomycin D at 0.8 μM	(■)
	" " Actinomycin D at 8.0 μM	(◆)
Acid Phosphatase	Untreated (◇)	
	Treated with Actinomycin D at 0.8 μM	(◆)
	" " Actinomycin D at 8.0 μM	(▲)

Alpha-haemolysin was not detected in treated cultures.



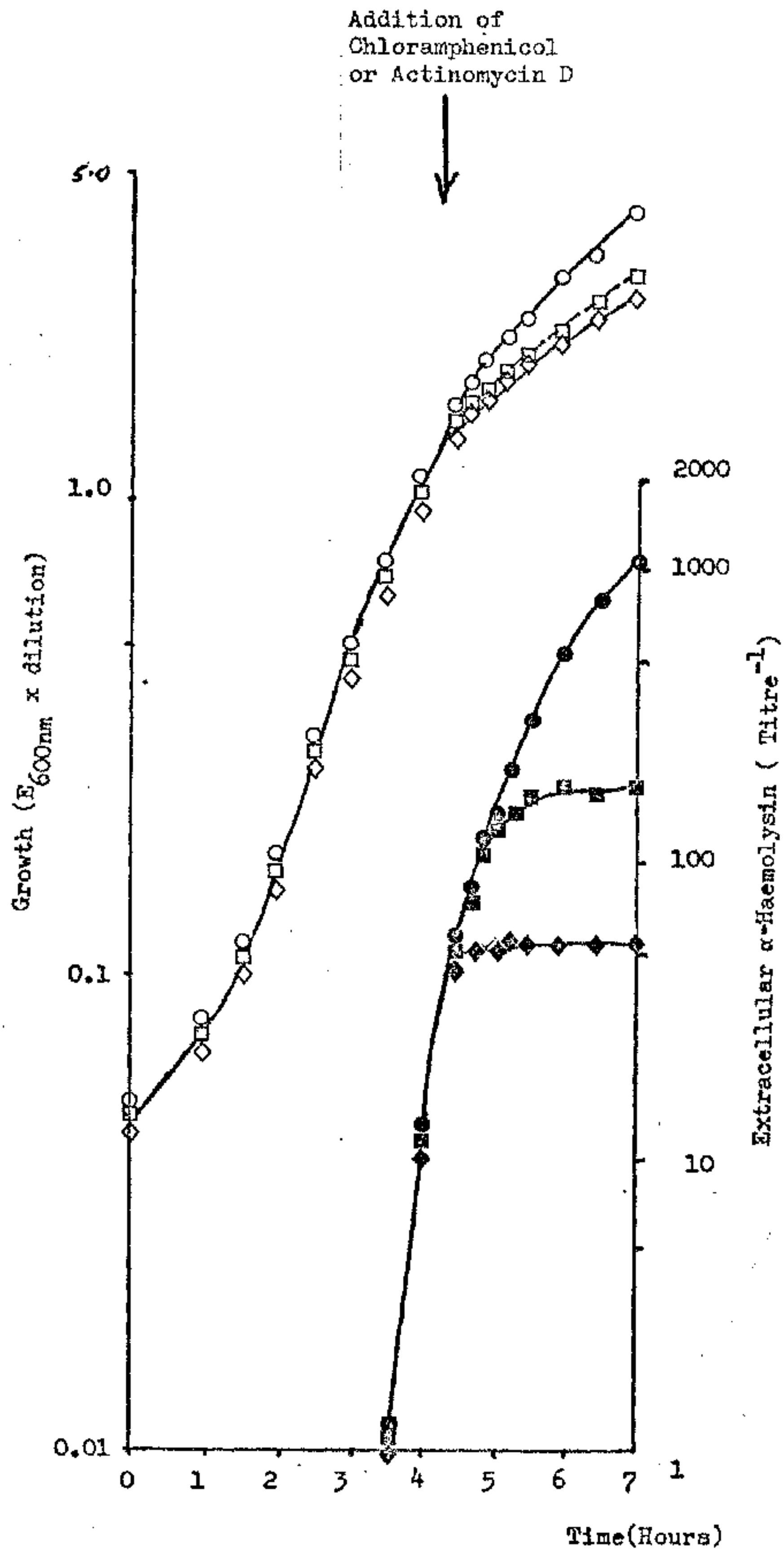
500
5.0
100
1.0
10
0.1
1
0.01
Growth (Extractable DNA µg.cm-3)
Growth (E600nm x dilution)
Actinomycin D
Addition
2000
1000
100
10
1
Extracellular Acid Phosphatase (Titre-1)
0
1
2
3
4
5
6
Time(Hours)

Figure 18

Effect of Chloramphenicol or Actinomycin D on Active Alpha-haemolysin Synthesis.

Conditions were as for Figure 3, except that Chloramphenicol (30 µM) or Actinomycin D (0.8 µM) was added at 4.5h.

E_{600nm}	Untreated	(○)
	Treated with Actinomycin D	(□)
	" " Chloramphenicol	(◇)
Alpha-haemolysin	Untreated	(●)
	Treated with Actinomycin D	(■)
	" " Chloramphenicol	(◆)



Addition of
Chloramphenicol
or Actinomycin D
5.0
1.0
0.1
0.01
Growth (E_{600nm} x dilution)
2000
1000
100
10
1
Extracellular α-Haemolysin ($Titre^{-1}$)
0
1
2
3
4
5
6
7
Time(Hours)

Actinomycin D and chloramphenicol had similar inhibitory effects on ^{E}600nm increase, resembling those obtained for addition at 2.5h (Figs. 16 & 17). Alpha-haemolysin synthesis was, however, differentially affected by these two treatments since chloramphenicol stopped alpha-haemolysin synthesis within 5min of addition to the culture, while actinomycin D treatment permitted synthesis to continue at a gradually reducing rate for 25-30 min, suggesting that durable mRNA was being used.

C.3.2. Compounds Affecting Membrane or Membrane Based Functions

Based on the report by Stouthamer (1979) the following compounds were chosen for study : Sodium Nitrite, Potassium Ferricyanide, Sodium Thiosulphate, Sodium Azide, Potassium Cyanide and 2,4.-Dinitrophenol. Conditions were the same as for experiments described in Section C.3.1. above and all cultures were assayed for ^{E}600nm, alpha-haemolysin and extracellular acid phosphatase. With the exception of Sodium Thiosulphate, all proved to be inhibitory as described individually below. Sodium Thiosulphate, at concentrations up to 30 mM had no effect on ^{E}600nm, alpha-haemolysin or acid phosphatase.

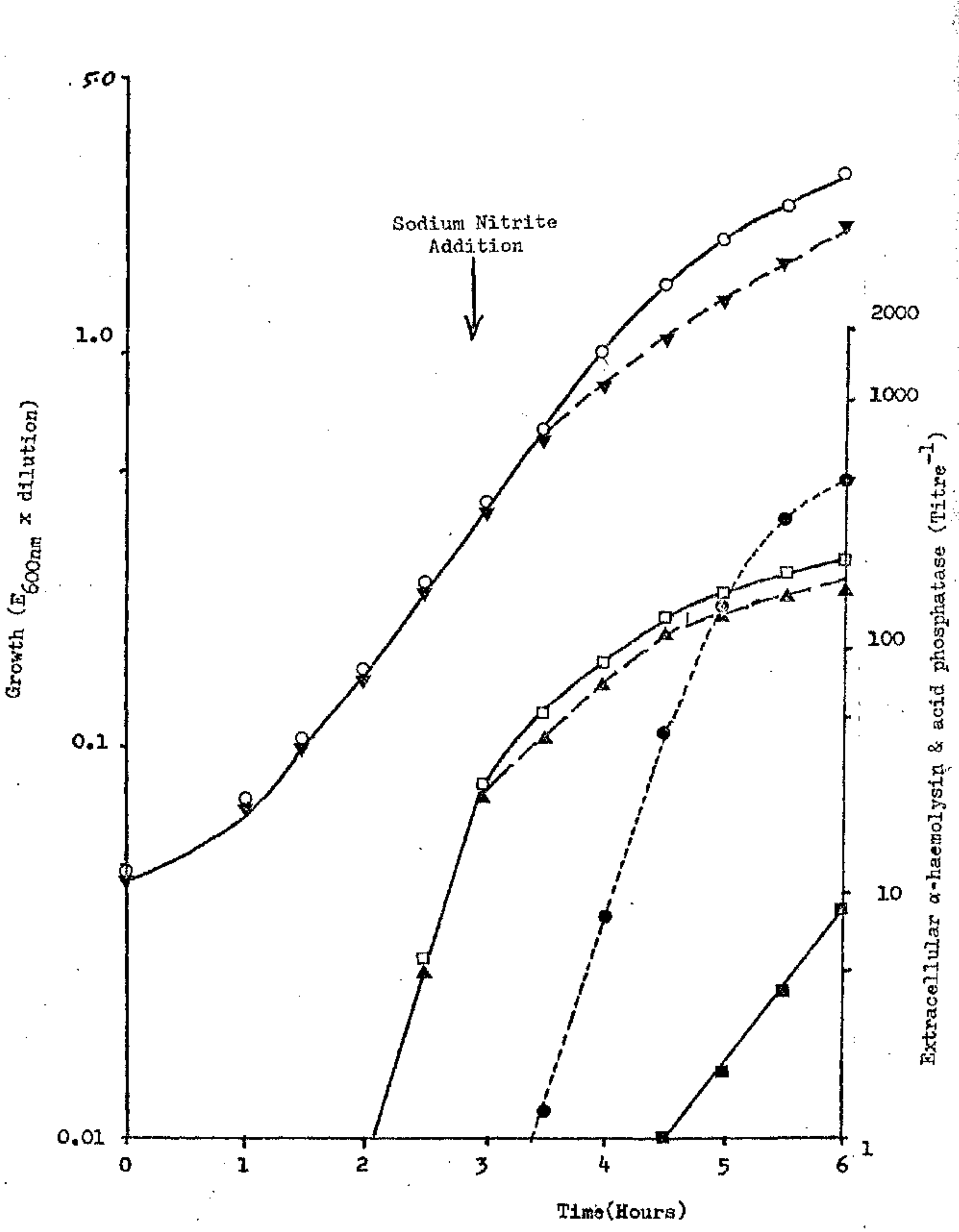
C.3.2.a. Sodium Nitrite At concentrations up to 15 mM, Sodium Nitrite had no detectable effect on any of the three parameters. Figure 19 shows the effect of 30 mM added at 2.5h. Inhibition of growth (^{E}600nm) and extracellular acid phosphatase production became detectable after 1h. The effect on ^{E}600nm was equivalent to a general reduction of about 25% by 6h so that the culture apparently entered the late log phase about 1h earlier than the control. Production of Acid phosphatase was similarly affected. Inhibition of alpha-haemolysin production resembled very closely that caused by 15mM phenethyl alcohol. The

Figure 19

Effect of 30 mM Sodium Nitrite on Growth (E_{600nm}), Alpha-haemolysin and Extracellular Acid Phosphatase Production.

Conditions were as for Figure 3 except that Sodium Nitrite was added at 2.5h to a final concentration of 30 mM.

E_{600nm}	Untreated	(○)
	Treated	(▼)
Acid Phosphatase	Untreated	(□)
	Treated	(▲)
Alpha-haemolysin	Untreated	(●)
	Treated	(■)



Sodium Nitrite Addition
5.0
1.0
0.1
0.01
Growth (E_{600nm} x dilution)
2000
1000
100
10
1
Extracellular α-haemolysin & acid phosphatase ($Titre^{-1}$)
0
1
2
3
4
5
6
Time(Hours)

treated culture had attained the same final levels as the controls after overnight incubation.

C.3.2.b. <u>Potassium Ferricyanide.</u> At concentrations up to 5 mM, Potassium Ferricyanide had no detectable effect on any of the three parameters. Figure 20 shows the effect of 15mM or 30 mM added at 2.5h. Inhibition of growth ($^{E}600nm$) became detectable after 1h. In both cases the effect was an apparent reduction in growth rate, leading to a drop of about 40% in the 15 mM culture and 50% in the 30 mM culture by 6h due to the culture entering a slower period of growth, similar to the late log phase, about 2h earlier than the control. Production of acid phosphatase was similarly affected, although it was difficult to distinguish between the effects of 15mM and 30 mM. Inhibition of alpha-haemolysin production was similar to, although perhaps slightly greater than, the effect produced by 15 mM phenethyl alcohol. There was no detectable alpha-haemolysin produced by the culture treated with 30 mM Potassium Ferricyanide. Both treated cultures had attained the same final levels as the controls after overnight incubation.

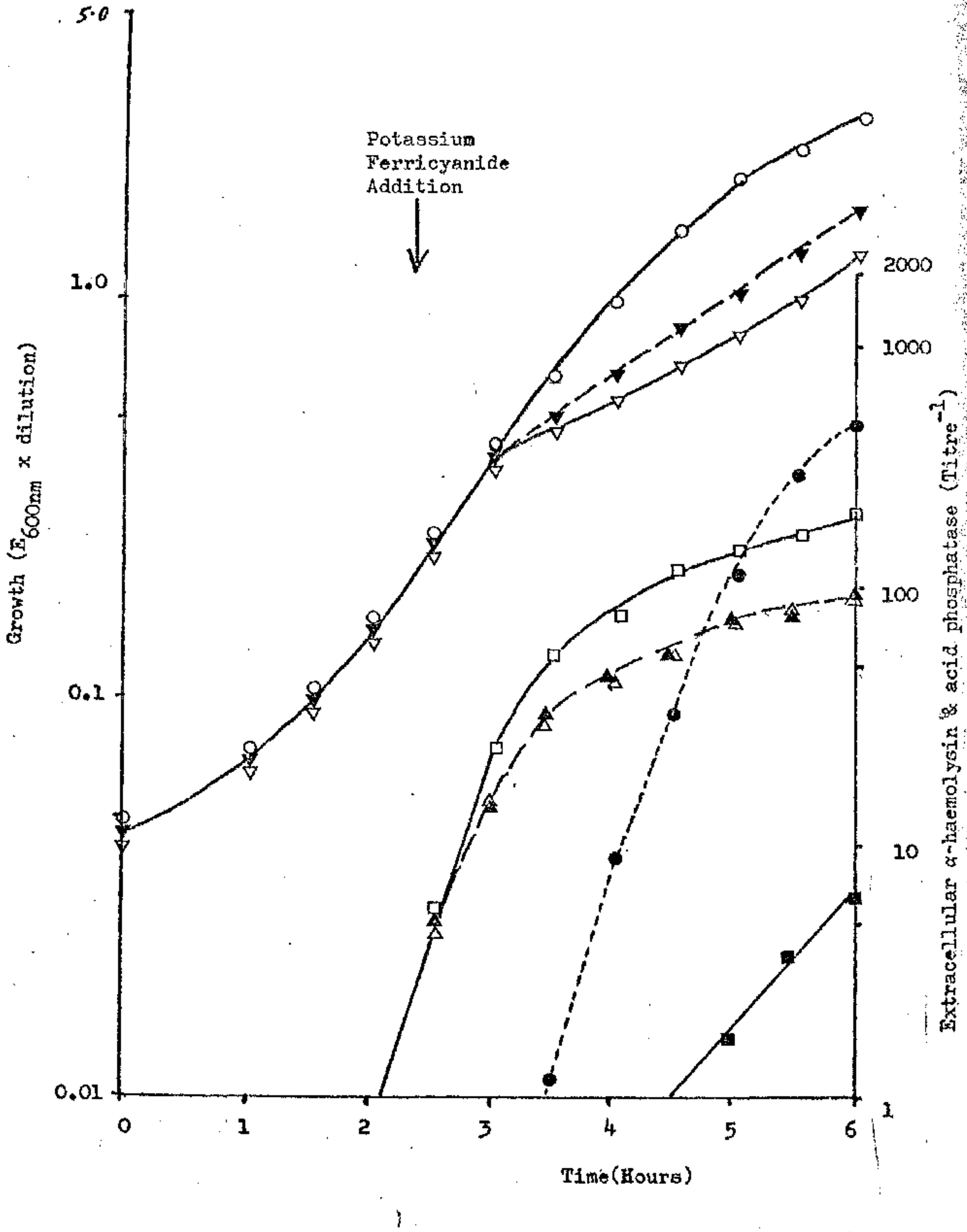
C.3.2.c. <u>Sodium Azide.</u> Sodium Azide at 1 mM had no detectable effect on any of the three parameters. However, as shown in Figure 21, addition to a final concentration of 2mM caused an immediate decline in growth rate, as measured by $^{E}600nm$, Leading to a drop of about 50% by 6h. Addition to a final concentration of 20 mM allowed the $^{E}600$ to increase slowly for about 90 min and then remain constant. Intermediate concentrations (not shown) had proportional effects. Inhibition of extracellular acid phosphatase production was similar to that of the $^{E}600nm$ except that phosphatase continued to increase slowly after

Figure 20

Effect of Potassium Ferricyanide on Growth (E_{600nm}), Alpha-haemolysin and Extracellular Acid Phosphatase Production.

