LOCAL ANAESTHETICS AS ANTIBACTERIAL AGENTS

A THESIS

Submitted in partial fulfilment of the requirements

for the Award of the Degree of

DOCTOR OF PHILOSOPHY

by

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LOUGHBOROUGH

NOVEMBER, 1981

ACKNOWLEDGEMENTS

I extend my special appreciation to my supervisor, Dr. W. G. Salt, for his unfailing enthusiasm, encouragement, and supervison, throughout this project. I benefited a great deal from his valuable assessments, the numerous discussions I had with him, and his professional work with the electron microscope. Also I thank Professor K. W. Bentley, Director of Research and Head of the Chemistry Department, and Dr. R. J. Stretton, Head of the Microbiology Section, for the use of the facilities in the department.

I am especially grateful for assistance from Mr. J. Brennan and Mr. F. Page, but also from all the other members of the academic and technical staff in the department.

Also I thank my colleagues, whose criticisms and discussions have been very beneficial.

I am grateful to Mrs. M. Brooks for efficiently typing this thesis.

My deepest gratitude goes to my family for their love to me throughout my life and to my beloved sons for adding zest to my life. I thank God who has given me patience and endurance to complete this work in spite of all the emotional hardships I have experienced through the death of my loving, supportive husband, during the course of this project.

D E D I C A T I O N

In loving memory of my MOTHER,
FATHER
and
HUSBAND

ORIGINALITY

All the work presented in this thesis has been carried out by the author except where acknowledged and has not previously been presented for a degree at this university or any other institution.

ABBREVATIONS

When appropriate the following abbreviations have been used in this thesis.

ca	About
CFM	Carbohydrate free medium
CMC	Critical micelle concentration
CPB	Cetylpyridinium bromide
CTAB	Cetyltrimethylammonium bromide
LA	Local anaesthetic
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
O	Oil
OD	Optical density
RPM	Revolutions per minute
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TCA	Trichloroacetic acid
TTC	2,3,5 triphenyltetrazolium chloride
W	Water
>	Greater than
<	Less than

Other abbreviations were defined as used in the text.

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INTRODUCTION

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SECTION ONE

LOCAL ANAESTHETICS

Unlike other drugs, anaesthetics are not usually used to cure or to lessen the distressful symptoms of an illness but rather are to be regarded as chemicals which place the patient into a state of aloofness and non-resistance to a surgical insult (Takman and Camougis, 1970).

A local anaesthetic (LA) interrupts the transmission of impulses in peripheral pain conducting nerves on which it acts directly. The resulting block may be partial or complete; though it should always be reversible. This requirement for reversibility precludes the use, as local anaesthetics, of compounds such as phenol that cause long-lasting nerve blockade due to irreversible nerve damage. Similarly compounds such as ethyl chloride whose action relies on local cooling are not strictly local anaesthetics though they do have a local anaesthetic-like action. The phenolics, however, raise an interesting dilemma. Their tissue damaging effects are concentration dependent and the induced anaesthesia is reversible when the agent is applied at very low concentrations. This conflict in action, local anaesthesia versus local toxicity, is one of the more difficult problems in the selection of a compound for use as a local anaesthetic.

1.1.1 Historical Review

Though this thesis is primarily concerned with local anaesthetics (LAs) as antibacterial agents, it is appropriate

to briefly outline the development of LAs.

The first LA to be discovered was cocaine, an alkaloid contained in large amounts (0.6 to 1.8%) in the leaves of Erythroxylon coca , a shrub growing in the Andes Mountains. Long before the time of Columbus, the Indians of South America were aware of the special properties of these leaves, which they used as a stimulant.

Niemann (1860) succeeded in isolating and purifying cocaine, and noted that it has a bitter taste and produces a numbing effect on the tongue, making it almost devoid of sensation. The molecular formula of cocaine (C_{17} H_{21} NO_4) was determined by Lossen (1865), who also showed its structure as benzoylmethylecgonine. Von Anrep (1880) studied its pharmacological actions. He observed that the skin became insensitive when cocaine was infiltrated subcutaneously, and recommended that the alkaloid be used clinically as a LA. This suggestion, however, was not acted upon, and credit for the introduction of cocaine into clinical use as a LA is usually given to Karl Koller. Koller (1884) exhaustively investigated the possibility of using cocaine to relieve pain in ophthalmic procedures. He showed that a drop of a 2% cocaine solution rendered the cornea and conjunctiva of the frog and the guinea pig insensitive to mechanical, chemical, and electrical stimulation. He repeated these experiments on himself, on his colleagues, and finally on his patients and demonstrated that cocaine was useful in relieving pain. Thus in 1884 the first practical LA was introduced into therapeutics.

The ensuing acceptance of cocaine as a LA was immediate, and in this way the history of local anaesthesia

differs sharply from that of general anaesthesia. Halstedt and Hall (1884) introduced LAs into dental practice and then by demonstrating that cocaine could stop transmission in nerve trunks, laid the foundation for nerve block anaesthesia in general surgery.

The curiosity and interest aroused by the various pharmacological properties of cocaine led to chemical manipulations of the molecule even before its structure had been ascertained. Thus the identification of ester functions in the largely unknown cocaine structure prompted uses of alcohols (other than methanol) and acids (other than benzoic acid) in the preparation of analogues and homologues from the hydrolysis products of cocaine (e.g. Merck, 1885; Einhorn, 1888). This chemical search resulted in the synthesis of procaine (Einhorn and Uhlfelder, 1904, 1909), which today is still widely employed. Clinical investigations still continue, however, because no available local anaesthetic is free of undersirable properties.

1.1.2 Pharmacology of Local Anaesthetics

In addition to blocking conduction in nerve axons in the peripheral nervous systems, LAs interfere with the function of all organs within which the conduction or transmission of impulses occurs. Thus, they have effects on the central nervous system (CNS), the neuromuscular junction, the autonomic ganglia, and all forms of muscle fibre (Ritchie and Cohen, 1975).

Following absorption, LAs may cause stimulation of the CNS, producing restlessness and tremor that may proceed the chronic convulsions. Central stimulation is followed by

depression and drowsiness, respiratory failure and coma (Takman and Camougis, 1970; Holroyd, 1978).

Also LAs act on the cardiovascular system. The primary site of action is the myocardium, where decreases in electrical excitability, conduction rate and force of contraction occur (Ritchie and Cohen, 1975; Holroyd, 1978).

The LAs also affect transmission at the neuromuscular junction. Harvey (1939) observed that the close intra-arterial injection of as little as 0.2 mg of procaine into the cat's tibialis anterior muscle reduced twitches and tetanic responses evoked by maximal motor-nerve volleys, as well as the response of the muscle to injected acetylcholine. The muscle, however, responded normally to direct electrical stimulation. Hirst and Wood (1971) suggested that procaine also diminishes the release of acetylcholine by the motor-nerve endings.

1.1.3 Sites of Application of Local Anaesthetics

Today LAs have wide clinical applications in dentistry, medicine, and veterinary science. The several locations at which a local anaesthetic may be applied (Wade, 1978; Holroyd,1978; Crossland, 1980; Bowman and Rand, 1980) are shown in Fig.1.1.

Surface or Topical Anaesthesia: The LA is applied in the form of a solution, ointment, cream or powder directly to the site at which anaesthesia is required. The method is restricted to mucous or damaged skin surfaces. Application of LA to unbroken skin will not normally show any effect. Topical anaesthesia proper is used to relieve pain or itching