Conditions were as for Figure 3, except that Potassium Ferricyanide was added at 2.5h to a final concentration of 15mM or 30mM

E_{600nm}	Untreated	(○)
	Treated with 15 mM	(▼)
	" " 30 mM	(▽)
Acid Phosphatase	Untreated	(□)
	Treated with 15 mM	(▲)
	" " 30 mM	(△)
Alpha-haemolysin	Untreated	(●)
	Treated with 15 mM	(■)



Potassium
Ferricyanide
Addition
5·0
1.0
0.1
0.01
Growth (E_{600nm} x dilution)
2000
1000
100
10
1
Extracellular α-haemolysin & acid phosphatase (Titre^{-1})
0
1
2
3
4
5
6
Time(Hours)

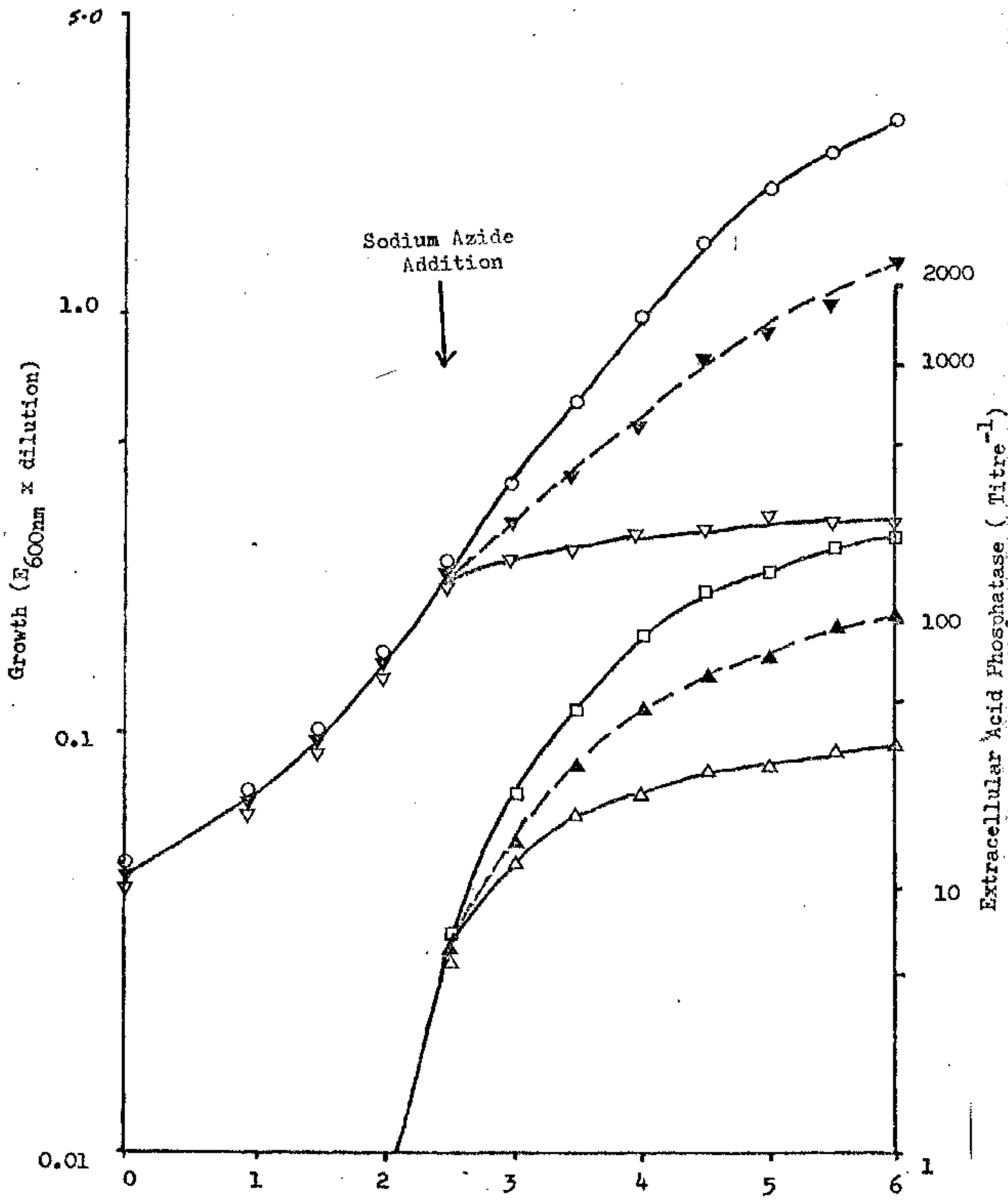
Figure 21

Effect of Sodium Azide on Growth (E_{600nm}), Alpha-haemolysin and Extracellular Acid Phosphatase Production.

Conditions were as for Figure 3, except that Sodium Azide was added at 2.5h to a final concentration of 2 mM or 20 mM

E_{600nm}	Untreated	(○)
	Treated with 2mM	(▼)
	" " 20 mM	(▽)
Acid Phosphatase	Untreated	(□)
	Treated with 2 mM	(▲)
	" " 20 mM	(△)

Alpha-haemolysin was not detected at Azide concentrations above 1 mM.



5·0
1.0
0.1
0.01
Growth (E_{600nm} x dilution)
Sodium Azide Addition
2000
1000
100
10
1
Extracellular Acid Phosphatase ($Titre^{-1}$)
0
1
2
3
4
5
6
Time(Hours)

the culture had ceased growing. Alpha-haemolysin was not produced by any of the treated cultures. However, cultures treated with 15 mM or less eventually attained the same levels as controls after overnight incubation.

C.3.2.d. <u>Potassium Cyanide</u>. As with the other compounds discussed above, Potassium Cyanide was increasingly inhibitory with increasing concentrations. Inhibition of growth rate, as measured by E_{600nm}, was immediate for all concentrations tested, as shown in Figure 22. Maximum depression due to 1 mM Potassium Cyanide was 40%. which was reached 2h post addition, but this had declined to only 25% by 6h, as the inhibited culture overhauled the control in its late log phase. Concentrations above 1 mM were proportionately more inhibitory with 5 mM, for example, causing a decline of 20% by 6h. Immediate and total cessation of growth was produced by 10 mM. Extracellular acid phosphatase fell in line with inhibition of E_{600nm}, although once again a slow increase persisted in the totally inhibited culture. Alpha-haemolysin production was anomalous in that although its first appearance in the presence of 1 mM Potassium cyanide was delayed about an hour, its rate of increase was not very different from that of the control. Alpha-haemolysin was not detected at higher concentrations although cultures treated with 2 mM or less eventually attained the same levels as controls after overnight incubation. Treatment with 2.5 mM or more led to death of the culture on incubation beyond 6h.

C.3.2.e. <u>2,4 Dinitro - Phenol</u>. As shown on Figure 23, addition of Dinitrophenol to a final concentration of 1mM caused inhibition of growth rate (as measured by E_{600nm}) within 1h resulting in a depression

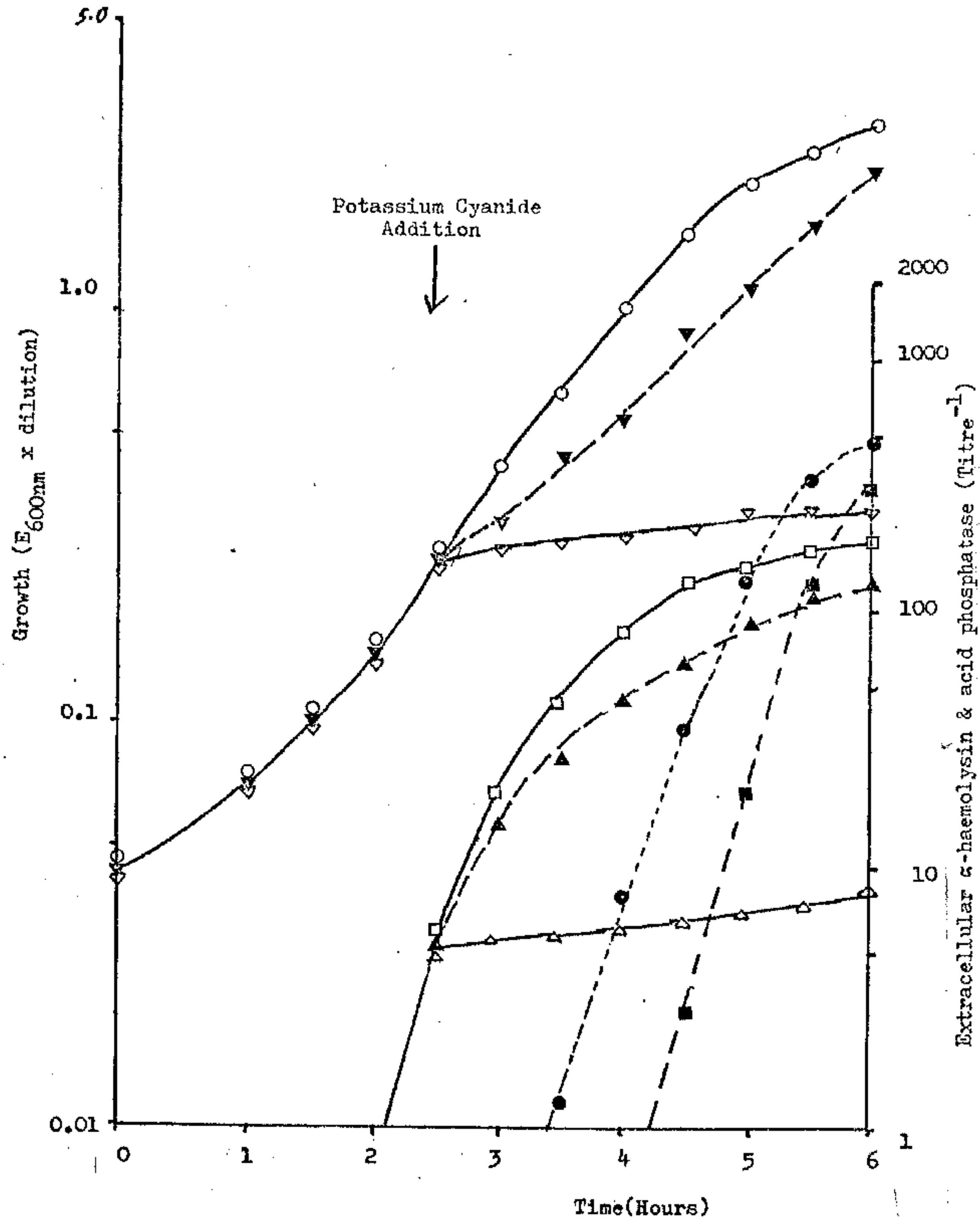
Figure 22

Effect of Potassium Cyanide on Growth (E_{600nm}), Alpha-haemolysin and Extracellular Acid Phosphatase Production.

Conditions were as for Figure 3, except that Potassium Cyanide was added at 2.5h to a final concentration of 1 mM or 10 mM.

E_{600nm}	Untreated	(○)
	Treated with 1 mM	(▼)
	" " 10 mM	(▽)
Acid Phosphatase	Untreated	(□)
	Treated with 1 mM	(▲)
	" " 10 mM	(△)
Alpha-haemolysin	Untreated	(●)
	Treated with 1 mM	(■)

Alpha-haemolysin was not detected at Cyanide concentrations above 1 mM.



Potassium Cyanide Addition
Growth (E_{600nm} x dilution)
Extracellular α-haemolysin & acid phosphatase (Titre^{-1})
Time(Hours)
5.0
1.0
0.1
0.01
2000
1000
100
10
1
0
1
2
3
4
5
6

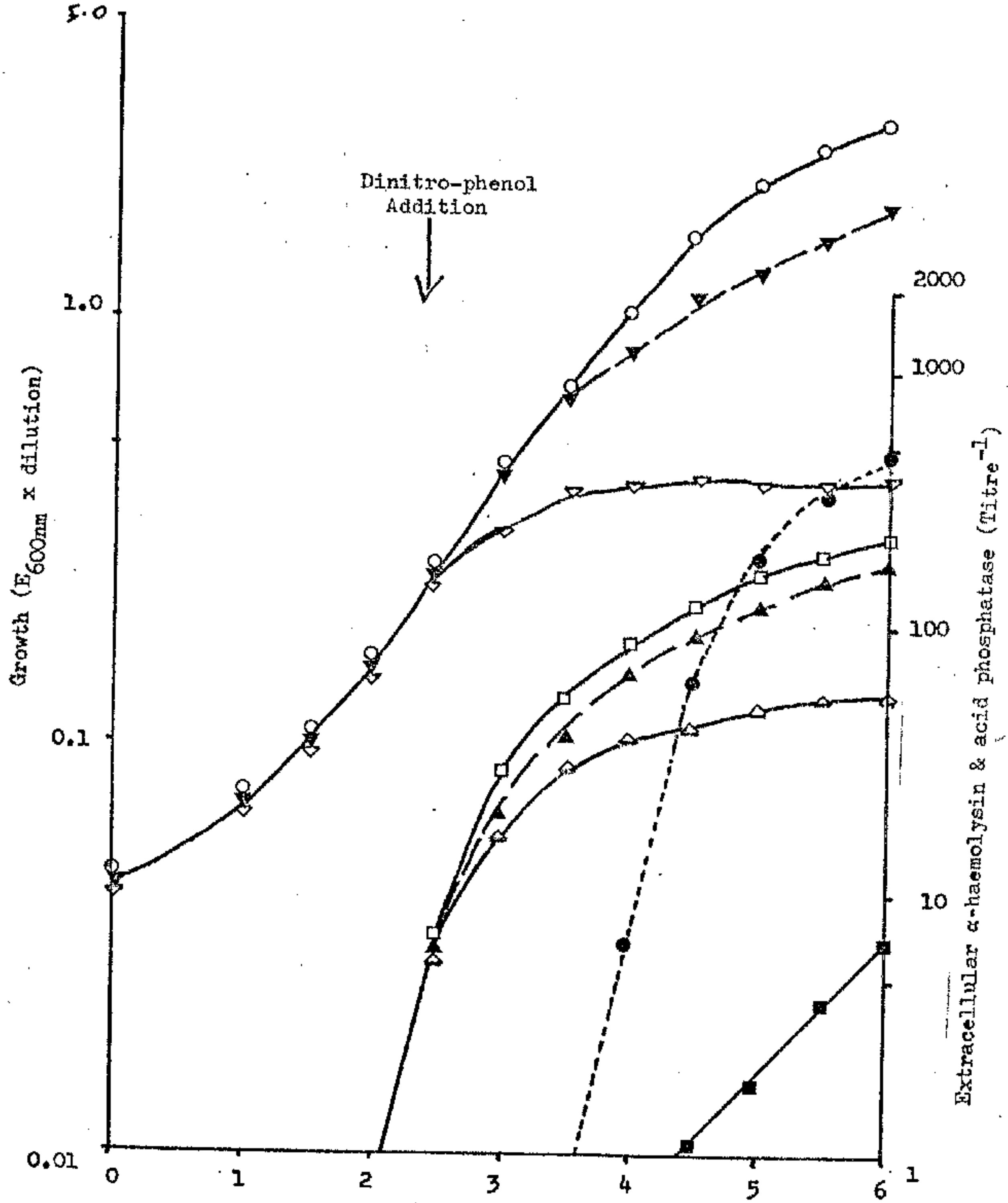
Figure 23

Effect of 2,4 - Dinitro - Phenol on Growth (^{E}600nm), Alpha-haemolysin and Extracellular Acid Phosphatase Production.

Conditions were the same as for Figure 3, except that 2,4-Dinitro-phenol was added at 2.5h to a final concentration of 1 mM or 2.5 mM.

^{E}600nm	Untreated	(○)
	Treated with 1 mM	(▼)
	" " 2.5 mM	(▽)
Acid Phosphatase	Untreated	(□)
	Treated with 1 mM	(▲)
	" " 2.5 mM	(△)
Alpha-haemolysin	Untreated	(●)
	Treated with 1 mM	(■)

No alpha-haemolysin was detected at concentrations above 1 mM.



Dinitro-phenol Addition
5.0
1.0
0.1
0.01
Growth (E_{600nm} x dilution)
2000
1000
100
10
1
Extracellular α-haemolysin & acid phosphatase (Titre^{-1})
0
1
2
3
4
5
6
Time(Hours)

of about 25% relative to the control by 6h. This was very similar to the effect of 15 mM phenethyl alcohol and inhibition of acid phosphatase and alpha haemolysin production was also very similar to that produced by 15 mM phenethyl alcohol. A final Dinitro-phenol concentration of 2.5 mM resulted in inhibition which was very similar to that produced by 30 mM phenethyl alcohol, i.e. growth rate immediately reduced and stopped after about 90 min. Acid phosphatase was similarly affected although a slow increase continued after total inhibition of growth. No alpha-haemolysin was produced. However, unlike phenethyl alcohol, this total inhibition was not readily reversible and cultures treated with 3 mM or more dinitro-phenol usually suffered losses during harvesting and above 5 mM death and culture lysis was usually detectable by 6 h. Titration of acid phosphatase and alpha haemolysin in culture supernates treated with 5 mM or more dinitrophenol was difficult or impossible, due to the colour of the reagent and its direct effect on erythrocytes. However, there is no reason to believe that these results would have differed from those which could be predicted, based on E_{600nm} and control results.

D. COMPARISON OF THE EFFECT OF PHENETHYL ALCOHOL AND DINITROPHENOL ON UPTAKE OF RADIOLABELLED AMINO ACIDS.

In order to determine whether the inhibitory effect of phenethyl-alcohol might be due to a membrane-directed reduction in the uptake of amino acids, the kinetics of uptake of some amino acids by intact cells in the presence or absence of the reagent, were determined. As a control 2-4, Dinitrophenol, which is known to inhibit amino acid transport by dissipating the membrane proton gradient (p.35), was used. Based on the results of growth experiments, shown on Figure 23, 1 mM was chosen as the Dinitrophenol concentration to compare with 15 mM phenethyl alcohol.

Washed cells suspended in P.B.S. at pH 7.0 were incubated for up to 7h to induce starved conditions before being introduced to the radio-labelled amino acid, but since transport kinetics were the same over the whole period, 30 min was used routinely. The amino acids, chosen to represent different groups (Kaback, 1974) were Glycine, L-Alanine, L-Glutamic acid, L-Glutamine, L-Lysine and L-Histidine, but the last two gave rates of uptake in the controls which were too low to make inhibition studies meaningful.

Labelled amino acid was added to 20 cm^3 cell suspension at zero time to give a final activity of $1\mu Ci.cm^{-3}$ and samples were taken at intervals, filtered to remove exogenous label and cell associated activity determined. The results are shown on Figure 24 which presents them as percentage uptake relative to the untreated controls. In the first 5 min, all treated cell suspensions exhibited a sharp decrease in uptake. After this, the relative uptake of the Dinitrophenol-treated suspensions rose due to saturation of the controls with amino acid. Uptake in the phenethyl alcohol-treated suspensions, however, continued to decline which was due to a reduction in the cell-associated label, indicating that label was effluxing from the cells under these conditions. The degree of efflux varied and was particularly marked for L-Glutamine.