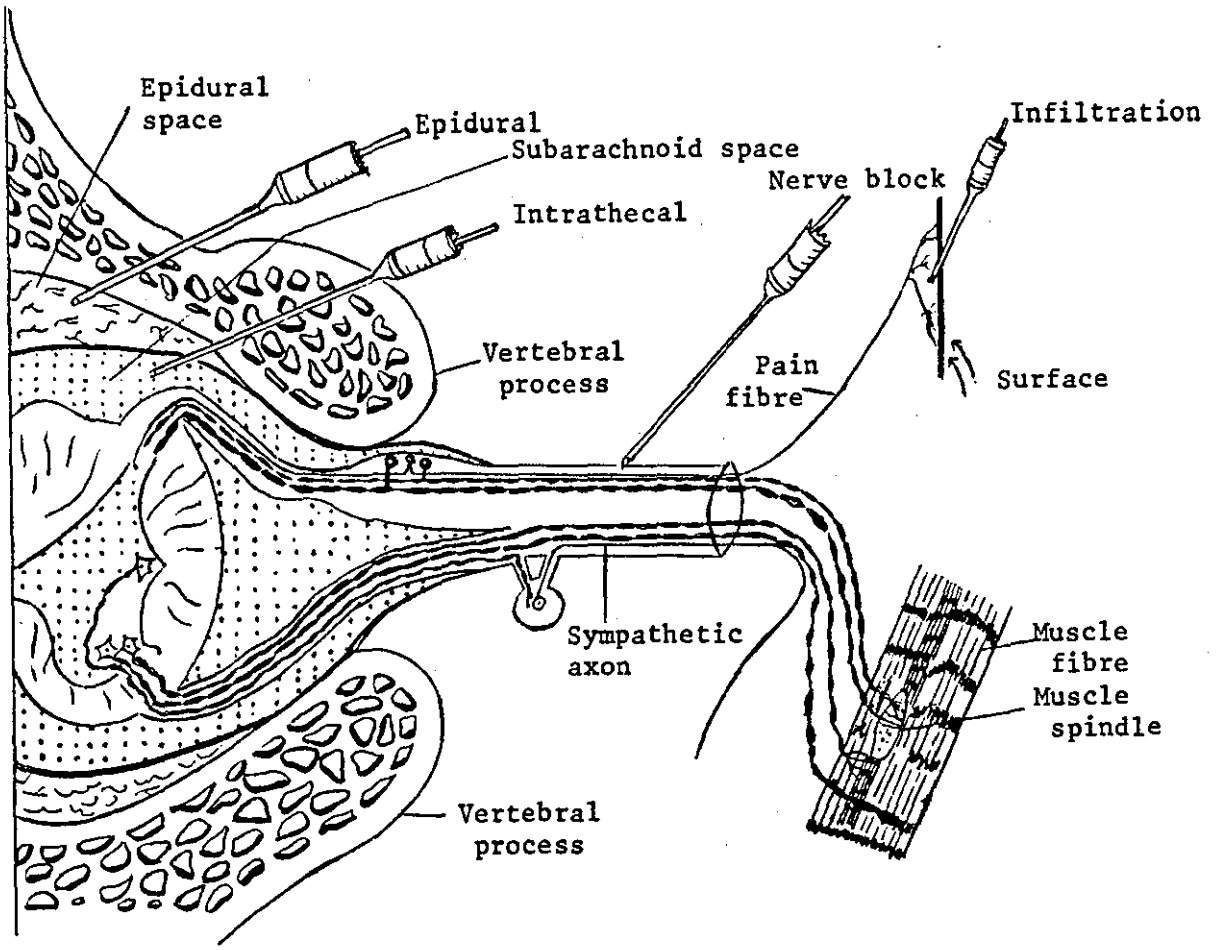


Fig 1.1 The routes of administration of local anaesthetics. Half of a cross section of the spinal column is shown with a spinal nerve.

(After Bowman and Rand, 1980)

in, for example, haemorrhoids, or to anaesthetise the cornea.

The LAs mainly used for surface anaesthesia are amethocaine (tetracaine), benzocaine, cocaine, lignocaine (lidocaine), and prilocaine.

Local or Infiltration Anaesthesia: A solution of the LA is injected subcutaneously at one or more sites in and around the area which is to be incised by the surgeon. The object is to ensure blockade of the finer nerve endings which supply the region. Infiltration anaesthesia is used for minor operations. It is also employed in the treatment of rheumatoid arthritis and muscular rheumatism. The LA is infiltrated around the joint or into the fibrositic nodules.

Lignocaine, mepivacaine, prilocaine and procaine are the compounds mainly used in this sort of anaesthesia.

Conduction or Regional Nerve Block Anaesthesia: The LA is injected as close as possible to the main nerve trunk in a limb supplying the area in which anaesthesia is required. Conduction in both motor and sensory fibres is blocked, enabling operations to be carried out on the limb. Higher concentrations of LA than are used in infiltration anaesthesia are required to produce anaesthesia by nerve block, because the fibres in the nerve trunk are considerably thicker than the fine nerve terminals encountered in the skin.

Epidural or Extradural Anaesthesia: It is a special

form of regional nerve block in which the solution is injected into the epidural space, and comes into contact with the dura covering the roots of spinal nerves. Some of the drug penetrates the dura, enters the subarachnoid space and anaesthetises the nerve roots. The rest of the solution diffuses outside the dura and reaches the paravertebral space causing additional blockade at this point. In Caudal anaesthesia, an epidural injection is made through the sacral hiatus.

For regional nerve block and epidural anaesthesia, the compounds mainly used are bupivacaine, lignocaine, mepivacaine, prilocaine and procaine.

Spinal or Subarachnoid Anaesthesia: The LA solution is injected into the subarachnoid space so that it reaches the roots of spinal nerves. The technique is used to induce anaesthesia in parts of the body up to the thoracic region. Abdominal or pelvic operations can then be carried out while the patient remains conscious.

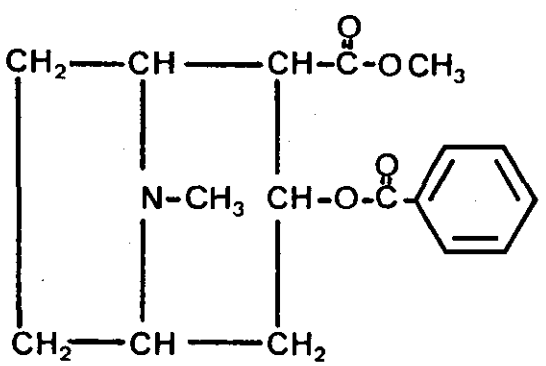
1.1.4 Properties Desirable in Local Anaesthetics

An ideal LA should combine several properties. It should be inexpensive, stable in solutions and capable of being sterilized by heat. In administration it should not be irritating to the tissue to which it is applied, nor should it cause any permanent damage to nerve structure; most LAs in common use fulfil these requirements. Systemic toxicity should be low, as the LA is eventually absorbed from its site of application. The LAs therapeutic index is of obvious importance in the evaluation of the efficacy and

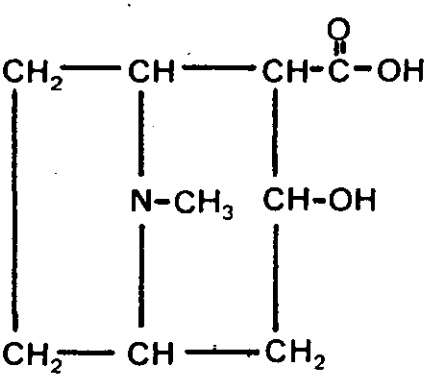
safety of these drugs. Anaesthesia should be of rapid onset and must last long enough to allow time for surgery, yet not so long as to need an extended period of recovery. Sometimes, for example, in the control of chronic pain, a LA action lasting for days or even weeks is required. Unfortunately, the available compounds employed for anaesthesia of such long duration, have high local toxicity.

1.1.5 Chemistry and Structure - Activity Relationships

Three years had elapsed after the introduction of cocaine as a LA when the first effort to correlate structure to activity was made. Filehne (1887), suggested that the effect was associated with the benzoic acid moiety of cocaine and that the ecgonine portion was of only slight importance (Fig. 1.2).



cocaine



ecgonine

Fig. 1.2 Structure of cocaine and ecgonine

The benzoic acid hypothesis was soon criticized (Poulsson, 1890). It was pointed out that benzoyl-ecgonine was inactive and that many analogoues of cocaine with other organic acids possess considerable anaesthetic potency (Stockman, 1885-1886).

The toxicity of several compounds related to cocaine

was found to be practically identical (Ehrlich, 1890) while only a few of them, which were esters of acids closely related to benzoic acid, were potent LAs. Later Ehrlich and Einhorn (1894) suggested that the ability to induce anaesthesia is a common property residing in widely differing organic compounds. The term "anaesthesiophor" introduced by Ehrlich (1890) has since been used widely to indicate the structural fragments of a molecule to which the LA effect could be assigned.

In an homologous series, LA parameters such as potency or duration usually increase with increasing molecular weight for the lower members until a maximum is reached. Further increase of number of carbon atoms decreases the anaesthetic effect (Abreu et al., 1955; Büchi, Cordes and Perlia, 1964; Ekenstam, 1966; Åberg et al., 1977).

Miescher (1932) demonstrated that effective LAs possess certain general structural characteristics and noted:

(1) Compounds with LA activity are oxygen-containing substituents that are bonded to either higher aliphatic and hydroaromatic groups or equally to electronegative groups, such as alkylene and trichloromethyl groups (narcotics), aromatic and unsaturated heterocyclic rings (e.g. benzoic acid, p-aminobenzoic acid, cinnamic acid, and quinoline-4-carboxylic acid derivatives).

(2) The carbonyl group exhibits a particularly satisfactory effect in the form of the ester group (procaine series), keto group (Falicaine series) and as the amide group in an α-position to an unsaturated ring (cinchocaine series).