E. LOCALIZATION OF PHOSPHATASE ACTIVITY

Cells from overnight culture in BS or HIB medium were fractionated according to the protocol shown in Figure 2 (p.79) and the fractions were assayed for phosphatase activity, as described below.

E.1. Determination of pH Range of Activity. Samples were assayed over the pH range 5.0-9.0. Similar patterns were obtained in each case and results for supernatant, intact protoplasts and solubilised proto-

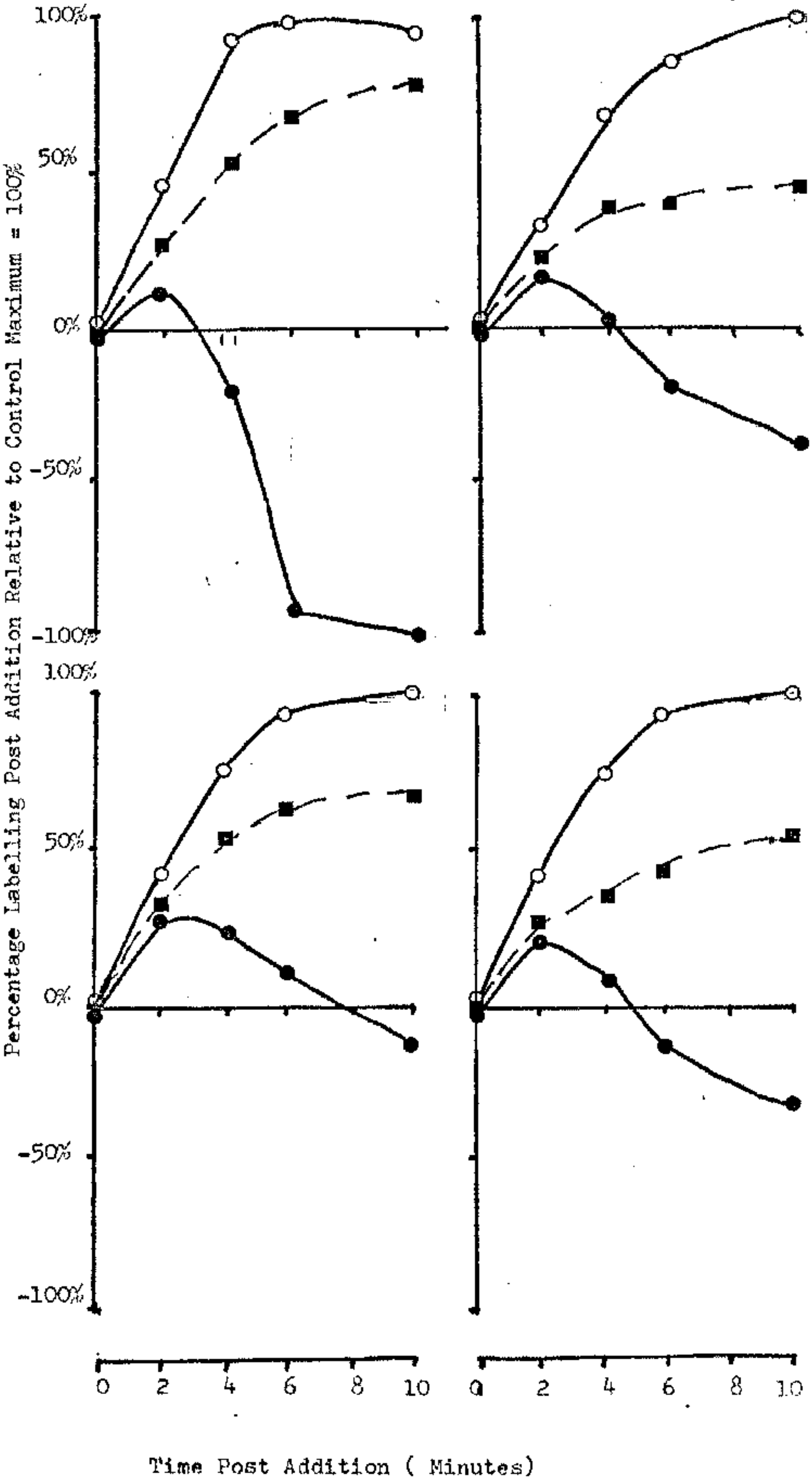
Figure 24

Percentage Uptake of L-Glutamic Acid, Glycine, L-Alanine and L-Glutamine Relative to Controls, in the Presence of 15mM Phenethyl Alcohol or 1mM 2,4 Dinitro-phenol

Cell suspensions ($20cm^3$) were presented with labelled amino acid at $1\mu Ci.cm^{-3}$ and sampled at intervals. The inhibition caused by 15mM phenethyl alcohol or 1mM dinitrophenol when added to actively transporting cells is represented as percentage uptake relative to control.

Control (Maximum = 100%) (○)
phenethyl alcohol (●)
dinitrophenol (■)

L-GLUTAMINE	GLYCINE
L-GLUTAMIC ACID	L-ALANINE


Percentage Labelling Post Addition Relative to Control Maximum = 100%
100%
50%
0%
-50%
-100%
100%
50%
0%
-50%
-100%
0
2
4
6
8
10
0
2
4
6
8
10
Time Post Addition (Minutes)

plasts are shown on Figure 25. The three peaks, in soluble or solubilised fractions, occurred at pH 6.1, 6.8 and 7.8 whereas in intact protoplasts the middle peak was at 7.1. In both cases, the main peak was at pH 6.1.

E.2. Distribution of Phosphatase Activity

The percentage distribution of phosphatase activity in the cell and its environment was calculated by determining the activity of each fraction and assigning the results to the most probable source. The results are shown in Table 7. These results clearly indicate that phosphatase activity was largely cell-associated although a significant amount of leakage was evident.

E.3. Release of Phosphatase from Whole Cells in the Absence of Growth.

To determine how tightly bound the phosphatase was, cells were harvested from 18h culture in HIB at 37°C and resuspended in fresh medium at 4°C. At intervals, samples were removed and assayed across the pH range 5.0-9.0. The results are shown in Figure 26.

Rapid release of phosphatase occurred, which continued at a decreasing rate for about 7h. Once again, the pH spectrum revealed three peaks at 6.1, 6.8 and 7.8 suggesting that the active material leaking from the suspension was the same as that normally detected in the culture supernate.

E.4. Localisation of Phosphatase Activity by Electron Microscopy

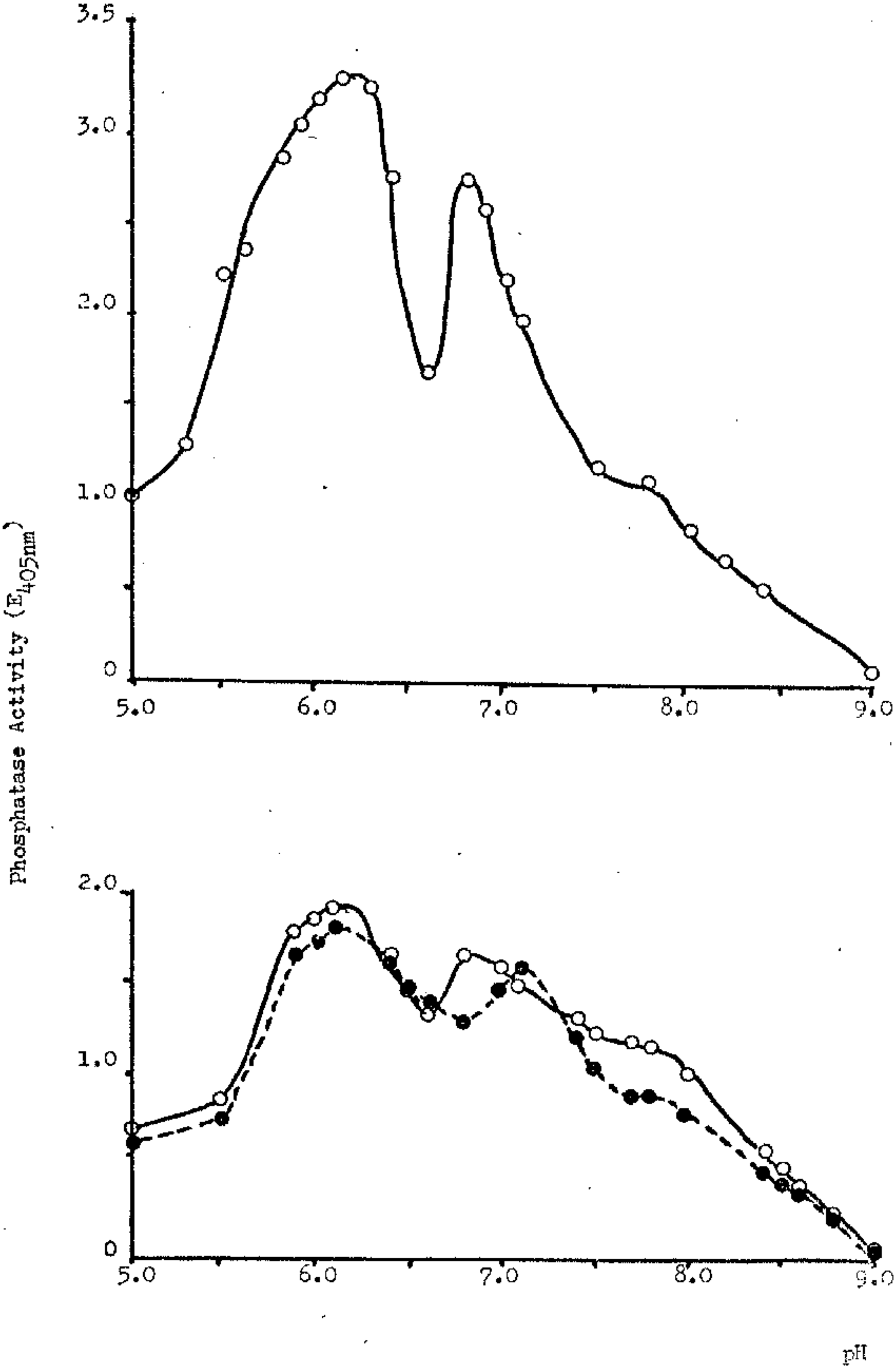
Cells from an overnight culture, either unwashed or washed once or twice, were stained by a method based on that used by Cheng *et al.*

Figure 25.

Activity of Phosphatase Over the pH Range 5-9

Samples were incubated with buffered 4-Nitrophenyl phosphate substrate for 30 min at 37°C and then the reaction terminated and the colour of the 4-Nitrophenol product intensified with 1M Na_2CO_2. Colour intensity was measured at 405nm and used as a measure of enzyme activity.

The upper figure shows activity of culture supernate. The lower figure shows activity of whole protoplasts (○) and protoplasts solubilised with Triton X-100 (●).



3.5
3.0
2.0
1.0
0
5.0
6.0
7.0
8.0
9.0
Phosphatase Activity (E_{405nm})
2.0
1.0
0
5.0
6.0
7.0
8.0
9.0
pH

Table 7

Percentage Distribution of Phosphatase Activity (pH 6.1).

Location *		Percentage of Whole Culture Activity	
Supernatant			40
	Wall	25.0	
Exterior to Protoplast			35
	Periplasm	10.0	
	Membrane	12.5	
Protoplast			25
	Cytoplasm	12.5	

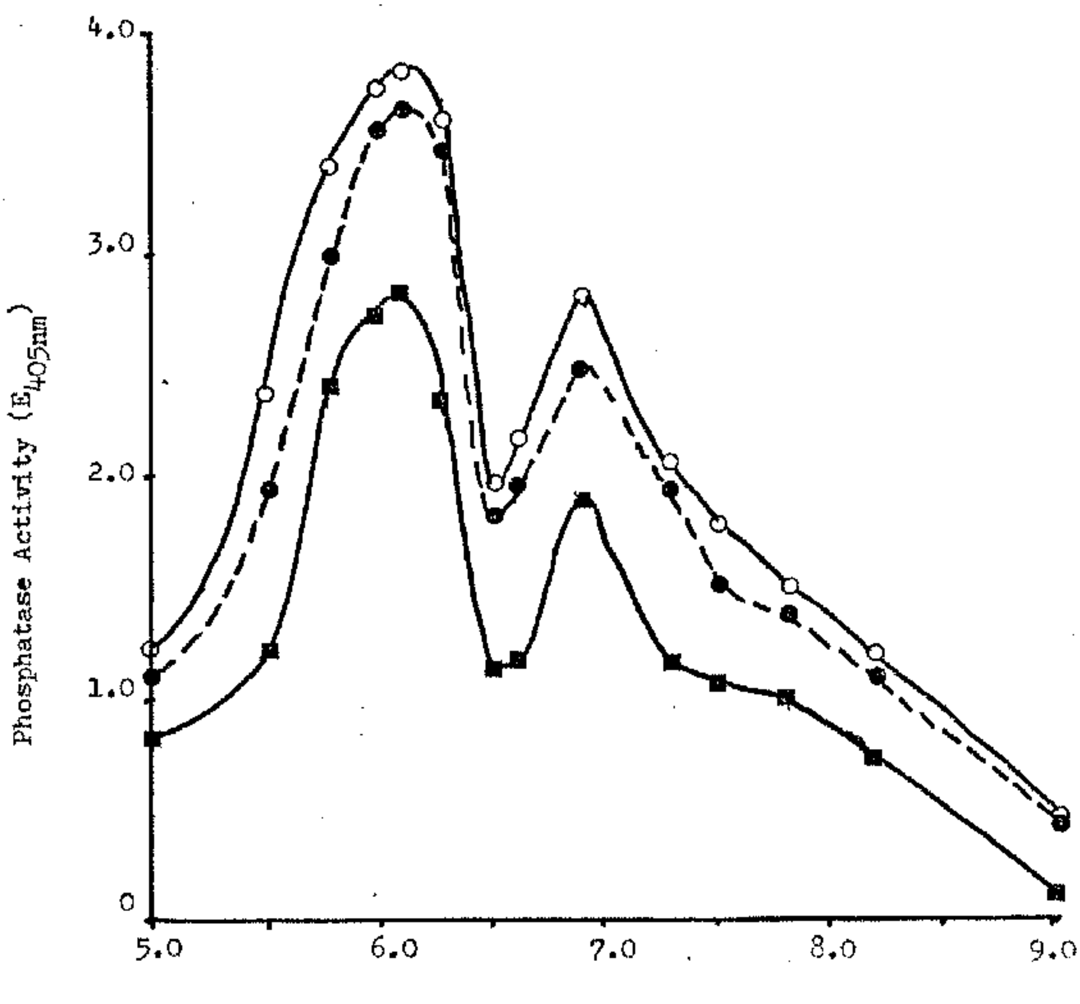
* Based on fractions derived as outlined on Figure 2

Figure 26

Release of Phosphatase from Whole Cells in the Absence of Growth.

Cells from overnight growth in HIB at $37^{\circ}C$ were suspended in fresh medium and incubated at $4^{\circ}C$. Samples were removed at intervals and Phosphatase activity assayed over the pH range 5 - 9.

Incubated for 0 hours	(○)
Incubated for 3 hours	(●)
Incubated for 7 hours	(■)



4.0
3.0
2.0
1.0
0
Phosphatase Activity (E_{405nm})
5.0
6.0
7.0
8.0
9.0
pH

Figure 27(a)

Electron Micrograph of Thin Section of Unwashed Cells Stained to Show Phosphatase Activity.

Thin sections prepared from unwashed cells from an overnight culture in HIB were stained to show phosphatase activity at pH 6.1. Phosphatase rich regions appear as electron dense patches.

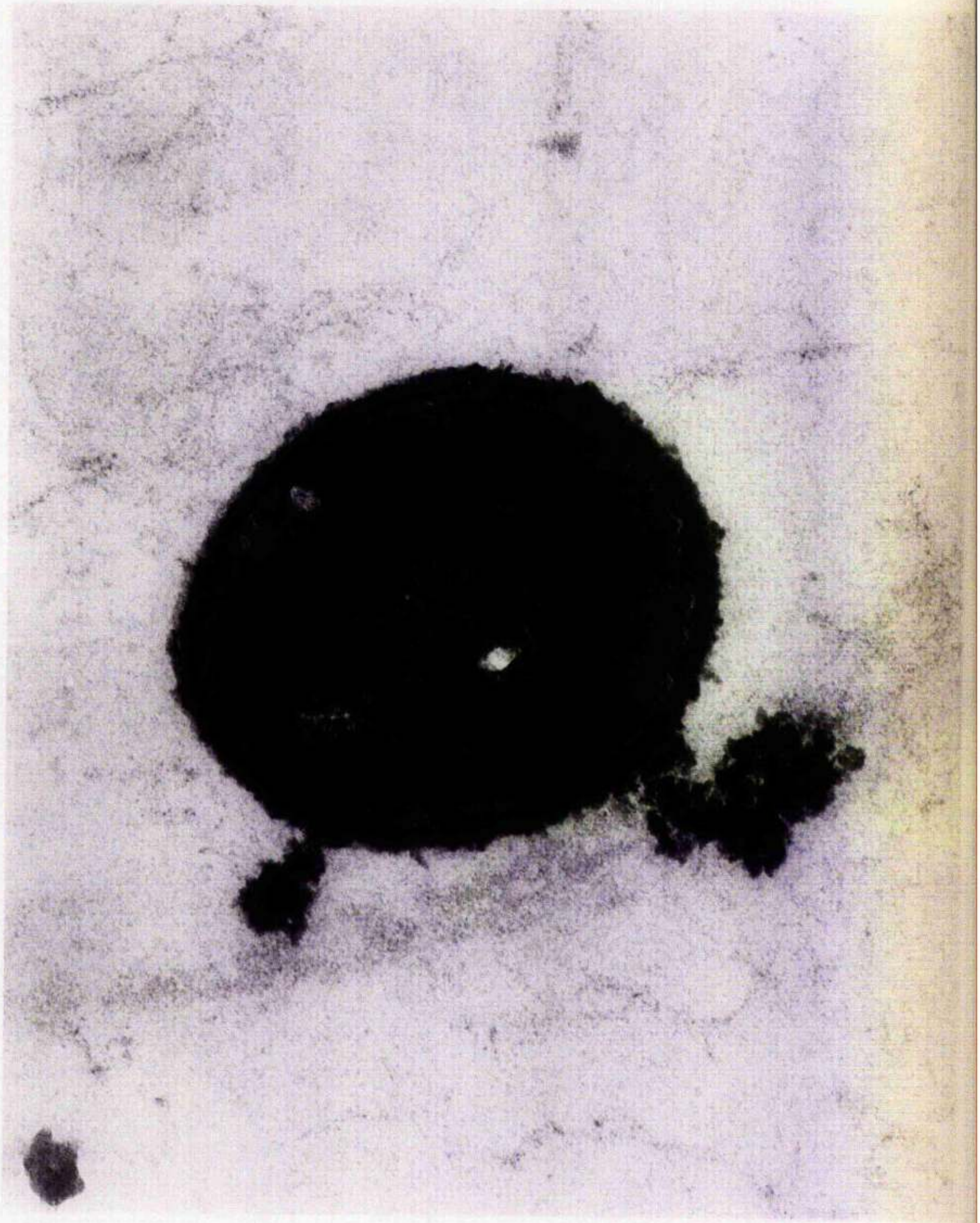


Figure 27 (b)

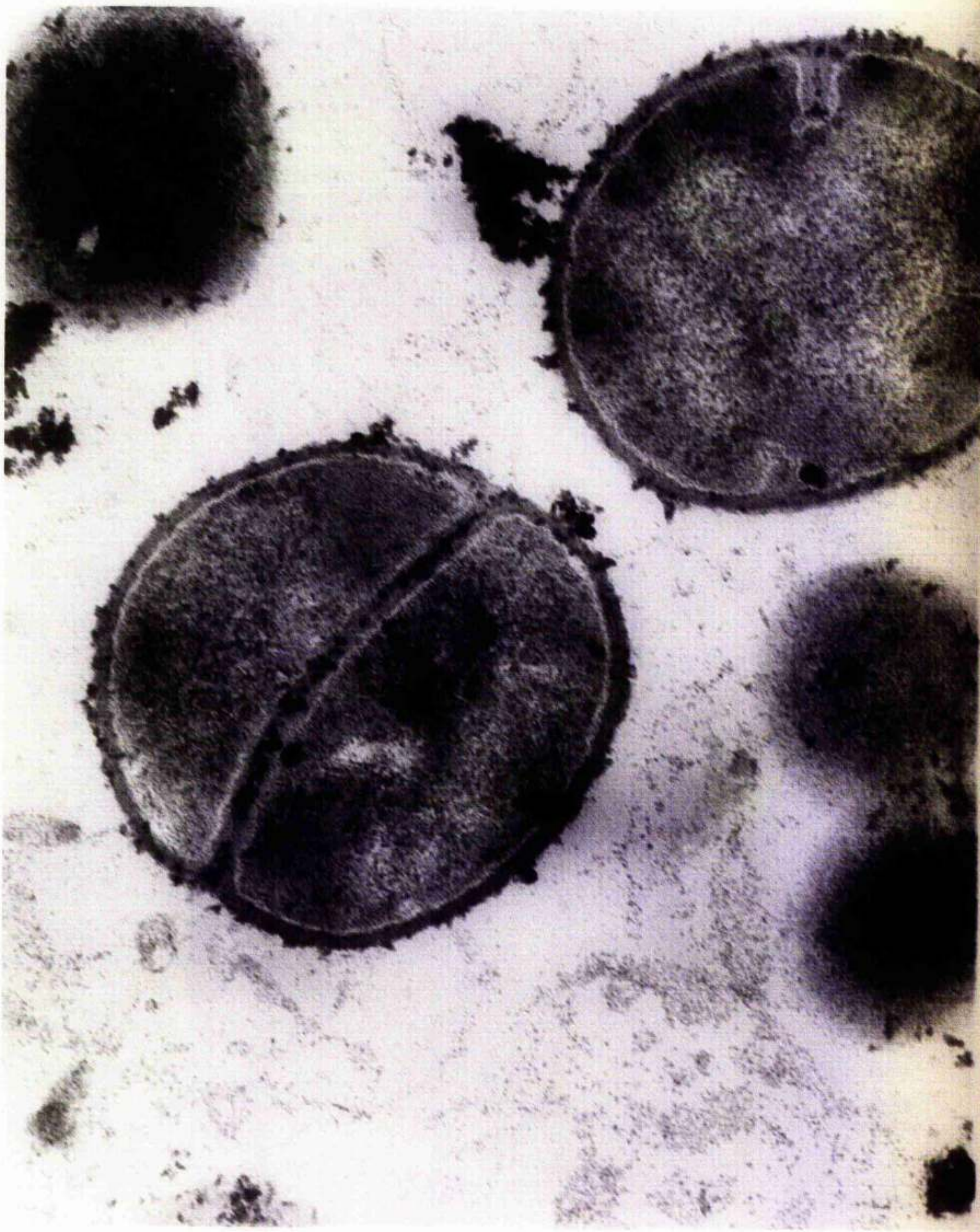
Electron Micrograph of Thin Section of Once Washed Cells Stained to Show Phosphatase Activity

The culture was identical to that used for Fig. 27(a) except that the cells were washed once in 20 mM Veronal Acetate Buffer before sectioning.

Figure 27 (c)

Electron Micrograph of Thin Section of Twice Washed Cells Stained to Show Phosphatase Activity.

The culture was identical to that used for Fig. 27(b) except that the cells were washed twice.



(1970) which showed the sites of phosphatase activity as electron dense regions of lead phosphate. Electron micrographs of thin sections prepared from these suspensions are shown in Figure 27. Phosphatase activity was seen to be concentrated in the wall, periplasm and membrane and as clumps in the cytoplasm. Washing reduced the peripheral activity considerably without reducing the number of cytoplasmic clumps. However, the general lightening of the cytoplasm suggested that considerable free activity was also present which could be easily washed out.

F. PRODUCTION, ISOLATION AND CHARACTERISATION OF VARIANTS MUTANT IN EXTRACELLULAR PROTEIN PRODUCTION.

F.1. Possibility of A Plasmid Location for the Alpha-haemolysin Genes.

As discussed before (pp 7,59) the spontaneous rate of loss of alpha-haemolysin is quite high and such rates are often associated with plasmid elimination. Since the locus of the alpha-haemolysin gene seemed to be in doubt, or perhaps strain variable, the spontaneous rate for NCTC 10344 was determined and also the effect of growth in Acriflavine (81 µm), Sodium Dodecyl Sulphate (70 or 140 µM) or growth at 42°C, all treatments designed to eliminate plasmids. With the exception of the altered conditions mentioned here, growth was carried out under the conditions used for production experiments (p 75). The frequency of non-haemolytic clones in each condition was determined. The normal frequency of spontaneous loss in these experiments was 0.02%. After growth in Acriflavine, the frequency was 0.05%, whereas in Sodium Dodecyl Sulphate, the frequency was 0.01% and at 42°C the frequency was 0.03%. These results suggested that no elimination was taking place, and this was supported by the observation that the non-haemolytic isolates were revertable at a frequency of 0.00001%.

F.2. Production of Alpha-haemolyson Deficient Mutants

The production of mutants deficient in alpha-haemolysin activity was carried out using a number of different mutagenic treatments.

F.2.1. Ultra-Violet Light

The colony forming unit of Staph. aureus is normally a small clump of cells and so attempts were made to disrupt these. Mechanical dispersion by vortexing or ultrasonic treatment proved to be effective in disrupting the clumps of cells. Typical killing curves are shown in Figure 28. Differences in sensitivity, due to age, were tested for by comparing cells from 18h (upper figures) or 48h (lower figures) incubations at 37°C on nutrient agar slopes. Sensitivity was assessed by determining the time to 1% survival and this is interpolated onto figure 28. Cells from 18h and 48h cultures were equally sensitive. Since UV killing follows 1-hit kinetics, the linear portion of the graphs has been extrapolated to demonstrate that all populations contained multiple genomes.

Samples for production of mutants were exposed in glass petri dishes until 1% survival and plated out on 3% sheep blood agar plates to give 50-100 c.f.u. per plate. After overnight incubation at 37°C, non-haemolytic isolates were subcultured onto sheep blood plates to check for stability of the mutation and confirmatory tests, as described on p. 80 were carried out to ensure that isolates were Staph. aureus.

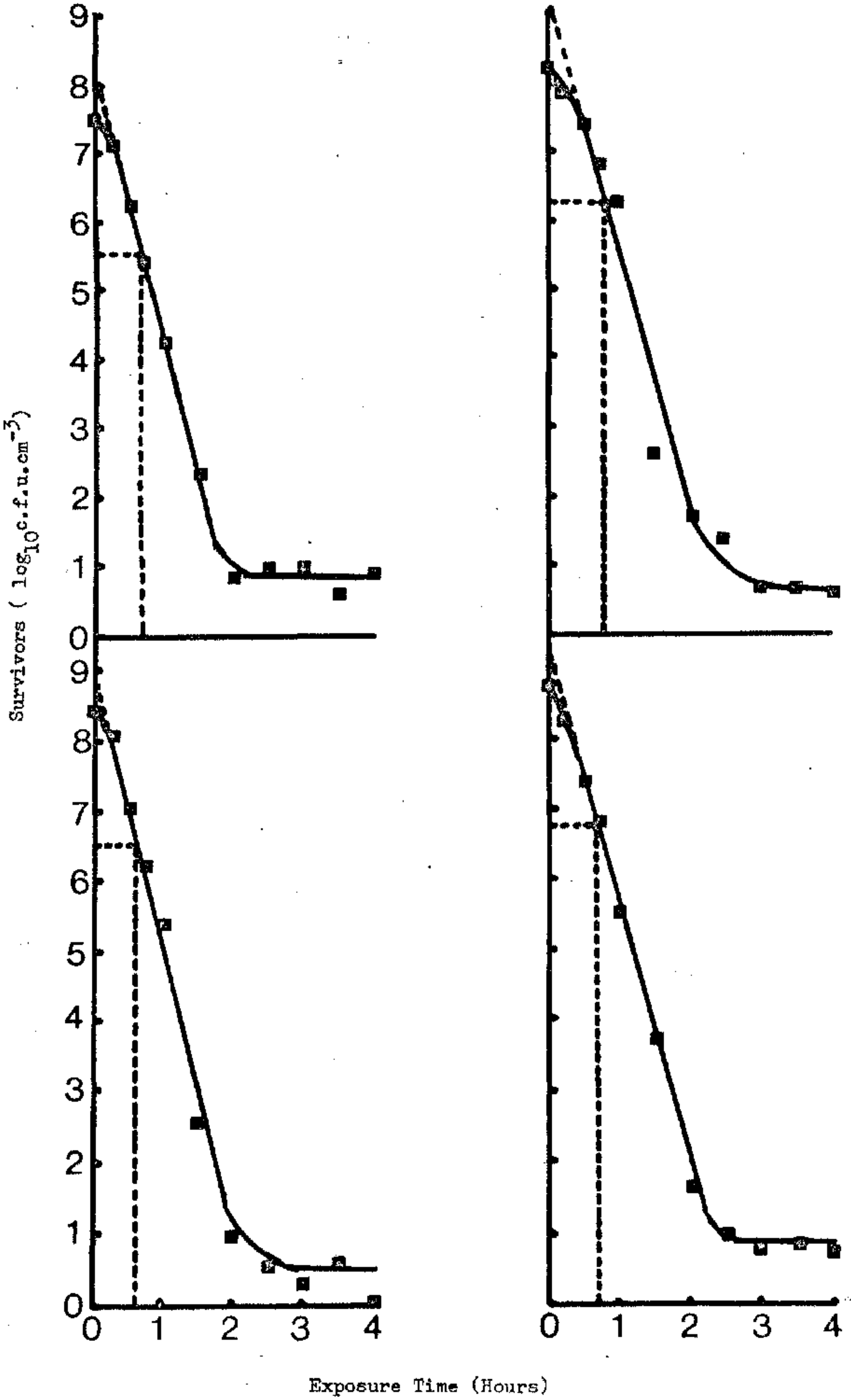
F.2. Chemical Mutagens

MNNG, 5-Bromouracil, 2'6 Amino purine, Ethylmethane Sulphonate and

Figure 23

Effect of Age and Clump Size on Survival After Exposure to U.V. Irradiation

Populations of Staph. aureus were prepared by suspending growth from blood agar base slope cultures incubated at 37°C for 18 hours (upper figures) or 48 hours (lower figures). Undispersed populations are shown at left and populations dispersed by vortex + beads are shown at right. Irradiation in 2 cm^3 saline. All curves have been extrapolated to permit calculation of the number of genomic units. In addition, time to 1% survival has been interpolated on all figures.


Survivors ($\log_{10}$c.f.u.cm^{-3})
Exposure Time (Hours)
0
1
2
3
4
5
6
7
8
9

Nitrous acid were also used as mutagens. After exposure to the mutagen, the cultures were plated out on 3% sheep blood agar plates to give 50-100 c.f.u. per plate and mutants isolated as for UV treated cultures above.

F.3. Characterisation of Mutants

The mutant clones isolated above were examined for ability to produce other extracellular activities and for normal growth characteristics.

F.3.1. Demonstration of Possible Pleiotropic Mutations. A total of 67 mutants were examined for all of the characters described previously (p.82-84). They had all retained the ability to ferment the carbohydrates, although some, which were very slow growing, could do so only at a reduced rate. The general pattern of mutants fell into five distinct groups (Table 8)& mutants of each group could be found after treatment with any of the mutagens. Colonial morphology for groups 1-4 was similar to that of wild type; group 5 mutant colonies were much smaller , about 1mm after 24h growth. Six spontaneous mutants were also examined and their patterns indicated that they were two group 1, one group 2 and three group 4.

Reversions to wild type were found in all cases although the back mutation rate was low (0.00001%).

F.3.2. Temperature Sensitivity. All of the mutants were examined for possible production of haemolysin at 30^{o}C, or for a haemolysin which was produced at 37^{o}C but was inactive at this temperature, but active at 30^{o}C. There was no evidence to suggest that these mutants were temperature sensitive.

F.3.3. Investigation of Possible Co-operative Effects. The mutants were cross streaked on 3% sheep blood agar plates to see if there was any evidence of co-operative effects. Some group 2 mutants showed slight haemolysis when cross-fed by some group 1 mutants although this

TABLE 8

Possible Pleiotropic Mutations of Alpha-haemolysin Deficient Isolates.

Group	Phenotype	Percentage of Mutants *
1	$H^-S^+C^+N^+L^+Ca^+G^+P^+B^+Car^+$ $H^-S^rC^+N^+L^+Ca^+G^+P^+B^+Car^+$ $H^-S^rC^rN^+L^+Ca^+G^+P^+B^+Car^+$	27
2	$H^-S^-C^-N^{\pm}L^{\pm}Ca^{\pm}G^{\pm}P^+B^+Car^+$	24
3.	$H^-S^-C^-N^-L^-Ca^-G^+P^+B^+Car^+$	9
4.	$H^-S^-C^+N^+L^+Ca^+G^-P^+B^+Car^+$	16
5	$H^-S^-C^-N^-L^-Ca^-G^-P^-B^-Car^+$	24

* 67 Alpha-haemolysin deficient mutants were examined.

KEY:

H - Alpha-haemolysin; S - Staphylokinase

C - Coagulase; N - Nuclease

L - Lipoprotein lipase; Ca - Caseinase

G - Gelatinase; P - Phosphatase

B - Bound Coagulase; Car - Carbohydrates fermented

\- Characteristic absent; r - Characteristic reduced

± . Characteristic not present or present at low levels.

observation was not always reproducible.

F.3.4. Replica Plating Study

Populations of Staph. aureus were exposed to UV as described previously, samples spread on plates of 3% sheep blood plates to give 50-100 colonies per plate and incubated for 12 h at 37°C. These were used as master plates for replica plating experiments, as described on page 86. Results are shown on Table 9.

Group 5 phenotypes were not detected in this experiment because the initial incubation period of 12h, necessary to ensure small colonies on the master plate, was too short to provide adequate clones of these mutants. Because of the nature of replicated colonies, it was not always possible to make a clear decision regarding the strength of a particular reaction. In these cases subjective decisions were made.

TABLE 9

Possible Pleiotropic Mutations in Alpha-haemolysin Deficient Mutants Compared by Replica Plating

Group	Phenotype	Percentage* of Mutants
1	$H^-S^+N^+L^+Ca^+G^+P^+$ $H^-S^+N^+L^+Ca^+G^+P^+$	42
2	$H^-S^-N^{\pm}L^{\pm}Ca^{\pm}G^{\pm}P^+$	33
3	$H^-S^-N^-L^-Ca^-G^+P^+$	16
4	$H^-S^-N^+L^+Ca^+G^-P^+$	10

* Mutants were H^- colonies on the master plate which were then examined on the replicas.

KEY : As for Table 8 except that Coagulase (C and B) and Carbohydrate Fermentation are not represented, due to lack of a suitable plate test.

DISCUSSION

DISCUSSION

A. INTRODUCTORY REMARKS

The aim of this work was to investigate the inhibitory action of phenethyl alcohol and to use this compound, and others, to investigate the control of extracellular protein production in *Staphylococcus aureus*. To provide contrast, alpha-haemolysin and acid phosphatase were chosen as the two extracellular proteins to be investigated, the former because there was strong evidence that it was a true extracellular protein (p.24) and the latter because there was reason to think that it might be a cell-associated enzyme whose release from the cell was accidental or, at least, incidental (p. 25). As will be discussed later (p. 181-182) the degree to which this enzyme activity was cell-associated has been determined, confirming its choice for this work. Since phenethyl alcohol has been shown to affect growth rate, DNA replication and membrane structure and function, attempts were also made to investigate these effects in *Staph. aureus*. In the following discussion individual sections will be considered separately and finally synthesised.