(3) In general, an increase in the local anaesthetic activity can be reached by:

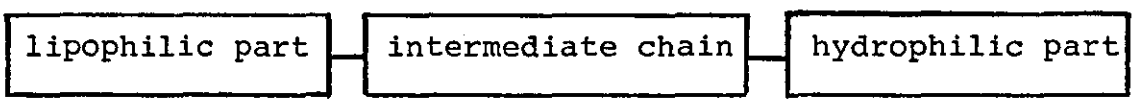
(a) introducing higher alkyl groups in the form of side chains,

(b) introducing alkoxy groups which improve the lipid solubility,

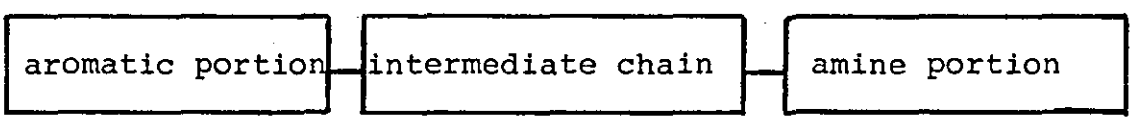
(c) introducing basic substituents which improve the basicity of compound as a whole, and

(d) accumulation of aromatic and heterocyclic rings.

Löfgren (1948) also gave a generally all-inclusive formulation of these relationships. His own results and his study of the literature led him to the conclusion, that almost all useful LA molecules can be arranged according to the following scheme:



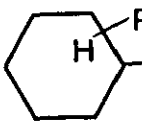
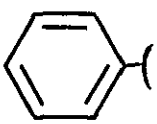
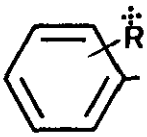
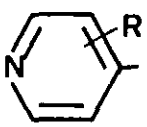
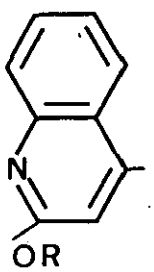
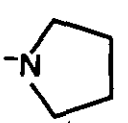
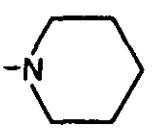
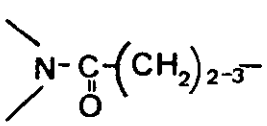
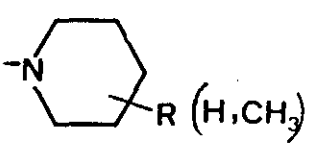
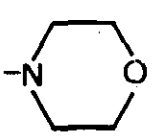
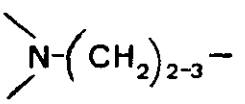
which usually can be expressed as:



Most of these compounds can, according to this point of view, be subsumed under the simple structure outlined in Table 1.1.

Aromatic radicals are primarily phenyl with different kinds of substitued phenyl groups, as well as 2-alkoxyquinoline groups. The connecting chain contains a hetero atom (O, N, or S) in an ether, alcohol, ketone, ester, amide,

Table 1.1 Chemical Structure of Local Anaesthetics

Lipophilic	Intermediate	Hydrophilic
Aromatic radical	Connecting chain	Amine group
(higher) C_nH_{2n+1}	$-O-(CH_2)_{2-3}-$	$-NH-C_nH_{2n+1}$
	$-CH(OH)-(CH_2)_{2-3}-$	$-NH-(CH_2)_{2-3}-OH$
	$-C(=O)-(CH_2)_{2-3}-$	$-NH-(CH_2)_{2-3}-$ 
	$-C(=O)-O-(CH_2)_{2-3}-$	$-N(C_nH_{2n+1})_2$
	$-C(=O)-S-(CH_2)_{2-3}-$	$-N[(CH_2)_{2-3}-OH]_2$
	$-C(=O)-NH-(CH_2)_{2-3}-$	$-N[(CH_2)_{2-3}-$  $]_2$
	$-NH-C(=O)-(CH_2)_{2-3}-$	 
		
	$-NH-C(=O)-O-(CH_2)_{2-3}-$	
		

($\dot{R}$ = H, $-NH_2$, $-NHR^1$, $-OH$, $-OR^1$, $-CH_3$, $-Cl$)

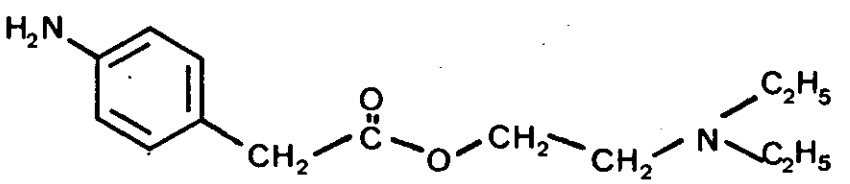
(After Büchi, et al, 1964)

amine or thio ester linkage. The amino group can be secondary or tertiary in nature and may carry alkyl, hydroxyalkyl, or aralkyl groups, or can be pyrrolidino, piperidino, pipecolino, or morpholino radicals.

Doerge (1971) mentioned some general examples which show that a change of structure changed the activity.

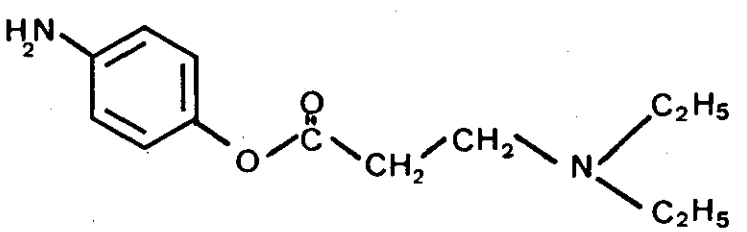
(a) The most desirable position of the amino group on the aromatic ring is para to a carboxy group.

(b) The introduction of a methylene group (CH_2) as in phenylacetic acid, will decrease the activity. The following structure (I) is practically inactive



(I)

(c) The carboxyl group must be conjugated with an aryl group. Therefore the following structure (II) has no LA activity.

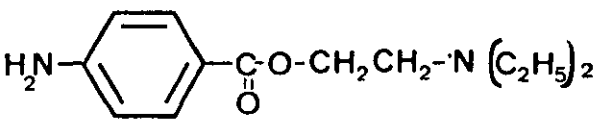
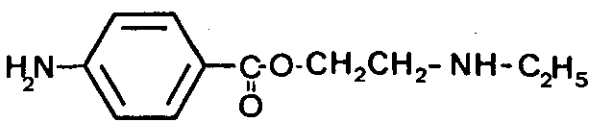
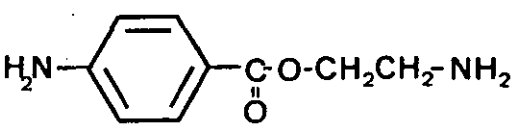
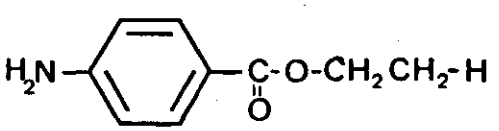
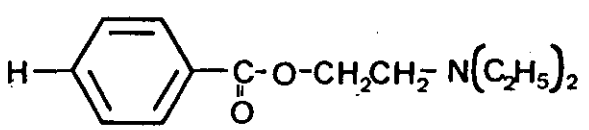
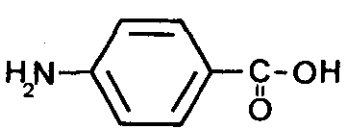
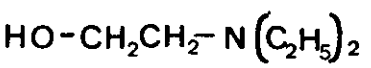
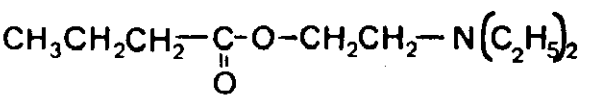
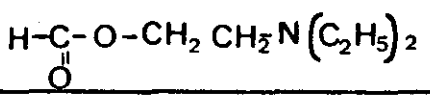


(II)

(d) The esters of p-aminobenzoic acid are more effective than are the esters of benzoic acid, p-hydroxybenzoic acid and p-alkoxybenzoic acid.

Similar observations were made by Büchi and Perlia (1972) who illustrated the relative importance of the LAs chemical constitution, by eliminating individual molecular functions and assessing LA effectiveness (Table 1.2).

Table 1.2 Influence of Chemical Change on Local Anaesthetic Activity of Procaine (+ = Active; - = Non-Active).

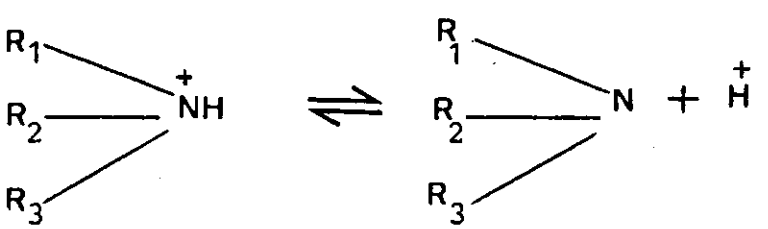
	LA Activity	
	Surface	Regional
	+	++
	(+)	+ (+)
	(+)	+
	++	-
	-	+
	-	-
	-	-
	-	-
	-	-

(After Büchi and Perlia, 1972)

1.1.6 Mechanism of Action as Local Anaesthetics

The LAs in the form of the unprotonated amine tend to be only slightly soluble and unstable in solution. Therefore, they are generally marketed in the form of their water-soluble salts, usually the hydrochlorides. In as much as the LAs are weak bases the salt solutions are quite acid, a condition that fortunately increases their stability and that of any accompanying vasoconstrictor substance with which LAs are often formulated. It has been predicted, that previous alkalinization of an anaesthetic solution (Gros, 1910; Fosdick et al., 1930; de Jong,1969), or the use of a salt of a weak acid such as the borate or carbonate salt of the LA (Bignon, 1892), will increase its clinical efficacy. However, objective tests have failed to substantiate this point (Ritchie and Ritchie, 1968), and more alkaline preparations have the disadvantage of being relatively unstable.