B. GROWTH AND EXTRACELLULAR PROTEIN PRODUCTION UNDER NON-INHIBITED CONDITIONS.

As a basis for future studies, the effect of media composition on growth and extracellular protein production was investigated, as this had been reported to be significant (p. 44-50). Since the majority of experiments were to be carried out in BS medium the effect of varying its composition was investigated.

B.1. Effects of Varying the Composition of BS Medium.

B.1.1. Effects on Growth.

Growth was measured by increase in E_{600nm}, increase in extractable DNA ($\mu g.cm^{-3}$) and increase in viable count ($c.f.u.cm^{-3}$), and the results of varying medium composition are shown in figures 3-6. BS medium is a complex medium containing glucose, yeast extract dialysate, casamino acids, nicotinic acid and thiamine.

An initial inoculumn of 1.10^{8} $c.f.u.cm^{-3}$ Staph.aureus grown aerobically in the complete medium at 37°C displayed a lag phase of about 1h. This was followed by an exponential phase lasting about 4h during which the population doubled approximately every 35 min. Growth rate then began to slow down, falling to one doubling every 90 min by 7h. This rate continued until the population entered the stationary phase at about 12h. This pattern was produced by all three of the measuring systems and was similar to that reported by other workers using complex growth media (p.44-45).

B.1.1.a. Role of Complex Nitrogen Sources. Reducing the concentration of either the casamino acids or the yeast extract dialysate by 50% produced quantitative changes in the growth rate of the culture (Figure 4). Since these two components account, in roughly equal amounts, for virtually all of the nutrient supply, it might be expected that the difference in growth rate would be about 25%, but the doubling time in early log phase was still about 35 min. However, transition to late log phase occurred about one generation earlier and the final stationary value (measured by $^{E}600nm$) was about 30% down. It seemed likely that this was due to a reduction in the concentration of some essential component or components which the cell was either unable or only poorly able to synthesise from other compounds. The minimal medium derived for this strain (p.67-68)contained Aspartic acid, Cysteine, Glycine, Histidine, Nicotinamide and Thiamine, which all appeared to be essential for growth. Of these six compounds, Cysteine & Histidine are probably the rarest in complex growth media, although this was not investigated.

Diluting the whole medium by 50% produced essentially the same effect as diluting either one by 50%. The reason for this is unclear since logically this medium should be more seriously depleted, and consequently should give a lower yield. This will be reconsidered later (p. 159-160).

Dilution of the medium beyond 50% led to obvious lengthening of the doubling time and lower growth yield. At 90% dilution, the culture grew for about five generations and then underwent an apparent lysis. The most likely explanation for this would appear to be a shortage of cell wall precursors, while other materials were still available, i.e.

a seriously unbalanced growth condition.

B.1.1.b. Role of Carbon and Energy Source. The normal carbon and energy source in BS medium is 14mM Glucose. However, changing glucose concentration or eliminating it from the medium had no apparent effect on doubling time or final growth yield. Replacement of Glucose by 14mM Acetate gave results which were similar to 50% reduction in Nitrogen source. The reason for this might be an increase in maintenance energy requirement or an increase in respiration rate, conditions discussed by Stouthamer (1979) (p.33-36). Inhibition in the presence of 14mM succinate was even more marked. Since this compound was presumably taken directly into the Citric Acid cycle, a stimulation of respiration rate seemed most probable (p.35).

B.1.2. Effects on Extracellular Protein Production. The extracellular level of alpha-haemolysin, acid phosphatase, gelatinase, staphylokinase and coagulase was assayed in all experiments. However, only the first two were consistently detected and since these had been chosen for more detailed study, most attention will be given to them. Two general points should be noted: Within the limits of the assays, alpha-haemolysin, gelatinase, staphylokinase, coagulase, behaved in a similar manner in the different growth media and none of them were detected until exponential phase was already established. Acid phosphatase activity was usually detectable just after exponential phase began, although if the inoculum was only washed once rather than twice, it was possible to detect residual acid phosphatase at zero time.

The first appearance of all the activities in BS medium was clearly related to the population density since the E_{600nm} of the culture at the time when one titre^{-1} unit for a particular protein could be detected was constant. Production of acid phosphatase began at E_{600nm} of 0.16 ± 0.02 and alpha haemolysin at E_{600nm} of 0.68 ± 0.11. Similar restricted conditions could be produced for extractable DNA or viable count although it was not possible to calculate confidence limits due to the restricted number of readings. Staphylokinase, gelatinase and coagulase were first detected at mean E_{600nm} of 0.23, 1.3 and 2.0 respectively. A similar relationship between E_{600nm} and appearance of alpha-haemolysin was reported by McNiven (1972).

The differences detected here might be real and certainly the assay of acid phosphatase must include some leakage of preformed material from the inoculum which is presumably not the case for the other proteins. If the other proteins were under some form of common control, as discussed later (p.189), it might be expected that all four would be detected together. However, the sensitivity of the different assays would be expected to vary due to differences in the Km and Vmax of the 'enzymes'. The absolute number of molecules of each protein might also vary even if they were under common transcriptional control due to secondary controls at this level or to translational controls.

B.1.2.a. Role of Complex Nitrogen Sources. Reducing the concentration of either the casamino acids or the yeast extract dialysate by 50% produced changes in line with the changes in growth parameters. Although there was a slight delay before the different components appeared, compared to controls, the E_{600nm} values at time of first appearance were similar to those in the complete medium. The same was true for medium which had been diluted 50%. In medium which had been diluted 60% only the phosphatase and alpha-haemolysin were detected. Phosphatase was apparently still produced in a manner consistent with growth rate, but the appearance of alpha-haemolysin was significantly delayed until E_{600nm} of 2.0 and production rate was reduced about 55%. Such a pronounced inhibition suggested that production of alpha-haemolysin, and by implication staphylokinase, gelatinase and coagulase, was controlled by central metabolism, either precursor supply or ATP supply being the likeliest contenders.

In medium which had been diluted 90% the phosphatase was the only activity to be detected. Cultures growing in this medium underwent an apparent massive lysis at 5.5h. Extracellular phosphatase activity continued to increase reflecting the release of cell associated activity from a cytoplasmic or periplasmic location. Extracellular levels of the other proteins did not appear during this lytic phase, suggesting that cell-associated material was not present.

B.1.2.b. Role of Carbon and Energy Source. As noted for growth, doubling, reducing or removing glucose from the medium had no apparent effect on synthesis, but its replacement by acetate or succinate at 14mM did. Acid phosphatase was altered in line with the changes in growth but alpha-haemolysin and the others were more significantly affected. The effect of acetate was to delay the first appearance of alpha-haemolysin until an E_{600nm} of 1.0 and to reduce production rate by about 30%. Succinate only delayed first appearance until an E_{600nm} of 0.82 but reduced production rate by about 40%. These results were difficult to fit into a general model. The lack of any effect due to glucose argued against any form of catabolite repression, but it was not impossible to consider that the way in which energy was produced within the cell was a controlling factor.

B.2. Growth and Extracellular Protein Production in Other Media.

B.2.1. Proprietory Media.

The growth rate, yield and extracellular protein production in complex, proprietory growth media was investigated. The rich media Heart Infusion Broth (HIB) and Trypticase Soya Broth (TSB) were similar to BS medium and 50% diluted BS medium respectively. In Lab Lemco Broth (LLB) which is nutritionally much poorer, the culture doubling time was about 60 min (Figure 7) and the total growth yield was only about 10^9 c.f.u.cm^{-3}. Since alpha-haemolysin was not usually detected until this population density was achieved, it might be expected that this activity would be absent. However, traces of haemolytic activity, although rarely more than two haemolytic units.m^3, could be found after the culture had effectively ceased growing. The first appearance was detectable at E_{600nm} of 0.5 which is much lower than usual. This was probably not significant and might be due to physical differences such as cell size, although this was not obvious under the microscope.

These results suggested that the nature of the medium components was less significant than the overall supply and this was investigated further.

B.2.2. Defined Media. A minimal medium was devised for strain NCTC 10344 which contained glycine, aspartic acid, cysteine, histidine, nicotinamide and thiamine as well as glucose and salts. In this medium, growth was very poor with doubling times of about 100 min and only acid phosphatase was detected. Yield of cells was poor reaching a maximum of about $1.5.10^8$ c.f.u.cm^{-3}. Minor additions did not significantly affect growth in this medium. However, addition of glutamic acid, glutamine, lysine and proline in combination, produced a significant stimulation in overall growth yield, doubling time was reduced to 60 min. and alpha-haemolysin was produced. These results implicated members of the glutamate family in the control of alpha-haemolysin production and tended to suggest that overall supply was less important than nature of the media components. However, in view of the central role which glutamate usually plays in amino acid and nucleotide synthesis, it was not too surprising that overall supply of this compound might be important.

B.3. General Conclusion on Non-inhibited Conditions.

Production of acid phosphatase appeared to be a growth linked event, whereas the appearance of alpha-haemolysin and possibly the other extracellular proteins, was dependent, not only on growth, but on adequate supplies of medium components for the maintenance of growth. The fact that growth and protein production was very similar in media which were made significantly different by alteration of the concentration of the components, made it seem probable that there was a natural upper limit to the assimilation rate. In BS medium, for example, the normal medium would be excessively loaded with metabolites so that the cells could obtain maximum levels of all precursors and would thus grow and produce extra-cellular proteins at the maximum rate. Even if some, or all, of the medium components were reduced by 50%. supply would still be sufficient to ensure maximum production, although overall yield might be affected by the shortage of some important or essential ingredient. Where the cells have to balance their growth requirements against supply, the ability to produce a phosphatase would be highly important to the maintenance of the supply. Other extracellular proteins might be of use in conditions of shortage, but since they represent a potentially large loss of material, it would probably be more advantageous to the cell to produce them only when there was an excess of precursor.

C. GROWTH AND EXTRACELLULAR PROTEIN PRODUCTION IN BS MEDIUM TREATED WITH VARIOUS POTENTIAL ANTIMETABOLITES.

The results discussed in this section were obtained with two aims in view; to deduce some information about the production of alpha-haemolysin and acid phosphatase by analysis of the effects of various antimetabolites and to deduce some information about the mode of action of phenethylalcohol by comparison with the results obtained from the other reagents.

C.1. Effects of Phenethyl Alcohol.

As described in the introduction (p.51-55) phenethyl alcohol has been shown to affect DNA synthesis in *E. coli*, possibly through an action on membrane located protein. A similar effect has been noted in *Staph. aureus* although the mechanism has not been determined. Inhibition of extracellular protein production was also noted but no detailed analysis has been reported. The implication in most cases has been that phenethyl alcohol directed its effect through the membrane and changes in membrane fluidity have been demonstrated in *E. coli*.

The two components of the system are the cells and the phenethyl alcohol, so initial investigations examined the effects when either of these was varied while the other remained constant.

C.1.1. Effects of Phenethyl Alcohol at Various Concentrations.

Experiments were set up, as for the media experiments above, using BS medium inoculated with 1.10^8 c.f.u.cm^{-3} of twice washed cells, incubated aerobically at 37°C. Phenethyl alcohol was added at concentrations from 0-45 mM to early log phase cultures at 2.5h.

C.1.1.a. Effects on Growth Rate. Figures 9(a) and (b) show the effect of phenethyl alcohol on growth, measured as E_{600nm} or extractable DNA ($\mu g.cm^{-3}$).

The degree of inhibition increased up to 30 mM and at this concentration, both parameters exhibited an increase of approximately 50% over 30-60 min, and then ceased to increase. Such behaviour would be consistent with a blockage of DNA replication at the initiation stage. The cycles of replication which were in progress at the time of phenethyl alcohol addition would go to completion, but no new cycles would begin. The cells were not permanently damaged by this treatment because removal of the inhibitor by centrifugation followed by resuspension in fresh medium at 37°C led to an immediate reinitiation of growth.

At concentrations of phenethyl alcohol above 30 mM, total inhibition of both growth parameters occurred in less than 30 min. The extractable DNA levels actually fell after such treatment, but since the external 260/280 ratio of the populations did not vary, it seemed likely that this fall was an artifact of the DNA assay system. Clearly the cells were severely damaged by treatment with such high concentrations of phenethyl alcohol since the recovery times after removal from inhibitor were greatly extended. The most likely cause was damage to the integrity of the membrane.

Inhibitory effects might be considered as either competitive or non-competitive with respect to the substrate on which the cells are growing if the equations derived by Pirt (1975) are employed.

He made the simplifying assumption that bacterial cells behaved as enzymes reacting on the growth-limiting substrate and then derived equations for the two different conditions by analogy with enzyme kinetics. Such an assumption follows naturally from the formal similarity between the mediated permeation of substrates into cells and classical enzyme kinetics (Finean, Coleman and Michell, 1974, p.29).

In <u>competitive inhibition</u> it is assumed that the inhibitor competes with the growth-limiting substrate for uptake by the biomass, although not necessarily by competing for the same site since many competitive inhibitors have been reported to function by associating with the mediation mechanism so as to restrict substrate transport without themselves penetrating the cell.

In <u>non-competitive inhibition</u> it is assumed that the inhibitor reacts with the biomass at some site other than that for uptake of the substrate without affecting affinity for the substrate. The equations are presented here (Pirt's numbers in brackets).

For competitive inhibition, the equation is :

(17.6) $$\mu = \mu_m S/S + (1 + \frac{i}{K_i}).K_S \quad \ldots\ldots\ldots\ldots \quad 1$$

and for non-competitive inhibition, the equation is :

(17.16) $$\mu = \mu_m S/(1 + \frac{i}{K_i}).(S + K_s) \quad \ldots\ldots\ldots\ldots \quad 2$$

Where μ is specific growth rate :

μ_m is maximum specific growth rate

S is substrate concentration

i is inhibitor concentration

K is dissociation constant for substrate reacting with biomass

K_i is dissociation constant for inhibitor reacting with biomass.

A graphical solution can be obtained most easily by reciprocal transformation of the equations thus :

(17.6) $$\frac{1}{\mu} = \frac{K_s\, i}{\mu_m S K_i} + \frac{1}{\mu_m} \quad \ldots\ldots\ldots\ldots \quad 3$$

(17.16) $$\frac{1}{\mu} = \frac{(S + K_s)\, i}{\mu_m\, S K_i} + \frac{1}{\mu_m} \quad \ldots\ldots\ldots\ldots \quad 4$$

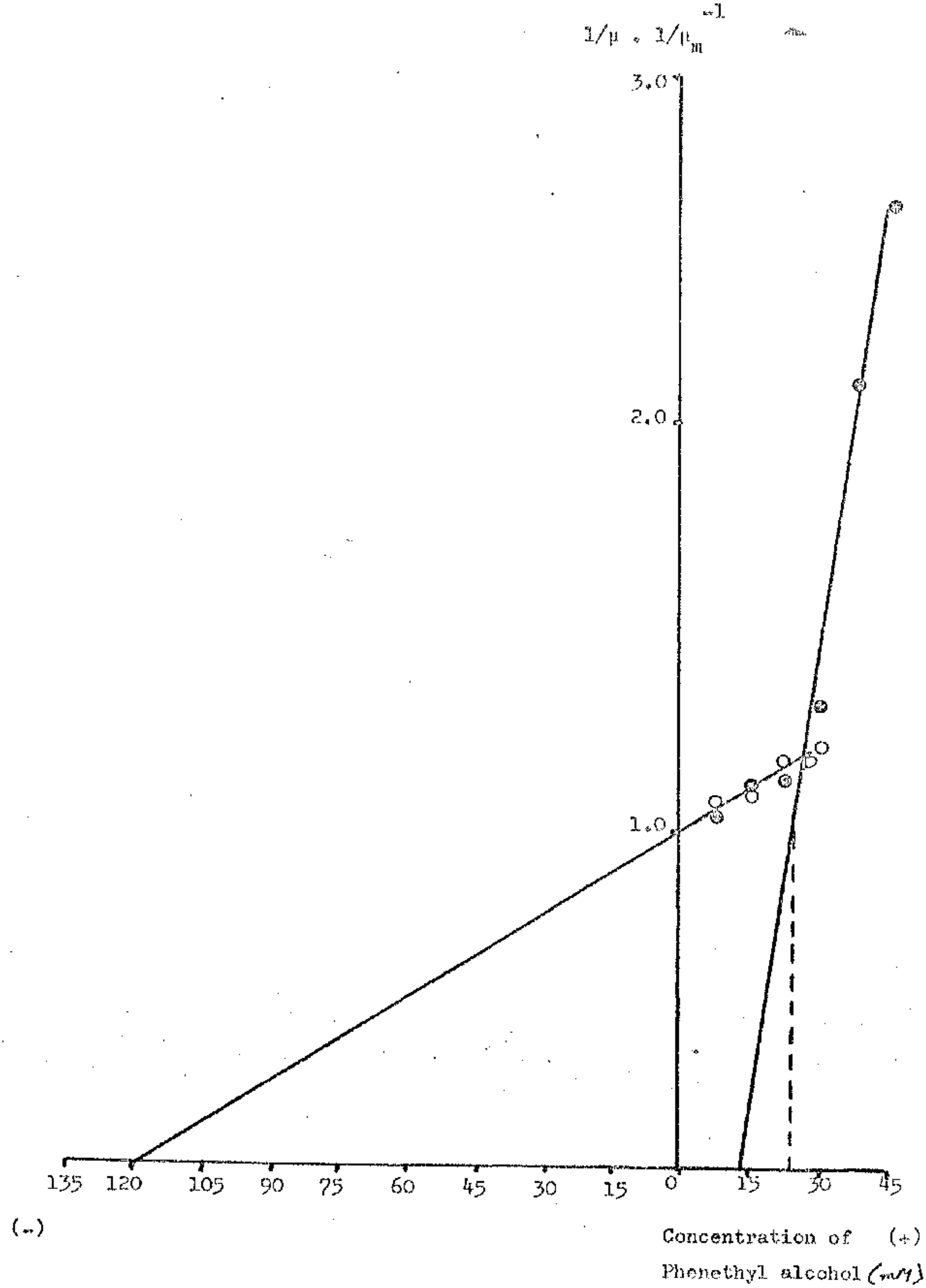
The change in substrate concentration during a short time interval will be negligible where $S >> K_s$ which would occur during exponential growth in BS medium. S can therefore be considered as a constant in both these equations and both then become equations for straight lines where $\frac{1}{\mu}$ is plotted against i. In both cases, the y-axis intercept (i.e. i = 0) is $\frac{1}{\mu_m}$; in other words the growth rate is maximum when there is no inhibitor present. The x-axis intercept in the case of equation 3 is at $\frac{-SK_i}{K_s}$ and for equation 4 is at $\frac{-SK_i}{(S + K_s)}$. Where $S >> K_s$ the latter then becomes $-K_i$. This indicates that non-competitive inhibition is independent of substrate concentration under these conditions. The x-intercept for equation 3 will therefore be large, whereas that for equation 4 will be small. A plot of $\frac{1}{\mu}$ against i would indicate whether the inhibition was competitive or non-competitive and such plots have been constructed. All showed the general form displayed on Figure 29 which was based on the data presented in Figure 9a,b. Values of $1/\mu$ (based on E_{600nm} or extractable DNA) were calculated for the period of 60 min post addition and converted to ratios of $1/\mu_m$ to allow direct comparison of the E_{600nm} and DNA values. The period of 60 min was chosen partly because inhibition by 30 mM or more was total after this time and partly because utilization of substrate caused deviation from linearity (as discussed above) in calculations based on later times.

Three distinct patterns are shown on Figure 29. The effect on rate of DNA synthesis was linear up to 30 mM but above this concentration, calculations could not be made due to membrane damage, as previously discussed (p. 161). This inhibition matched that shown by total biomass (E_{600nm}) up to about 22.5 mM. Extrapolation of this line to the x-axis intercepted at about -120 mM, indicating that the inhibition

Figure 29

Determination of the Nature of the Inhibitory Effect of Phenethyl Alcohol on Growth of Staph. aureus.

Inhibitory effects of phenethyl alcohol on growth of Staph. aureus may be either competitive or non-competitive. From a plot of the reciprocal of growth rate ($\frac{1}{\mu}$) against inhibitor concentration (i), the x-intercept can be used to determine which kind of inhibition is happening in any particular case. In the figure, the inhibitory effect of different phenethyl alcohol concentrations on increase of E_{600nm} or extractable DNA is assessed. So that both E_{600nm} and DNA can be compared directly, the ratio $^{1}/\mu . {}^{1}/\mu_{0}^{-1}$ is plotted. Such plots turn out to be bi-phasic when μ is measured by E_{600} (●) and to be linear up to 30 mM phenethyl alcohol when μ is measured by intracellular DNA concentration (○). Extrapolation of the two lines to the x-axis gives intercepts of - 120 mM and + 15 mM. The latter can be assumed to be - 10 mM if a new datum at 25 mM is taken (dotted line). These indicate competitive inhibition at low concentrations and non-competitive inhibition at high concentrations.



$1/\mu - 1/\mu_m^{-1}$
3.0
2.0
1.0
135 120 105 90 75 60 45 30 15 0 15 30 45
(-)
Concentration of (+)
Phenethyl alcohol (mM)

observed was competitive since this value was too high to be K_i. It was concluded that low concentrations of phenethyl alcohol interacted competitively with a substrate or substrates directly concerned with rate of growth. Above 22.5 mM, inhibition of E_{600nm} ceased to conform to this pattern, but when the values of $\frac{1}{\mu}$ for 30-45 mM were considered in isolation, it could be seen that they were related in a linear manner. When this line was extrapolated back to the x-axis, it passed through $\frac{1}{\mu_m}$ at about 25 mM and intercepted the x-axis at about 15 mM. This could be ascribed to a non-competitive inhibition starting at 25 mM with a value for K_i of 10 mM. This value of K_i would, of course, be distinct from the inhibitor constant for the competitive effect discussed above. It was concluded that high concentrations of phenethyl alcohol caused non-competitive inhibition, possibly by disrupting of membrane integrity. As discussed on p.38-40 a number of inhibitors have been shown to cause competitive inhibition of amino acid transport. The possibility that phenethyl alcohol might affect amino acid transport in Staph. aureus is considered later (p.179-180).

C.1.1.b. Effects on Extracellular Protein Production. A comparison of alpha-haemolysin and acid phosphatase production in the treated cultures was carried out.

In the presence of 15 mM phenethyl alcohol, the production of alpha-haemolysin was severely inhibited (Fig. 9c). The initial appearance was delayed until an E_{600nm} of 1.25 ± 0.2 which was double the value for the normal medium (p.156) and the rate of synthesis was reduced about 80% or more. At higher concentrations, alpha-haemolysin was not produced, so clearly alpha-haemolysin production was more sensitive to phenethyl alcohol than growth. It was possible that release of preformed alpha-haemolysin was being blocked by the treatment, but lysis of treated cells with lysostaphin did not reveal any cell-associated activity. In addition, cells which had been removed from inhibition by centrifugation had to grow for 30-60 min before alpha-haemolysin was detected.

Production of acid phosphatase was reduced by phenethyl alcohol treatment in line with the inhibition of growth, although extra-cellular levels continued to rise slowly even after growth had ceased in cultures treated with 30 mM or more. The most likely reason for this increase was leakage of preformed material, a matter which is discussed in more detail later (p.181).

C.1.2. Effect of Population Density. Different sizes of population were exposed to phenethyl alcohol at fixed concentrations of 15 mM or 30 mM in early log phase. The population sizes were varied by using initial inocula of 2.10^{8} $c.f.u.cm^{-3}$ or 3.10^{8} $c.f.u.cm^{-3}$. Under these conditions growth was similar to the standard pattern obtained using an inoculum of 1.10^{8} $c.f.u.cm^{-3}$ except that transitions to late log phase and early stationary phase were advanced in time because population densities appropriate to these phases were obtained earlier. The results are shown in Fig. 10a.b.

C.1.2.a. Effect on Growth Rate. Addition of 15 mM phenethyl alcohol depressed the growth rates, as measured by E_{600nm}, by around 20% in both cases, i.e. the reduction was similar to that for the lower standard inoculum. Addition of 30 mM phenethyl alcohol led to a reduction in growth rate leading to about 50% increase over 40 min., followed by a cessation of growth, once again reflecting the pattern for the lower inoculum.

C.1.2.b. Effect on Extracellular Protein Production. Acid phosphatase production was reduced in line with growth rate in both these experiments although the usual slow increase was noted after growth had stopped in the 30 mM treated culture. In the culture obtained from an inoculum of 2.10^{8} c.f.u.cm^{-3}, the first appearance of alpha-haemolysin (at an E_{600nm} of 0.78) coincided with the addition of inhibitor, whereas in the other culture, alpha-haemolysin had been detectable (at an E_{600nm} of 0.72) about 30 min before treatment. These population densities, at the time of first appearance, were within the normal range, which was further evidence that production of the haemolysin was controlled by growth conditions. In both cases production following treatment with 15 mM phenethyl alcohol continued, but the rate was reduced by about 95%. This indicated that control of synthesis was fairly precise since less accurate control would lead to either a period of continuing high production or to an immediate shut down, followed by a later re-initiation. Addition of 30 mM phenethyl alcohol caused an immediate reduction in the rate of production of alpha-haemolysin (Fig. 10b) over a 30 min period and thereafter the extracellular level remained constant, about 17% above that at time of addition. This slight increase might indicate a declining mRNA production, i.e. transcription continuing at a reducing rate for 30 min or a declining protein production, i.e. transcription absent and preformed messengers being translated, or some combination of the two. This will be reconsidered later (p. 175).

C.1.3. Effect of Culture Age. The usual phases of growth covered by these experiments have been early log phase, late log phase and early stationary phase, since these are the phases during which growth and extracellular protein production were most marked. However, in the majority of experiments, phenethyl alcohol has been added to early log phase cultures. To investigate the role which phase of growth played in the inhibitory effects, phenethyl alcohol at 15 mM or 30 mM was added to cultures in late log phase and early stationary phase (approx. 5h and 6.5h respectively, for BS medium inoculated with 1.10^{8} c.f.u.cm^{-3}) and the results are shown on Fig. 11a,b,c,d.

C.1.3.a. Effect on Growth Rate. Because the cultures were beginning to slow down at the time or before the inhibitor was added, the calculation of effects on growth rate was less easy. In late log phase, 15 mM phenethyl alcohol caused an apparent reduction in growth rate of about 20-25% in the 90 min period post-addition. However, beyond this time, the slowing down of the control made such estimates meaningless. The behaviour during the period which could be assessed was very similar to that displayed by 15 mM phenethyl alcohol treatment in early log phase. Addition of 30 mM phenethyl alcohol led to cessation of growth after about 60 min, during which time the culture density only increased by about 25%. In early stationary phase, the effects were similar, except that reduction in growth rate was only about 10%, following treatment with 15 mM phenethyl alcohol and cessation of growth in the 30 mM treated culture after about 60 min only encompassed an increase of 15%. It seemed certain that the variation in effect was due to the phase of growth, i.e. to the growth rate rather than to the population density.

C.1.3.b. Effect on Extracellular Protein Production. Once again extracellular acid phosphatase production declined in line with the growth rate, whereas alpha-haemolysin production was more sensitive.

Addition of 15 mM phenethyl alcohol to late log phase cultures caused a reduction of about 80% in the production rate of the alpha-haemolysin, but only 70% in the early stationary phase. These variations were ascribed to the alpha-haemolysin levels in the controls approaching final titre and therefore confusing the calculation. Addition of 30 mM phenethyl alcohol caused almost immediate cessation of increase of extracellular alpha-haemolysin with only an increase of about 10% during the first 30 min being detected.

The production rate at time of inhibition seemed to be the only factor which affected the degree of inhibition and it was concluded that variations in the level of inhibition were therefore not due directly to cell concentration.

C.1.4. Comparison of the Inhibitory Effect of Phenethyl Alcohol and Fluorophenylalanine Analogues. Because of the possibility that phenylalanine might be an important factor in the control of alpha-haemolysin synthesis (p.41-42) and because phenethyl alcohol was a structural analogue of phenylalanine, a comparison of the effect of fluorophenylalanine analogues with that of phenethyl alcohol was undertaken. As shown in Fig. 12, the observations of Mathieu et al. (1970) that fluorophenylalanines inhibited growth and alpha-haemolysin production were confirmed, as was the observation that meta-fluorophenylalanine was more inhibitory towards alpha-haemolysin production than either the ortho- or the para- isomers. All of these effects were eliminated by excess phenylalanine. Phenethyl alcohol at 15 mM was approximately as inhibitory as 80 μM of L-meta-fluorophenylalanine to both growth and extracellular protein production, but phenylalanine could not reverse this effect. The incorporation of analogue and phenethyl alcohol together proved interesting, since, in this case, the phenethyl alcohol effect alone seemed to be operating. Removal of the cells from joint inhibition by centrifugation and resuspension in fresh medium restored the culture to normal, suggesting that the 15 mM phenethyl alcohol had prevented the intracellular accumulation of analogue.

C.1.5. Investigation of the Effect of L -Histidine on Phenethyl Alcohol Inhibition of Growth and Extracellular Protein Production.

Histidine had also been implicated in the control of alpha-haemolysin synthesis (p.46). The minimal medium devised for NCTC 10344 included histidine which appeared to be an absolute growth requirement. It was therefore not possible to directly reproduce the conditions employed by Dalen (1973 a,b). However, an attempt was made to overcome the effect of phenethyl alcohol on alpha-haemolysin synthesis by the addition of L-histidine to BS medium at concentrations up to 2 mM. As shown on Fig. 13, concentrations above 1 mM L-histidine did appear to partially stimulate alpha-haemolysin synthesis in the presence of 15 mM phenethyl alcohol. Since this stimulation was effected without a concommitant stimulation of growth it was gratuitous in contrast to the non-gratuitous effect in minimal medium described by Dalen (1973a,b). This result implicated L-histidine in the inhibitory effect due to phenethyl alcohol and made it seem likely that inhibition of amino acid uptake was involved.

C.2. Inhibition of Growth and Extracellular Protein Production by Phenethyl Alcohol Analogues.

The effect of phenethyl alcohol on growth and extracellular protein production discussed above were presumably a function of its molecular structure. To try to identify which parts of the molecule were important, a number of phenethyl alcohol analogues were tested in the same system, ie. addition at 15 mM during early log phase (2.5h) and the results shown in Fig. 14. The compounds tested fell into four groups as shown in Figure 29. Group 1 comprised only 2-Phenyl Acetic Acid, a compound which was crystaline at room temperature and which might be expected to be a natural metabolite. It was therefore not surprising that this compound produced no effect on either growth or protein production. The Group 2 compounds, Phenethylamide and Phenoxyethanol, both had fairly polar side chains and both caused slight stimulation of growth rate, measured by E_{600nm}, coupled with a decrease of about 30% in the rate of alpha-haemolysin synthesis. It seemed likely that the stimulation of growth was an artifact and that there was essentially no effect on growth. The inhibition of alpha-haemolysin synthesis by these two compounds highlights the sensitivity of this system to minor changes in the environment. 2-Phenethyl alcohol itself was a member of the third group which also contained 1-Phenethyl alcohol and 3-Phenyl propanol which have very similar structures and very similar polarity; they reduced growth rate by about 35% and alpha-haemolysin synthesis about 95%. Group 4 again comprised closely related compounds, in this case 2-Phenyl Ethyl Chloride and 2-Phenyl Ethyl Bromide. These compounds prevented any synthesis of alpha-haemolysin and inhibited growth rate by about 62%. Treated cells showed almost 50% loss during centrifugation. As judged (Fig. 30) by the molecular structure and properties of the various compounds (Handbook of Chemistry and Physics, 1973) the inhibitory effects clearly increased as the polarity of the side chain decreased, suggesting an involvement of the membrane in the inhibitory effects.

Figure 30

Structure of Phenethyl Alcohol Analogues

Phenethyl alcohol and its analogues fell into four groups according to their effect on growth rate and alpha-haemolysin synthesis.

When added at 15 mM :

Group 1 produced no effect on either.

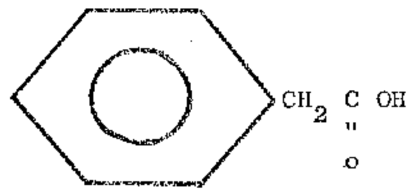
Group 2 slightly stimulated growth, but depressed alpha-haemolysin synthesis by about 30%

Group 3 inhibited growth rate by 30-40% and alpha-haemolysin synthesis by about 95%.

Group 4 inhibited growth rate by about 62% and totally inhibited alpha-haemolysin syntheses.

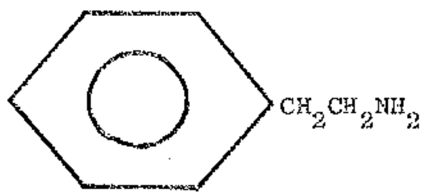
The structures are shown opposite.

GROUP 1.

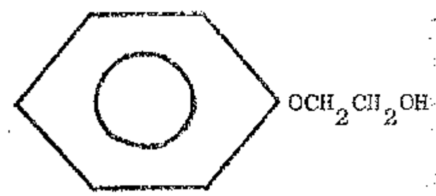


2- Phenyl Acetic Acid

GROUP 2.

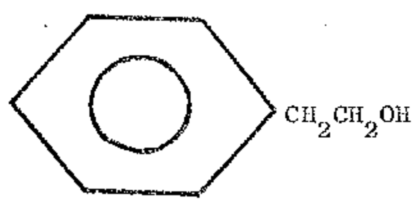


2- Phenyl Ethyl Amine

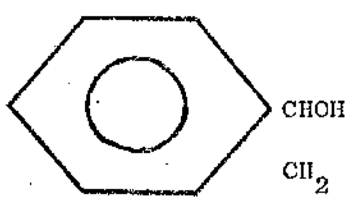


2- Phenoxyethanol

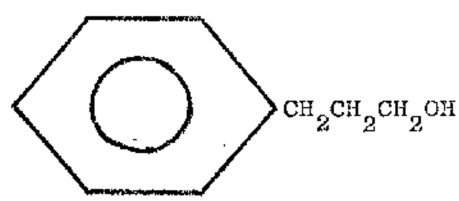
GROUP 3.



2- Phenyl Ethanol (Phenethyl Alcohol)

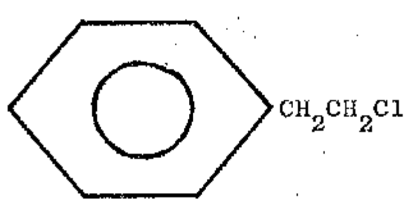


1- Phenyl Ethanol

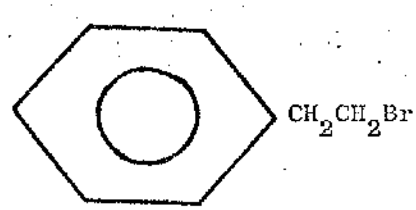


3- Phenyl Propanol

GROUP 4.



2- Phenyl Ethyl Chloride



2- Phenyl Ethyl Bromide

C.3. Inhibition of Growth and Extracellular Protein Synthesis by Antimetabolites Which Were Structurally Unrelated to Phenethyl Alcohol.

To provide further information on extracellular protein production and to provide a basis for comparison of the effects of phenethyl alcohol a number of antimetabolites, whose mode of action had been proposed, were chosen and their effects assessed.

C.3.1. Compounds Affecting Nucleic Acid and Protein Synthesis.