The most useful LAs are secondary or tertiary amines which will exist as uncharged or positively charged ions depending on the pH of the solution and on the dissociation constant (pK_a) of the compound. The ionization of a typical LA may be depicted as follows:



The more alkaline the solution, the greater is the proportion of anaesthetic in the uncharged form.

The pK_a's of most LAs in common use lie between 8.0 and 9.0, so that only 5 to 20% will be in the form of unprotonated amine at the physiological pH. This fraction, although small, is important because the drug usually has to diffuse through connective tissue and other cellular membranes to get to its site of action. It is generally agreed that it can do so only in the form of uncharged amine with its high oil/water partition coefficient (de Jong, 1969; Narahashi et al., 1970; Holroyd, 1978).

There is every indication that LAs act on any part of the nervous system and on every part of nerve fibre (Vandam, 1960; Hsia and Boggs, 1975; Strichartz, 1976). Their main site of action, however, is the membrane (Straub, 1956; Shanes, 1958; Takman and Camougis, 1970; Seeman, 1972; Lee, 1976) and there is seemingly little direct action of physiological importance on the axoplasm (Ritchie and Cohen, 1975). Those axoplasmic effects, such as effect on the giant axons of squid that do occur may be secondary to the membrane action (de Jong, 1969).

LAs block impulse conduction by interfering with the movement of sodium ions and to a lesser extent potassium ions through the membrane (Vandam, 1960, Rothstein, 1968; de Jong, 1969; Ritchie, 1975).

Many different types of drugs, such as certain biotoxins, alcohols, the volatile and gaseous general anaesthetics, organic acids and bases, and certain substituted quaternary ammonium ions can block conduction of the nervous impulse (Ritchie, 1975). Because of the diversity of these molecular structures, Ritchie (1975) suggested that more than one mechanism is involved in the production of nerve block. However, Staiman and Seeman (1975) have shown that

the effects of mixtures of LAs are close to being additive, suggesting a single mode of action for the wide range of LA molecules. Only one group, however, has emerged as being clinically useful in producing local anaesthesia, namely certain aromatic amines (such as lignocaine, procaine, cocaine, amethocaine).

Numerous theories have been proposed for the mode of action of LAs. They may be summarized as follows (Büchi and Perlia, 1972; Ritchie, 1975; Lee, 1976; 1979; Boggs et al., 1976). Local anaesthetics may:

(1) interfere with chemicals involved in nerve conduction;

(2) alter the density of fixed charges on the surface of the membrane;

(3) cause an expansion of some region of the membrane that is critical for conduction;

(4) react with some specific receptor in the nerve membrane;

(5) cause protein perturbation;

(6) close the sodium channel by changing the lipid molecules surrounding it from the gel, to the fluid, liquid crystalline phase.

SECTION TWO

GENERAL CONCEPTS OF ANTIMICROBIAL ACTIVITY

It would be quite wrong to discuss the antibacterial activity of any chemical compounds without considering the structure of their likely targets. In this project, LAs are being considered as antibacterial agents and their target is thus the components of the bacterial cell.

1.2.1. Structure of Bacterial Cell

Bacteria are prokaryotic and unicellular. The essential features of a very "generalised" bacterial cell are shown in Fig. 1.3.

Three fundamental divisions of the bacterial cell occur in all species: cell wall, cell or cytoplasmic membrane, and cytoplasm. Two types of thread like appendages may be found growing from bacterial cells, namely, flagella and fimbriae or pili (including the sex or F-pilus). Surface adherants (capsules, slime and extracellular substances) can be seen in some bacteria (Hugo, 1977; Murray, 1978).

Flagella are threads of protein often 12-15μm long which start as a small but complex basal organ at the cytoplasmic membrane. They are responsible for the movement of motile bacteria (Murray, 1978; Stanier et al., 1980).

Pili are organs of attachment. They are responsible for haemagglutination in bacteria and also for inter cell adhesiveness giving rise to clumping. The sex or F-pilus is part of a primitive genetic exchange system in some bacterial

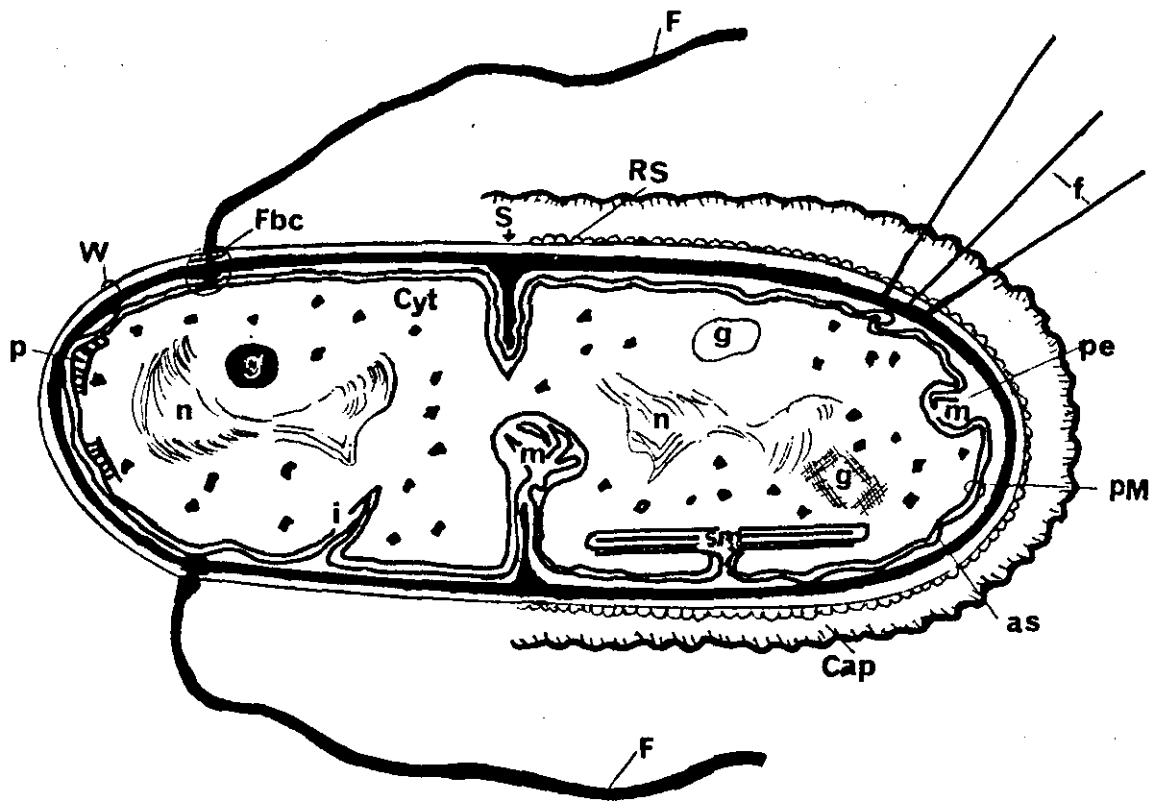


Fig. 1.3 Diagram of a dividing rod-shaped bacterium to illustrate anatomical relations of various structures: as, adhesion sites cap, capsule; cyt, cytoplasm containing ribosomes; F,flagella with 'hook' attachment to Fbc, the flagellar basal complex, f, fimbriae (common pili); g, inclusion granules and ribosome-free cytoplasm; i, simple membrane intrusion; m, mesosome ; n, nucleoplasms; PM, plasma membrane; p, polar membrane; pe, periplasmic space; RS, regularly structured outer wall layer (s); S, septum; sm, stacked membranes; W, cell wall.
(After Murray, 1978).

species. Through them part of the genetic material may be passed from one cell to another, thus giving rise to a simple form of sexual reproduction.

Some species of bacteria accumulate material as a coating of varying degrees of looseness. If the material is reasonably discrete it is called a capsule; if loosely bound to the surface it is called slime. Their function is likely to be protective for both the Gram-positive and Gram-negative bacteria (Meadow, 1978).

According to Schott and Young (1972) the nature of the exterior surface of bacteria (the cell wall) is of considerable interest because it is the first region of the cell with which nutrient, antibacterial agents or any other substances come to contact.

Bacterial cell walls, especially those of Gram-positive organisms, are fairly robust structures which protect the cell from osmotic lysis. Mitchell and Moyle (1959) found that solute concentrations in Staphylococcus aureus cells were capable of generating an osmotic pressure of about 20 atmospheres. Such a high pressure is capable of bringing about the osmotic lysis of the cytoplasmic membrane and it is the cell wall with its great tensile strength which prevent this.

The main difference between Gram-negative and Gram-positive bacteria lies in the composition of the cell envelope (cell membrane + cell wall). It consists of a basic structure of alternating N-acetylglucosamine and N-acetyl-3-O-1-carboxylethyl-glucosamine molecules, giving a polysaccharide backbone. This is then cross-linked by peptide chains, the nature of which is different in each species.

This basic peptidoglycan (murein), is attached by covalent links to teichoic acid molecules (in the case of Gram-positive bacteria, Fig. 1.4), or lipoprotein molecules and a layer of lipopolysaccharide (in the case of Gram-negative bacteria, Fig. 1.5).

In Gram-negative bacteria the complex outer layers beyond the murein sacculus must protect the organism to a certain extent from the action of toxic chemicals and may act as a crude "outer membrane" (Brown, 1975).

In addition, periplasmic spaces within the cell envelope may constitute chemical defence zones in which enzymes such as penicillinases may be found (Stanier et al, 1980).

The cell membrane is a very delicate structure found beneath and in some places, firmly attached to the cell wall. It is the site for very many physiological processes and is, therefore, prone to damage induced by chemicals.

Chemically the bacterial membrane is similar to other biological membranes (Salton, 1971). It consists of phospholipid and protein, though carbohydrates, may also be present (Weibull, 1957; Salton, 1967; Estrugo et al., 1972; Hugo, 1977).

A "unit membrane" structure was proposed by Danielli and Davson (1935), and in various modifications still forms the basis for many membrane models (Fig. 1.6(a , b)). This model as modified by Robertson (1962), proposes a three layered structure; protein-lipid-protein. The two lipid monolayers occupy the middle layer with their hydrocarbon chains faced end-to-end and their hydrophilic ends pointing outwards contacting protein dense layer. This triple-layer

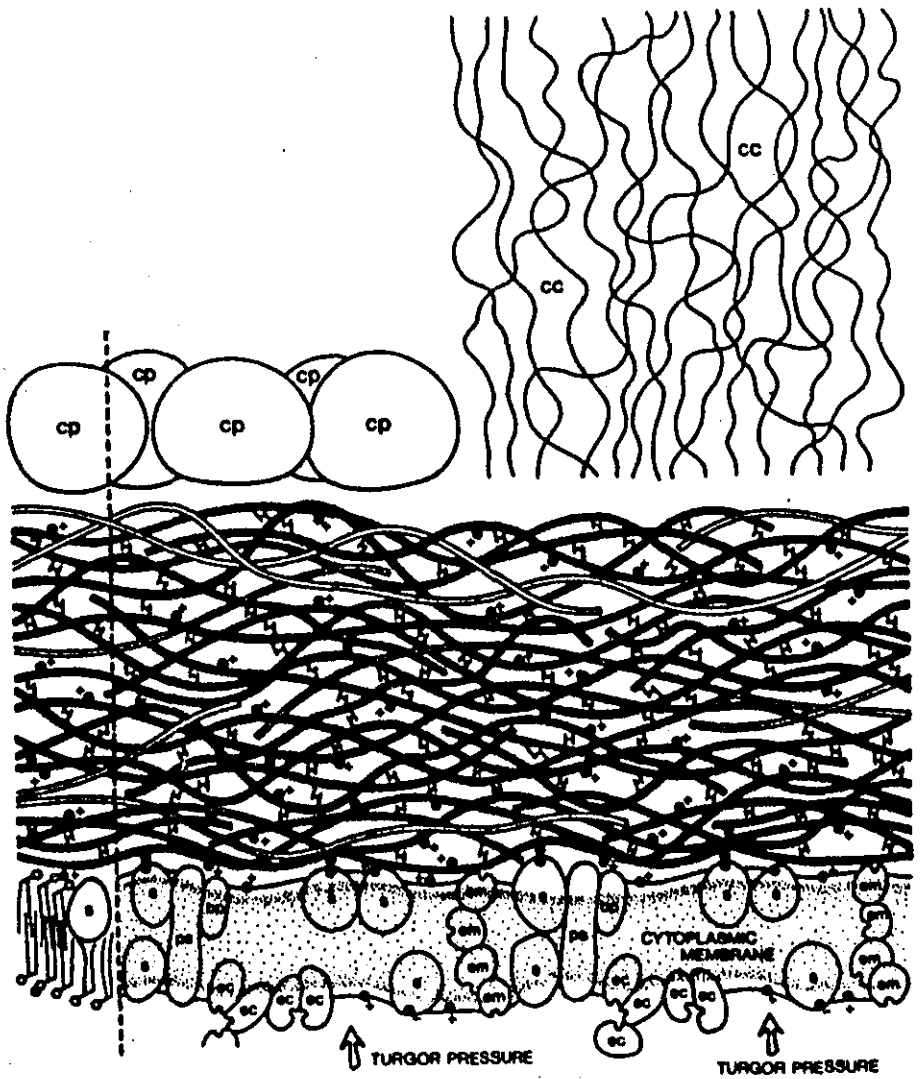


Fig 1.4 Diagram of Gram-positive cell envelope. +, Free cation; ⊕ bound cation; - Free anion; ⊖ bound anion; ⊖—⊕, adhesion point due to ionic bound; ;;;, hydrophobic zone; =, cross linking polypeptide in the peptidoglycan; —, polysaccharide; }, enzymatically active protein; =, teichoic and teichuronic acid polymers; ~O, phospholipid; bp, binding protein; em, cytoplasmic membrane enzyme which synthesizes macromolecular components of the cell wall; ec, cytoplasmic membrane enzyme whose function is directed to the cytoplasm; ep, periplasmic enzyme; es, surface enzyme; ps, permease; s, structural protein of cytoplasmic membrane; cc, capsular carbohydrate; cp, capsular protein.
(After Costerton, 1977).