C.3.1.a. Mitomycin C. This antibiotic causes DNA damage and is an effective inducer of lysogenic phage (p.43). When 3 µM was added to early log phase cultures at 2.5h (Fig. 15) a reduction in growth rate, measured by E_{600nm}, was detected and increase stopped after 2h. The cells then began a slow lysis. Extractable DNA proved to be inefficient as a measure of intracellular DNA in this system because DNA levels apparently fell immediately after addition of the inhibitor. Since E_{600nm} was still increasing, the loss of DNA must have occurred during the initial washing stage of the assay. This was probably due to a combination of membrane damage and phage induction. Treated cells were osmotically unstable and the majority lysed during centrifugation. However, clones derived from survivors were apparently normal in their extracellular protein production which suggested either that these were not 'cured' cell lines or that the extracellular proteins routinely tested for were not associated with lysogenic phage. A more detailed examination of this area would be interesting, perhaps using tritiated thymidine to assay for DNA synthesis.

Alpha-haemolysin was not synthesised by the treated cells, but since lysis ensued at an E_{600nm} of 0.74, this was expected. Release of acid phosphatase was interesting since this continued at an increased rate well into the lytic phase, reflecting release of preformed material from the cells.

C.3.1.b. Chloramphenicol This antibiotic blocks translation at the 70S ribosome (p.42). The degree of inhibition was concentration-dependent and the results shown on Fig. 16 were for 30 µM and 80 µM which resembled the effects on growth, measured by E_{600nm}, of 22.5 mM and 30 mM phenethyl alcohol. Inhibition of DNA synthesis by 80 µm chloramphenicol was rather anomalous in that it resembled that caused by 30 µM for about 2h, but then became total. The total DNA had approximately doubled in that time. This might reflect a variable control of initiation of DNA replication or a variability in the sysceptibility of different members of the population. In any case, first step resistance to chloramphenicol in NCTC 10344 occurred at about 20 µM (Wheller, unpublished) and cultures treated with this concentration, or above, did not grow beyond 9h in BS medium.

Acid phosphatase production was inhibited by all concentrations, approximately in line with the inhibition of E_{600nm}, although continued release of preformed material made this much less noticeable. Alpha-haemolysin was not detected in any cultures treated with chloramphenicol, suggesting that this protein was abnormally sensitive to such treatment. This finding contrasted with that reported by Hinton and Orr (1960) but was in general agreement with results of Mates (1974b) and Gemmel and Shible (1976).

C.3.1.c. Actinomycin D. This antibiotic blocks transcription at low concentrations and DNA replication at high concentrations (p.42), probably by cross linking DNA. The degree of inhibition was concentration-dependent and the results shown on Fig. 17 were for 0.8 µM and 8 µM.

At 0.8 μM, inhibition of all parameters was similar to that displayed by 22.5 mM phenethyl alcohol and 30 μM Chloramphenicol and at 8 μM total inhibition ensued within 90 min for E_{600nm} and 40 min for DNA, E_{600nm} having approximately doubled, and DNA having increased by about 50% before stopping. These results were consistent with the inhibitory mechanism proposed for Actinomycin D.

Alpha-haemolysin was not detected in these experiments and acid phosphatase production was inhibited in line with growth which was also consistent with the known mode of action.

C.3.1.d. Estimation of mRNA Durability by Comparison of Chloramphenicol and Actinomycin D on Active Alpha-haemolysin Synthesis. Katsuno and Kondo (1973) estimated the durability of the mRNA for enterotoxin B by comparison of cultures treated with these two antibiotics (p.42) so it was decided to investigate the durability of the alpha-haemolysin mRNA by the same method. Duplicate cultures in which alpha-haemolysin was being synthesised were treated with either 30 μM Chloramphenicol or 0.8 μM Actinomycin D and levels of alpha-haemolysin and total cell mass (E_{600nm}) were monitored. The results, shown in Fig. 18, indicate quite clearly that these two antibiotics, whilst inhibiting growth in a similar manner, displayed a very dissimilar effect on alpha-haemolysin synthesis. Addition of chloramphenicol, which blocks translation, caused total inhibition of alpha-haemolysin within 5 min, indicating that translation and extrusion were probably very tightly linked. Addition of actinomycin D, on the other hand, allowed synthesis to continue at a declining rate over a 30 min period, which was consistent with the view that the mRNA was of a more durable variety than that concerned with general cell protein synthesis. Since E_{600nm} continued to increase where alpha-haemolysin had been totally blocked, this was further evidence for the additional sensitivity of alpha-haemolysin synthesis to such interference. Such durability might be due to either a more resistant type of mRNA, for example, a modified 5' terminus consistent with the translation-extrusion model (p.28) or an enlarged pool of mRNA for extracellular proteins (p.30). However, the latter would still require some mechanism for reducing turnover of the mRNA, so the former might be more generally applicable.

Acid phosphatase was also examined in this system, although the results are not shown in Fig. 18. The general results were consistent with the effect on cell growth, but probably due to the usual leakage of preformed acid phosphatase, the results were not very clear cut.

C.3.2. Compounds Affecting Membrane or Membrane Based Functions.

C.3.2.a. Sodium Nitrite The precise mode of action of this compound is unknown, but it has been shown to reduce Y_{ATP} (Stouthamer, 1979). The effect of 30 mM sodium nitrite is shown on Fig. 19. Inhibition of growth, measured by E_{600nm}, acid phosphatase production and alpha-haemolysin synthesis was similar to that produced by 15 mM phenethyl alcohol. Since the molecular structure of the two compounds is very different, the mode of action cannot have been identical but the final result in metabolic terms would appear to be similar.

C.3.2.b. Potassium Ferricyanide. Figure 20 shows the effect of 15 mM and 30 mM potassium ferricyanide. The inhibitory effects of 15 mM were very similar to that produced by 15 mM phenethyl alcohol, which might at first suggest some relationship. However, 30 mM was not much more inhibitory than 15 mM except on alpha-haemolysin synthesis, which was detectable in 15 mM but not in 30 mM up to 6h. However, in one experiment which was continued for 8h, alpha-haemolysin was detected at about 7h and all treated cultures had attained control levels overnight. The results suggested that the number of potential target sites for action were both limited and non-lethal, but the nature of such targets was difficult to envisage. It would appear, however, that the mode of action was different from that of phenethyl alcohol.

C.3.2.c. Sodium Azide. The inhibitory effects of sodium azide are shown on Fig. 21. There was no easy relationship between inhibitor concentration and degree of inhibition. 1 mM had no effect on any of the parameters tested, whereas 2 mM produced 50% reduction in growth (E_{600nm}) and acid phosphatase production. It also prevented the appearance of alpha-haemolysin up to 6h although it was eventually produced and had attained control levels after overnight incubation. Inhibition increased with concentration thereafter and resembled that caused by 30 mM phenethyl alcohol at 20 mM. The initial large jump in effect between 1 and 2 mM might reflect some tolerance on the part of the cells, perhaps due to an exclusion of the inhibitor until external inhibitor concentration had reached a critical level at which passive diffusion was able to drive the azide molecules through to their site of action. Whatever the reason, the action did not resemble that of phenethyl alcohol.

C.3.2.d. Potassium Cyanide. This compound was immediately inhibitory to all three parameters at 1 mM (Fig.22). Inhibition of growth (E_{600nm}) increased with concentration and became total at 10 mM where E_{600nm} increased by no more than 10% post addition. Acid phosphatase release was inhibited in line with inhibition of E_{600nm} although, as usual, it continued even in totally inhibited cultures. At concentrations above 1 mM no alpha-haemolysin was detected, but in the presence of 1 mM cyanide, the rate of production was similar to that of the control and although initial appearance was delayed about 1h, production nevertheless began at an E_{600nm} of 0.62 which was in the normal range at which production began in untreated cultures. Since alpha-haemolysin synthesis, which had been found to be extremely sensitive to all other reagents, was less sensitive to potassium cyanide, this suggested that the mode of action of potassium cyanide was different from the other compounds described in this section and certainly different from phenethyl alcohol. Since cyanide would be interfering with respiration, it was concluded that respiration rates were less closely related to alpha-haemolysin production than other aspects of central metabolism. This might be related to the observation that alpha-haemolysin was produced most rapidly during the late log phase when respiration rate might be reduced, although media depletion has been proposed as the reason for this transition (Holme & Arvidson, 1976) (p.45).

C.3.2.e. <u>2,4-Dinitrophenol.</u> The inhibitory effects of dinitrophenol are shown in Figure 23. The inhibition of growth, measured by E_{600nm}, was concentration dependent up to 2.5 mM. At 1 mM inhibition resembled that due to 15 mM phenethyl alcohol and at 2.5 mM it resembled that due to 30 mM phenethyl alcohol. The same relationship applied to the inhibition of acid phosphatase release and alpha-haemolysin production. Although phenethyl alcohol was clearly weaker in its action than dinitrophenol, the dependence on concentration followed the same pattern. This compound (p. 33) is known to dissipate the membrane proton gradient and this is suggested to be its main mode of action. Concentrations of dinitrophenol above 2.5 mM caused irreversible damage to many of the treated cells and above 5 mM lysis usually ensued. This suggested that higher concentrations were causing serious membrane damage. An examination of these results using the system described for Fig. 28 indicated a two-phase effect, yielding K_i values of 10 mM and 2 mM.

These would appear to have been due to non-competitive inhibitions. Inhibitions due to phenethyl alcohol and dinitrophenol would therefore seem to be related, but not identical processes.

D. <u>COMPARISON OF THE EFFECT OF PHENETHYL ALCOHOL AND DINITROPHENOL ON UPTAKE OF RADIOLABELLED AMINO ACID.</u>

The results presented in Section C have indicated a number of aspects of phenethyl alcohol inhibition which will be reviewed in detail in section H below. However, there was a definite suggestion that phenethyl alcohol was affecting amino acid transport in some way so a comparison of the uptake of various labelled amino acids in the presence or absence of phenethyl alcohol was instituted. As a control, the effect of 2,4-Dinitrophenol was also investigated. The concentrations used, based on the results obtained from Figure 9 and Figure 23, were 15 mM and 1 mM respectively and the results are shown on Fig. 24.

The very low uptake of L-lysine and L-histidine was anomalous, particularly since L-histidine appeared to be an absolute growth requirment for NCTC 10344.

The other four amino acids, glycine, L-alanine, L-glutamic acid and L-glutamine were all taken up rapidly. Addition of 1mM dinitrophenol caused an apparent small efflux of label from the cells, but thereafter, uptake continued at a reduced rate. Saturation of the controls made the relative uptake in the presence of dinitrophenol look more than it was, in fact, and the probability that no active transport was taking place cannot be excluded, the small net increase being due to a facilitated diffusion as suggested by Christensen (1979). The effect of 15 mM phenethyl alcohol was much more dramatic. A rapid reduction in cell-associated label happened immediately and thereafter continued to fall. Quantitative differences between the effects of the two different inhibitors were found in all cases, this being most dramatic for L-glutamine where virtually all of the cell-associated label was lost within 2 min of the addition of phenethyl alcohol, whereas no more than 10% was lost by the dinitrophenol treated culture in the same time period. Therefore, although both these inhibitors appear capable of disrupting amino acid transport, their mode of action would appear to be different.

Efflux of L-methionine from Myococcus xanthis in the presence of 17 mM phenethyl alcohol has been noted by Jones (1979), but no reason was adduced. As reviewed on p.36-38 uptake of amino acids would appear to be an active, energy dependent, process. In. E. coli mutants which lacked the specific transport system for branched chain amino acids could not retain leucin (Anderson and Ozender, 1978), similar results have been noted for other amino acids (p.38) and I have suggested that a separate excretory process might be responsible, perhaps related to the counter-transport system proposed by Christensen (1979).

Non-competitive inhibition of amino acid transport by uncoupling agents such as dinitrophenol has been observed, but as reviewed on p.38-40 competitive effects due to other mechanisms have been noted for a variety of inhibitors. The most likely explanation for these observations was the production of unstable conformations of the transport proteins (p.39). However, the inhibition due to tributyl tin chloride was associated with leakage of amino acid from the cell (Singh & Bragg, 1979) which was suggested to be due to a transmembrane OH^--anion exchange. The observation of leakage was therefore not confined to phenethyl alcohol, but the explanation might have to be different in this case because direct chemical linkage to the membrane transport proteins or charge effects would be unlikely.

E. LOCALISATION OF PHOSPHATASE ACTIVITY

Phosphatase was chosen as an example of a cell associated protein because the evidence for association seemed good (p.25) and because its activity could be conveniently assayed. The degree of cell-association was, however, uncertain and details such as number of enzymes with this activity and pH optimum were unconfirmed. An attempt was therefore made to establish the pH spectrum of the cell-free activity and the cell-associated activity, the proportion of activity which was cell-free and the location of the activity within the cell.

The pH spectrum for activity was similar for cell-free and cell-associated activity (Fig. 25) and indicated a three component system with pH optima at 6.1, 6.8 and 7.8 for soluble enzyme and 6.1, 7.1 and 7.8 for intact protoplasts. The variation in the central peak value might have been due to conformational changes associated with the change from a possibly non-polar membrane location to a polar environment outside the cell. Alternatively, the enzyme might have been attached to the surface of the membrane by a loop of polypeptide which had to be cleaved (p.30). However, Triton X-100 solubilised protoplasts displayed the typical central peak at pH 6.8 so an environmental effect seemed more likely. The same spectrum of activity was released from non-metabolising cells suspended in cold (4^{o}C) medium (Fig.26) suggesting that all three activities were being released in concert. The three pH optimum peaks, presumably reflecting three enzymes, did not correspond to the 5.2-5.3 optimum noted by Malvaux and San Clemente (1969a,b). The enzyme which they studied was inhibited by Iodoacetate and EDTA and stimulated by Cu(II)ions. These effects were not noted for any of the pH optima described here (Whyte, pers. comm; Wheller and White, 1979) so this difference was possibly due to strain variation. Certainly the Oxford strain of <u>Staph. aureus</u> appeared to have only one pH optimum at 5.4-5.6 (Stevenson, unpublished). The results presented in Fig. 25 and 26 do not exclude the possibility of a minor component with an optimum at pH 5.3. The majority of the activity, about 60%, was associated with the pH 6.1 peak and so this was the activity routinely assessed.

Fractionation of the cells, using the protocol in Fig. 2 (p.77) yielded the results shown in Table 6. The majority of the enzyme (60%) would appear to be cell associated, but more than half of this was external to the protoplast and was therefore easily removed. Much of this was located in a loosely bound for attached to the wall, possibly to teiochic acid, as suggested by Arvidson (1976) for the 5.2 enzyme.

The cell associated activity was localised by electron microscopy using a technique which marked the site of enzyme activity with lead phosphate. There was considerable activity in the whole cell (Fig. 27a) but much of the loosely bound material could be removed by washing and the majority of the remaining activity appeared to be cytoplasmic. Whether the cytoplasmic enzyme(s) and the extracellular enzyme(s) were the same or not was not determined. The translation-exclusion model involving a signal sequence (reviewed on p.28-31) would presumably exclude such a possibility, but a different translational control system could be operating in this case.

The evidence presented here confirms the choice of acid-phosphatase as a cell-associated enzyme whose production could be usefully contrasted with that of the alpha-haemolysin.

F. INVESTIGATION OF EXTRACELLULAR PROTEIN PRODUCTION BY MUTATIONAL STUDIES

F.1. Possibility of a Plasmid Locus for the Alpha-haemolysin Genes.

The location of the genes for various extracellular proteins has been a matter for controversy which does not seem to have been resolved. From the work reviewed on p.59-63 it seemed clear that the location of the genes was strain variable. Attempted elimination of plasmids carrying determinants for alpha-haemolysin activity by SDS, Acriflavine or elevated temperatures, did not confirm that these genes were located on a plasmid in strain NCTC 10344.

F.2. Pleiotropic Mutation of the Extracellular Protein Genes.

Pleiotropic mutations are those in which an apparently single gene mutation causes changes in the activity of other gene products. As reviewed on p.56-59 pleiotropic mutations have been frequently noted in studies on Staph. aureus extracellular proteins. In this study, clones mutant in alpha-haemolysin production were isolated after treatment with UV, MNNG, 5BU, 2,6AP, EMS and NA and characterised with respect to a number of extra-cellular activities and to general metabolism. The results are shown in Table 7. On the basis of different patterns of pleiotropy the mutants were assigned to 5 groups, although it was clear that some overlap might occur.

F.2.1.a. Group 1 Mutants: Mutants in this group had lost the ability to produce alpha-haemolysin but had retained the ability to produce all of the other proteins, 'though not necessarily at wild-type levels. Some of the mutants also produced cross reacting material to anti-alpha haemolysin. It was therefore fairly certain that they were mutant in the structural gene for alpha-haemolysin. Since most of these mutants also displayed decreased ability to produce staphylokinase and coagulase, it was possible that these genes were contiguous. The order of the genes would not, of course, be determinable from these results and some members of the group might actually be original mutations in the coagulase or staphylokinase genes rather than the alpha-haemolysin gene.

F.2.1.b. <u>Group 2 Mutants:</u> Most of the mutants in this group had lost all of the activities tested, except phosphatase and bound coagulase A few mutants which showed low levels of activity for some of the characters and no activity for the remainder were also included, on the assumption that these were "leaky" for the characters concerned. As discussed previously (p.181-182) phosphatase was not a true extracellular protein and therefore mutants in this group might lack some feature which is unique to the true extracellular protein. The best candidate would appear to be some part of the excretion system, since extracellular proteins and cell associated proteins might well be controlled by two different excretion systems. There are several levels at which this might occur, including ribosomal selection of the signal codons, membrane recognition of signal sequence, cleavage at the outer surface of the membrane (possibly linked to a feed-back system such as that proposed by Saman & Hague, 1970) or even a mechanism for release of phosphatase which does not rely on a translation-extrusion mechanism. The possibility also existed that the "leaky" mutants were, in fact, wild types which would link them with phosphatase.