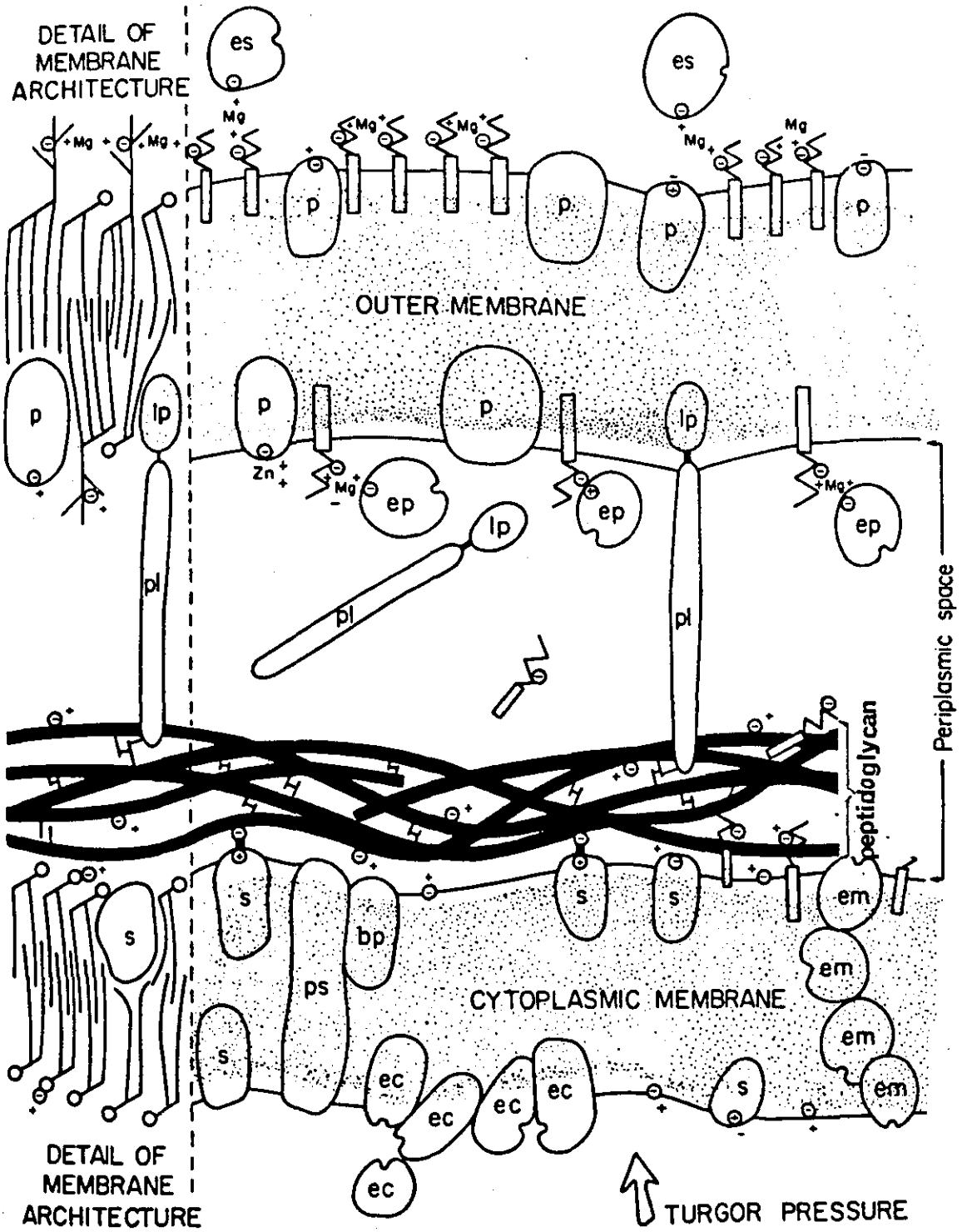


Fig. 1.5 Diagram of Gram-negative cell envelope.
+, Free cation; ⊕,bound cation ;-,Free anion ;⊖,bound anion; ⊖-⊕,adhesion point due to ionic bond;. :::,hydrophobic zone;)-(,covalent bond;≻≺,cross linked polypeptide; ▬,polysaccharide; ʃ,enzymatically active protein;⊖,lipopolysaccharide;⌐o, phospholipid; bp, binding protein; s and p,protein components of membranes; em, cytoplasmic membrane enzyme which synthesizes macromolecular components of the cell wall; ec,cytoplasmic membrane enzyme whose function is directed to the cytoplasm; ep,periplasmic enzyme; es,surface enzyme; ps,permease; pl and lp,protein and lipid components of lipoprotein bridges between outer membrane and peptidoglycan wall. After Costerton et al (1974).

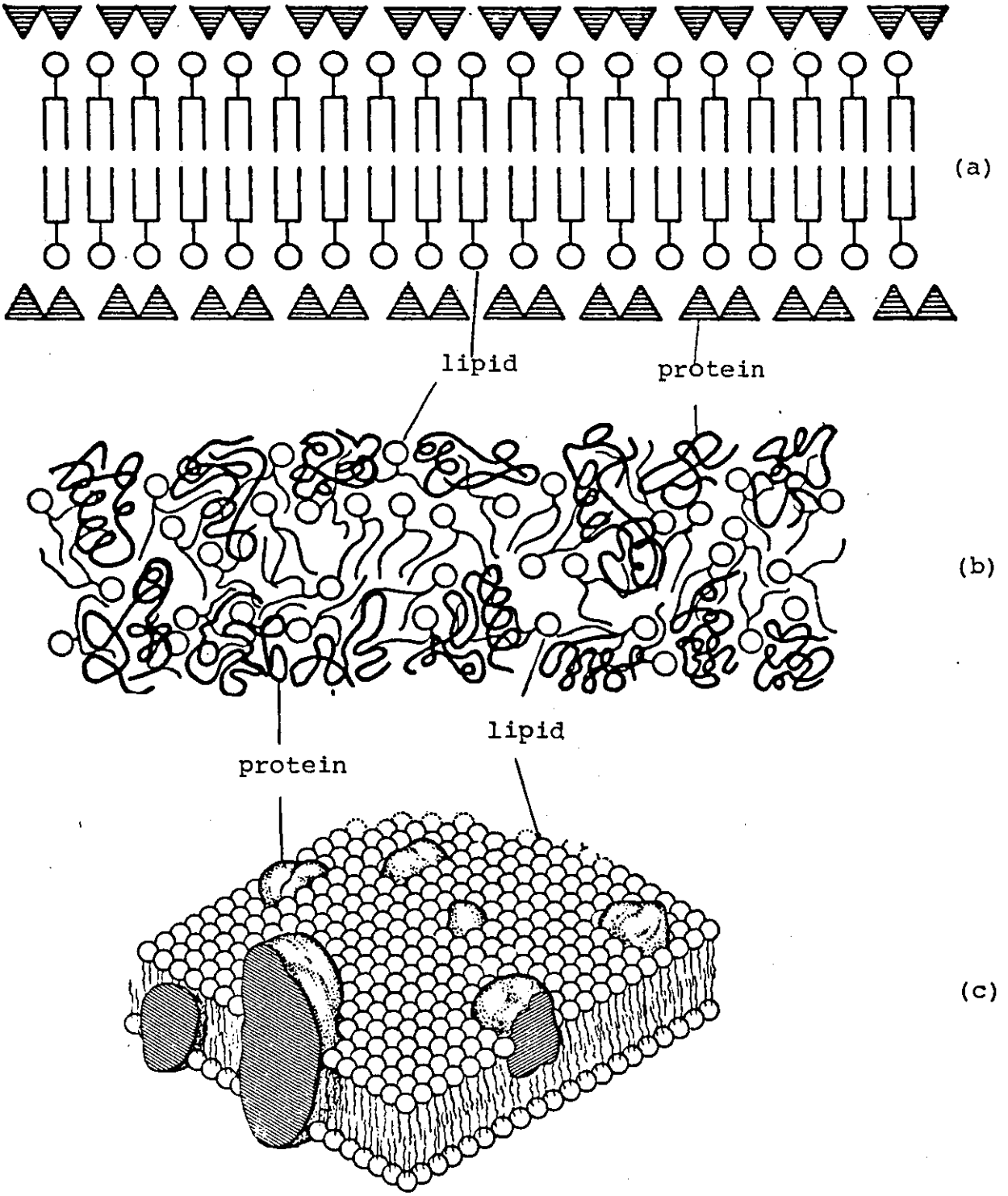


Fig 1.6: Models of membrane structure

(a) After Danielli and Davson (1935)

(b) After Stoeckenius and Engelman (1969)

(c) After Singer and Nicolson (1972)

sandwich is thus posed between internal and external environments and passage from one to the other would involve transport through: protein, lipid and protein. The hydrophobic layer, or core, would form an osmotic barrier, protein could form binding sites and/or enzymes involved in transport processes. This membrane model has been subjected to much study and criticism; the arguments have been assembled and reviewed by Stoeckenius and Engelman (1969).

The modern theory of the structure of cell membrane is of a phospholipid bilayer, in which protein molecules are embedded. This is thought to be common to a variety of membrane ranging from the cytoplasmic membrane of prokaryotes, to those of erythrocytes and mitochondria (Singer and Nicolson , 1972; Fig. 1.6(c)), though some chemical differences are apparent in the components of membranes from different sources.

Certain enzymes and especially the electron transport chain which are located in the membrane are responsible for transport systems which utilise electrochemical potential of the proton to power it. Salton (1971), listed the following as membrane associated enzymes: succinate, malate and lactate dehydrogenases, cytochromes, cytochrome oxidase, NADH oxidase , mannosyldiglyceride synthetase and ATPase. Also permeases, the group of enzymes implicated in the active transport of metabolites are located in the membrane (Cohn and Monod, 1957). In addition the plasma membrane is intimately involved in the biosynthesis of all cellular elements external to it, such as cell-wall mucopeptides, lipopolysaccharides and teichoic acids, and it apparently ensures the equal partitioning of the genome among daughter

cells at division (Harold, 1970).

Despite the complexity of some microbial envelopes, the plasma membrane appears to be in all cases the main osmotic barrier between internal and external media of the cell. The barrier is permeable to water. Other substances cross the membrane as a result of specific mechanisms, which fall into three classes (Harold, 1970; Gale et al., 1972; Franklin and Snow, 1975):

Passive diffusion: In this case the substance diffuses into the organism by passing down a concentration or electrical gradient. This process obeys the laws of diffusion and is normally terminated when the concentration of substance outside the cell is balanced by the concentration inside the cell. The substance must be sufficiently soluble in the hydrophobic medium of the membrane to allow it to shuttle across the barrier.

Facilitated diffusion: This occurs when substances which are insoluble in the hydrophobic barrier of the cell membrane react with a carrier within the membrane. The carrier-substance complex is then shuttled across the membrane, following which the complex is broken down and the substance released into the cell (Fig. 1.7).

Active transport: In this case, uptake is by carrier-mediated transport which unlike facilitated diffusion cannot pass across the membrane in the absence of a source of energy (Fig. 1.8). Inhibition of energy generation or coupling results in immediate and direct inhibition of transport.

The nature of the coupling of energy production to transport is still a matter for debate. There are three major hypotheses:

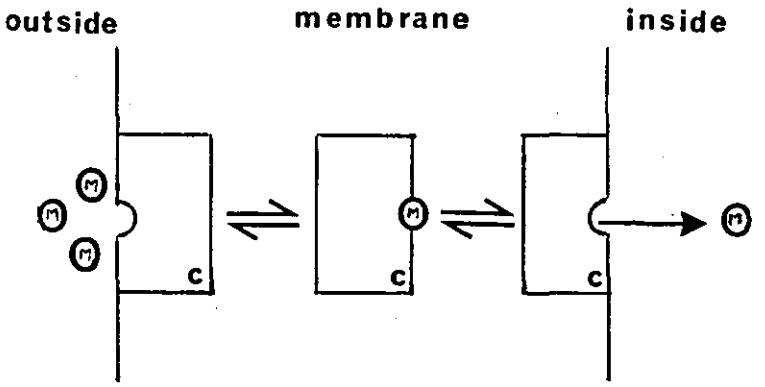


Fig 1.7 Facilitated diffusion: Metabolite (M) binds to mobile carrier (C) at the outer face of the membrane. The CM complex diffuses through the membrane and dissociates at the inner surface, releasing M into the cell interior.

(After Harrison and Lunt, 1975).

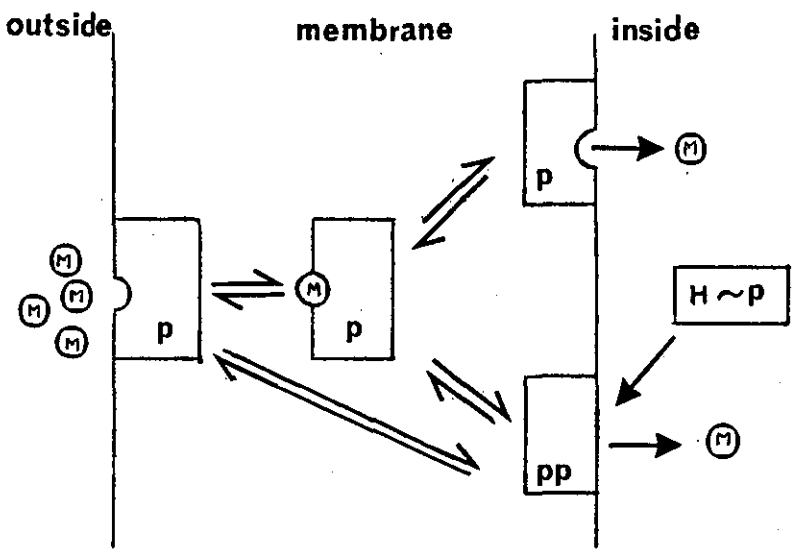


Fig 1.8 Active transport: the permease model: Metabolite (M) binds to permease (P) at the outer face of the membrane. The PM complex diffuses through the membrane and at the inner surface P is phosphorylated by the 'high energy' compound (H~P), resulting in a modified permease (PP) and in release of M. Alternatively the permease can act as a facilitated diffusion carrier as shown in the upper part of the scheme.

(After Harrison and Lunt, 1975).

(a) Chemical coupling hypothesis: Electron transfer leads to the formation of a covalent high-energy intermediate that serves as a precursor of ATP (Slater, 1966).

(b) Conformational coupling hypothesis: This is a variant of the chemical coupling hypothesis. It proposes that conformational changes in membrane proteins are mediated by ATP and are responsible for driving translocation processes (Green et al., 1968 ; Young et al., 1971).

(c) The Chemiosmotic - coupling hypothesis: (Mitchell, 1967; 1968; 1970). Which proposes that, the membrane is impermeable to H^+ and $O\bar{H}$, that the electron transport system in the membrane sets up a proton gradient across the membrane, and that the proton gradient then provides the source of energy to drive transport processes (Fig. 1.9).

Mitchell's chemiosmotic theory is substantiated by the finding that uncoupling agents such as 2,4-dinitrophenol (DNP) act as specific conductors of protons across bacterial, mitochondrial, and artificial membranes, thereby modifying part of the proton motive force across the membrane (Mitchell, 1961; 1970), and that these uncouplers are able to discharge transport processes (Hamilton, 1968).

The cytoplasm, which is a semifluid material, contains all the remaining components necessary for the life of the cell. These include those enzymes that are not present in membrane, reserves of food material, structures involved in the synthesis of protein (ribosomes and associated m-RNA and t-RNA) and the genetic material responsible for the inheritable character of the cell (DNA).

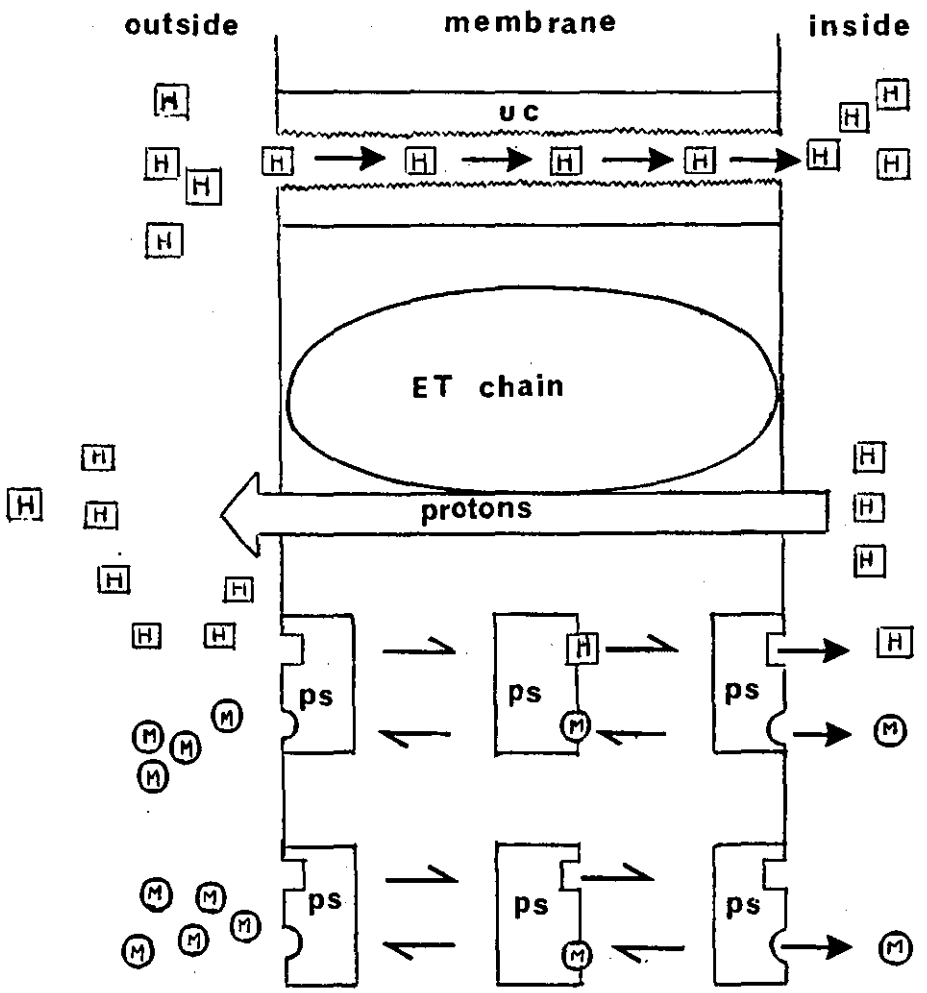


Fig 1.9 Active transport: The chemiosmotic model: The electron transport chain (ET chain), produces a transmembrane gradient. Proton Symport (PS) combines with metabolite (M) and protons (H). Protons run down their concentration gradient and power the active uptake of M. Uncoupler (UC) short-circuits the proton return and diminishes the power source. Under these conditions PS can still function as a facilitated diffusion carrier as shown in the lower part of the diagram.

(After Harrison and Lunt, 1975).

1.2.2 Drug-Cell Interaction

The first apparent interaction of a drug with the whole cell is when the compound is adsorbed onto the cell surface. The uptake of the drugs by cells have been measured by many workers as an initial study of their mode of action. Many drugs are readily taken up by bacteria, the amount adsorbed increasing with increasing drug concentration. The extent of adsorption of any drug by bacteria is likely to be governed by three principles factors, namely: drug concentration, bacterial cell numbers, and the time that the drug and the cell are in contact.

The drug-cell adsorption isotherm may show a point of inflection or change in slope, corresponding to a paritcular biological response (e.g. Salt and Wiseman, 1968), and the overall shape of the isotherm may give some indication of the interaction involved in the uptake process.

Giles et al. (1960) and Giles and Smith (1974) classified isotherms obtained for adsorption from solution into four basic patterns. According to the nature of slope of the initial portion of the curve, the isotherms are grouped as S, L, H and C types, plus an additional special case, the Z shape. Numerous workers have made use of this system to interpret drug cell interactions. Some example of the adsorption of antibacterial substances by micro-organisms that have been reported in literature are: uptake of cetrimide by Staphylococcus aureus and Escherichia coli (Salton, 1951), chlorhexidine by Staph. aureus and E. coli (Hugo & Longworth, 1964), cetyltrimethylammonium bromide (CTAB) by E. coli (Salt and Wiseman, 1968, 1970), benzyl and phenethyl alcohols by E. coli (Lang and Rye, 1972), some p-hydroxybenzoate esters by E. coli (Lang and Rye, 1973), 3-and 4-

chlorophenol by Pseudomonas aeruginosa (Gilbert and Brown,1978).

Following adsorption, secondary processes will occur leading to the inhibition of the reproductive and metabolic process of the cell. Wilson et al. (1975), summarized the target for antibacterial attack in the bacterial cell as in Fig. 1.10.