F.2.1.c. <u>Group 3 Mutants:</u> These mutants had completely lost all the characters examined, except gelatinase and phosphatase, and were therefore distinct from Group 2 mutants. One possible mutation which could produce this result was loss of function of a transcriptional control system, either by mutation of a regulator or an operator site, although such controls have not been directly demonstrated for the extracellular proteins. These mutants might have been defective in some part of the metabolic control which seemed to be implicated by the results presented in p.87 - 135 and which is discussed more fully below (p.188-190). However, the growth of all of these mutants on plates appeared normal, so the change could not have been very extreme.

F.2.1.d. Group 4 Mutants : These mutants had lost alpha-haemolysin, staphylokinase and gelatinase activities, but were otherwise wild-type. At first glance, these mutants did not conform to the pattern which was appearing. However, in the syntrophic experiments, it was sometimes noted that Group 4 mutants could exhibit slight haemolysis when cross-fed by Group 1 mutants. These mutants might, therefore, lack an extra-cellular 'activator' for converting alpha-haemolysin, staphylokinase and gelatinase into their active forms. Alternatively, a specific enzyme involved in cleavage of the signal sequence linking the protein to the membrane might have been involved. Wiseman & Caird (1970, 1972) and Wiseman et al. (1975) have suggested that the alpha-haemolysin was excreted as a proto-toxin, but their evidence was equivocal and no evidence for protomeric forms of the other two has been presented. Gelatinase itself might function as a cleavage enzyme so that this group would be structural mutants in the gelatinase gene, but no evidence for this was produced. A preliminary search for mutants with the primary lesion in the gelatinase gene has so far proved unproductive.

F.2.1.e. Group 5 Mutants : The mutants in this group were all very slow growing, and they lacked all of the activities tested for. In addition, they fermented carbohydrates only slowly and so they were considered to be "dwarf" mutants of the type described previously (p. 55).

F.2.2. Relationship of Previous Observations of Pleiotropic Mutants to this Study. As noted previously (p.56-59), pleiotropic mutations were frequencly detected in studies of this type and these are summarised in Table 3. It is difficult to relate these studies directly to each other and to the work reported here, since the observations were produced under different circumstances and the activities of the proteins were usually expressed in a qualitative manner. However, an attempt has been made to assign the mutants described in Table 3 to one of the five groups described above. Mutants considered to be in Group 1 are 2, 3, 6a, 6b, 8b, 8c, 8d and 8e; Mutant considered to be in Group 2 is 8h; Mutant considered to be in Group 3, 7a (possibly should be Group 2). Mutants considered to be in Group 5 are 6e, 8a. The other mutants did not conform sufficiently closely to the model to be assigned in this way. They might be mutations in the structural genes of proteins other than the alpha-haemolysin. Pleiotropy due to polar effects could then explain the following mutants : 1, 6d, 7b, 8f and 8g and might explain 6c and 7c.

Although the assignment of the mutants to 5 distinct groups was only a tentative arrangement, it was interesting to note that the results of other workers were compatible with this hypothesis. A possible model structure involving the 5 gene groups proposed here will be presented below, when all of the available information has been discussed.

G. GENERAL CONCLUSIONS

The results presented and discussed above were obtained in an attempt to answer some questions about the mode of action of the antimetabolic phenethyl alcohol and the control of extracellular protein production. Conclusions about these two topics are discussed below.

G.1. The Mode of Action of Phenethyl Alcohol.

Phenethyl alcohol inhibited growth and extracellular protein production in Staph. aureus and the inhibition was concentration dependent. At concentrations up to 25 mM the inhibition of growth was due to a competitive inhibition of substrate transport which was related to an efflux of amino acids from the intracellular pools. This form of inhibition was similar to that produced by membrane-directed inhibitors but the mode of action was apparently different from almost all of the other reagents reviewed (p.38-40) or discussed (p.173-179) above. The effect of various analogues of phenethyl alcohol (p.170-172) indicated that polarity of the side chain was important and the amphipathic nature of the molecule was likely to disrupt the membrane, which compared with the proposed mechanism for mono-esterified 1,2 diols (Akedo et al., 1971). The compound whose action most resembled that of phenethyl alcohol in this study was dinitrophenol but the inhibitory effect of this reagent, in common with most other uncoupling agents (p.38), was non-competitive (p.178 -179). At concentrations above 25 mM the phenethyl alcohol inhibition was non-competitive. This was accompanied by increasing damage to the integrity of the membrane resulting at concentrations above 30 mM in lysis of many of the cells. All of the inhibitory effects were independent of population density and largely independent of growth rate and growth phase. The effects of low concentrations were also rapidly reversible (p.160-170). All of these results are consistent with a mode of action in which phenethyl alcohol penetrates into the membrane and disrupts the structure because of its amphipathic nature. This disruption has several consequences. The major effect is to reduce the uptake of amino acids by disorientating the transport proteins. This is accompanied by alterations in general permeability. The result of these membrane directed changes is a reduction in the supply of amino acids to the cell's central metabolism with a consequent reduction in growth rate and extracellular protein production. Disruptions of energy production may also be a factor, although no evidence for this is presented.

G.2. Production of Extracellular Proteins.

The extracellular proteins can be divided into those which are truly extracellular and those which are cell-associated but leak out into the medium. Alpha-haemolysin was chosen as an example of a truly extracellular protein (p.24) and all of the evidence presented here supported this conclusion. Acid phosphatase was chosen as an example of a cell-associated protein (p.25). Since the evidence for this was less certain experiments were carried out to determine the proportion of extracellular versus cell-associated activity. The evidence suggested that there were at least three enzymes, all cell-associated, which displayed phosphatase activity, the major component having a pH optimum of 6.1. The experiments were mainly concerned with production in various media with or without additional antimetabolites, but some mutational studies were also carried out.

G.2.1. Batch Culture Experiments. The maximum growth rate attainable by the cultures was a doubling time of 35min in BS medium either complete or diluted up to 50%. At doubling times in excess of 60 min there was no production of alpha-haemolysin but this could be explained by the final growth yield which did not exceed the E_{600nm} of 0.68 $\pm$ 0.11 which was the starting condition for alpha-haemolysin production in all the uninhibited media. Production of alpha-haemolysin was not growth linked and the most rapid period of synthesis occurred after the growth rate had begun to slow down. The reason for this common observation (p. 47) had been suggested by Coleman et al. (1975) and Abbas-Ali and Coleman (1977)) to be due to competition for nucleotides. Since the medium would be becoming depleted by this stage, the reason for increase in the rate of excretion of the alpha-haemolysin might be an artifact, which related to an increase in the extracellular degradative enzyme production. This would, of course, require a common control mechanism for such proteins. The fact that alpha-haemolysin production was more sensitive to many media changes than was growth was interesting since it suggested some form of central metabolic control. The inhibitory effects of Acetate or Succinate (p.155) might reflect an effect on ATP production or general respiration and, in this context, it was noted that Potassium Cyanide (p.177-178), unlike the other antimetabolites, was not inhibitory to alpha-haemolysin production. Indeed, since the growth rate was actually reduced whereas the alpha-haemolysin rate was almost normal, the effect might best be described as a stimulation of alpha-haemolysin synthesis. A depletion of available oxygen, i.e. inhibition of respiration, might therefore be a major controlling factor.

The result of comparing chloramphenicol with actinomycin D treatment clearly indicated that the mRNA for the alpha haemolysin was more durable than might be expected for normal cell proteins. This was most probably due to the modified 5' signal codons required for excretion. No intracellular accumulation of alpha-haemolysin was detected in any of the treated cultures, making it fairly certain that this protein was excreted by a translation-extrusion system.

Depletion of the amino acid supply by treatment with phenethyl alcohol, dinitrophenol or other antimetabolites which might have this effect, was probably the major reason for the high sensitivity of alpha-haemolysin synthesis to these compounds, providing further evidence that central metabolism was the source of the control of expression of the alpha-haemolysin gene.

The production of acid phosphatase, by contrast, behaved in a manner consistent with normal, perhaps constitutive, cytoplasmic enzymes. Although its release into the medium was non-growth linked this was almost certainly due to leakage of preformed enzyme. Activity was always present and no evidence for any selective control was found. The possibility that the activity was released from the cell by a mechanism other than translation-extrusion could not be ruled out.

In some experiments, coagulase, staphylokinase and gelatinase were also assayed. These reacted in a manner which linked them with the alpha-haemolysin. Although they appeared at different times in the growth cycle, this might merely reflect a failure of the assay system and more refined assays, perhaps using immunological techniques would be very useful in trying to clarify this.

The results of all of these experiments led to the conclusion that the truly extracellular proteins were under a separate control system from the other cellular activities and that this control was exerted to ensure that the cell's central metabolic needs were not put at risk by the production of these proteins.

G.2.2. Mutation Studies. The production of pleiotropic mutants (p. 183-186) enabled an assignment of several genes or gene groups to be made by an analysis of the most probably site of point mutation which would lead to the pleiotropy. The results suggested that Phosphatase and Bound Coagulase were not related genetically to the other activities and that there were common controls of both the transcription and the translation of the genetic information for the extracellular proteins. This hypothetical arrangement is summarised in Fig. 31 which was based on the following assumptions :

(i) There is a common excretion system. This might take the form of a membrane protein or proteins whose function is to bind signal peptides for restricted groups of polypeptides.

(ii) Phosphatase is not within the system designated at (i), nor is it under the same control system as the true extracellular proteins (iv). Transport through the membrane might require a different protein which recognises a different signal peptide.

(iii) Gelatinase is probably not part of the system either, although it could be an extracellular activator (v).

(iv) The true extracellular proteins have a common control system which is affected by amino acid supply.

(v) Alpha-haemolysin, staphylokinase and perhaps gelatinase all require activation.

(vi) The genes are presented bunched in an operon, but control and genetic location may be more diffuse.

This model is presented as a hypothesis which can be used to further examine aspects of control in the production of the extracellular proteins. Its physiological constraints are based on the results presented here.

FIGURE 31

Summary Diagram to Show the Relationships [illegible]

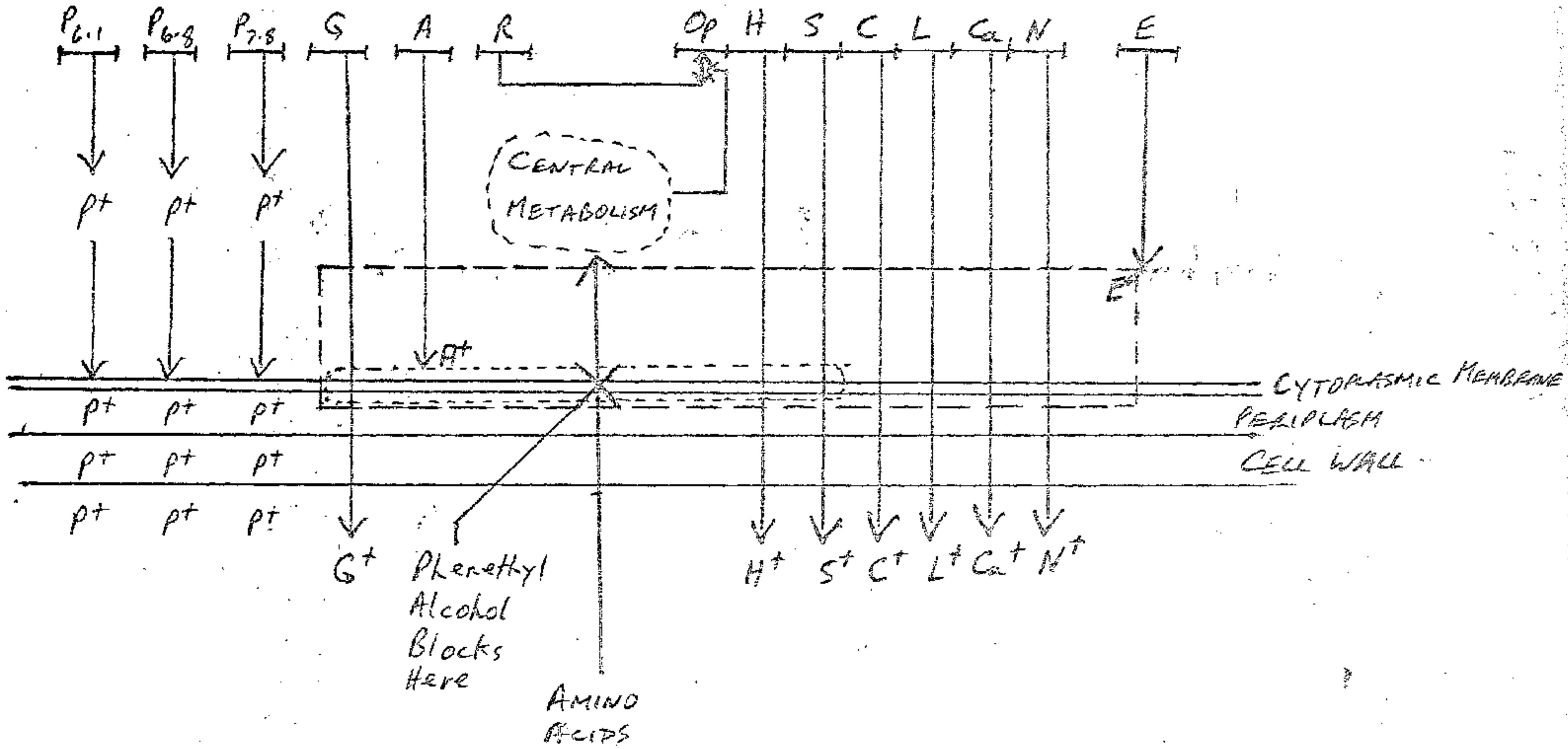
[illegible] Examined Here.

Key to Symbols used :

Genes : P: Phosphatases; R: Regulator; Op: Operator/Promoter; α: Alpha-haemolysin; S: Staphylokinase; C: Coagulase; L: Lipoprotein Lipase; Ca: Caseinase; N: Nuclease; G: Gelatinase; A: Extracellular Activator; E: Membrane Excretion System.

Proteins : The symbols above are used with a superscript (+);

The model shows the three proposed phosphatase genes and their gene products are shown leaking from a cytoplasmic location. Gelatinase, Alpha-haemolysin and Staphylokinase are shown as requiring an Activation step. Control of most of these productions is suggested as a by-product of precursor supply to central metabolism.



P6.1
P6.8
P7.8
G
A
R
Op
H
S
C
L
Ca
N
E
CENTRAL METABOLISM
p+
A+
E+
CYTOPLASMIC MEMBRANE
PERIPLASM
CELL WALL
G+
Phenethyl Alcohol Blocks Here
AMINO ACIDS
H+
S+
C+
L+
Ca+
N+

NOTE ADDED IN PROOF.

There is a natural tendency to continually update and revise the literature section. I have tried to resist this temptation since it seemed likely to lead to additional delays which would be counter-productive. However the recent publication of an extensive review edited by Easmon and Adlam (1983) cannot be ignored and the following is a brief summary of points from the book which have fairly direct relevance to the substance of this thesis. This last restriction means that recent information on structure and mode of action of the extracellular proteins has been omitted.

Plasmid and/or Chromosomal Location.

Distribution of the Staph. aureus genetic complement would seem to be 80-90% for the circular main genome, with 10-20% being plasmid or lysogenic phage DNA (E & A: 63-119). As discussed in this thesis Epidermolytic Toxin (ET), Coagulase and Enterotoxin B were the only extracellular proteins which seemed to be coded for by plasmid genes, at least in some strains, with conflicting evidence for some others notably the alpha- and beta- haemolysins and Staphylokinase. This situation is largely unchanged: ETA appeared to be always chromosomally located whereas ETB was always located on a plasmid and the genes were not homologous (E & A: 610-612); the location of the coagulase gene(s) was strain variable (E & A:530-534); the structural gene for the enterotoxinB, however, was not located on the proposed plasmid and the suggested linkage with methicillin resistance might be due to the latter being located on a transposon (E & A:98); the alpha-haemolysin gene might also be on a transposon (E & A:637).

Observations of Pleiotropy.

Additional studies have again revealed the common appearance of pleiotropic mutations. No general mechanism has been confirmed but membrane directed effects are suggested to be important (E & A: 697,796-799).

Factors Affecting Extracellular Protein Production.

In general the production of extracellular proteins appeared to increase after the end of the exponential phase(E & A:796-799) including the proteases (E & A:780-790), Nuclease (E & A: 761), Staphylokinase and Membrane Damaging Toxins (E & A:640-642). Protein A production did not conform to this pattern (E & A:432-433). Production od Coagulase might be like the general model or like that of Protein A (E & A:530-534; 796-799). Assessment of phosphatase production was affected by the binding of the enzyme to the cell surface. This would appear to be strain and medium variable and might be due to precipitation,i.e. non-specific (E & A:775-778). A general control model has been proposed in which the promoter for transcription of most extracellular protein genes is switched from down to up by two activators designated EX and S, with the concentration or activity of the latter varying with growth conditions. S might also cause the reverse switch in the case of the genes for protein A and coagulase. Control might be affected by oxygen tension and precursor supply might also be involved. Loss of production capability during continuous culture might reflect changes in EX (E & A:637, 796-799). This model is not inconsistent with the one described in this thesis.

Since this insertion is being made it would be foolish to ignore the observation by Tweten, Christianson and Iandolo (1983) that the alpha-haemolysin is synthesised as a larger molecule which is processed to the active form by removal of an apparent signal sequence, and that this processing is blocked by dinitrophenol treatment.

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Page numbers quoted in the text refer to contributions by the following:
63-119,98: Poston,S.M. and Naidoo,J.L.
432-433: Forsgren,A., Ghetie,V., Lindmark,R. and Sjoquist,J.
530-534: Jeljaszewicz,J., Switalski,L.M. and Adlam,C.
610-612: Arbuthnott,J.P.
637,640-642: Mollby,R.
697 Wadstrom,T.
761,775-778,780-790,796-799:Arvidson,S.O.

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