Hugo (1976) included essentially the same targets for chemical inactivation of the bacterial cell in three groups: 1) the cell wall, 2) the cytoplasmic membrane and 3) the cytoplasmic contents. Cytoplasmic membrane, includes the membrane itself and membrane associated enzymes. Within the cytoplasm, targets (both soluble and insoluble) include the ribosomes, the nucleic acids, cytoplasmic enzymes and general cytoplasm proteins which may be coagulated.

The wall is a prime target for chemical inactivation. Any drug which impairs the structure or synthesis of the cell wall (e.g. the penicillins) will indirectly facilitate membrane damage. Division may be impaired as may enzyme function (Hugo, 1976). Lysis of the cell often follows cell wall damage (Hugo, 1967).

Chemicals may cause damage to the cell membrane and its associated biochemical processes without any direct effect on the cell wall. This would include:

a) induction of leakage, so that small molecular weight substances such as amino acids, purines, pyrimidines, sugars and cations, especially K^+, leave the cell,

b) the inhibition of membrane enzymes especially adenosine triphosphatase (ATPase), and

c) the attenuation of the membrane electrochemical potential set up by the extrusion of H^+ during

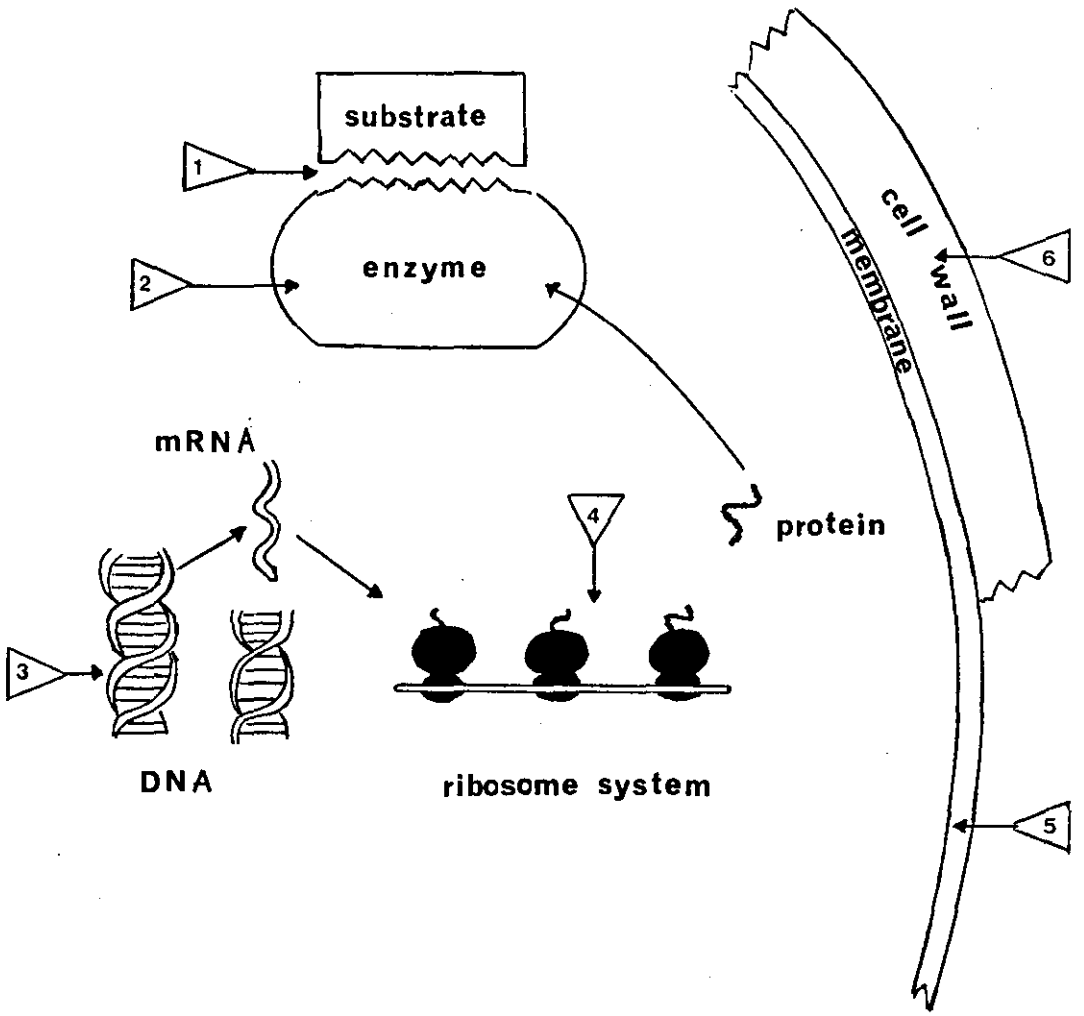


Fig. 1.10 Targets for antibacterial attack in the bacterial cells

1: active enzyme centre

2: allosteric changes in enzymes

3: DNA

4: the ribsome system responsible for protein synthesis

5: the cell membrane

6: the cell wall

(After Wilson et al, 1975)

metabolism.

Membrane damage has been detected after treating cells with many different compounds. For example, early studies of haemolysis showed that it could be induced by simple detergents (Schulman and Rideal, 1937; Pethica and Schulman, 1953), and by analogy Kuhn and Bielig (1940) suggested that quaternary ammonium compounds (QACs) because of their ability to dissociate conjugated proteins, might act on the bacterial membrane (a lipo-protein conjugate) and damage it in a manner resembling haemolysis.

Many other drugs have been shown to promote leakage from bacterial cells. For example, leakage from: P.aeruginosa by polymyxin (Newton, 1953); E. coli and Staph. aureus by chlorhexidine (Hugo and Longworth, 1964 ; 1965); Micrococcus lysodeikticus by chlorhexidine (Rye and Wiseman, 1964; 1965); E.coli by chlorhexidine (Rye and Wiseman, 1966); E. coli by CTAB (Salt and Wiseman, 1968); E.coli by novobiocin (Morris and Russell, 1970); E. coli and Staph. aureus by Fentichlor (Hugo and Bloomfield, 1971a); Staph. aureus by Triton X45 and Triton X100 (Lamikanra and Allwood, 1977a).

Many enzymes are attached to membrane. Membrane-bound ATPase has been implicated in the utilization of the trans-membrane pH and proton gradient to power energy dependent transport in microbial cells as postulated in Mitchell's chemiosmotic theory. Harold et al (1969) showed that chlorhexidine and DIO9, inhibited this enzyme in Streptococcus faecalis.

The membrane-bound electron transport chain of Bacillus megaterium was shown to be inhibited by hexa-

chlorophene (Frederick et al , 1974).

Several workers have shown that a group of chemicals will attenuate the membrane electrochemical potential generated by metabolism and, as a result, inhibit anabolic reactions dependent on it. These are uncoupling agents, some of which are also used as disinfectants. The classical uncoupling drug is 2,4-dinitrophenol. Its action as an uncoupler and an inhibitor of substrate uptake has been known for many years (Harold, 1970; Gale,et al, 1972). Similarly Hugo and Bloomfield(1971b) demonstrated that bacteriostatic concentrations of Fentichlor inhibited energy dependent uptake of substrates by E.coli and Staph. aureus. Later Bloomfield (1974) showed that Fentichlor was able to dissipate the membrane pH gradient in Staph. aureus.

High concentrations of many antimicrobial agents, have been shown to cause protein precipitation. This observation led Hugo (1967) to ascribe rapid antibacterial induced cell death, to general protein precipitation. In rapid cell death, subtle long term effects like the leakage of cytoplasmic material tend to be masked by the dramatic precipitation of protein which is observed.

There are two types of proteins, structural and enzymic. Both are characterised by the specifity of their structure and anything that disturbs the delicate balance of charges on the surface of the structure will bring about rapid denaturation. Enzymic proteins have highly specific contoured surfaces which carry more charges than those on structural proteins. For this reason, they are more prone to precipitation than the more stable structural proteins.

Precipitation has been observed microscopically, by

light scattering techniques and by the effect of drug on cell free extract (Hugo and Longworth, 1964; 1965; 1966; Hugo, 1967; Hugo and Daltrey, 1974; Silva et al., 1976).

A number of antibacterial dyestuffs belonging to the acridine series have been shown to impair the function of bacterial nucleic acids. Dyes such as proflavine fit, or intercalate, into the double-stranded DNA helix and thereby prevent its function (Franklin and Snow, 1975).

Although the ribosomes are a target for many antibiotics (e.g. Vazquez, 1966; Modolell and Davis, 1970; Cundliffe, 1972), they are not normally a specific target for chemical disinfectants.

1.2.3 Antimicrobial Activity of Local Anaesthetics

Complications, including infection, following the clinical use of local anaesthetics have been reported (Massey Dawkins, 1969). The common use of LAs during dental procedures has led to concern over the possible introduction of microorganisms along the path of the needle during infiltration (Streitfeld and Zinner, 1958; Winther and Praphailong, 1969; Zaidi and Healy, 1977) and this has given rise to much discussion over the need for disinfection of the oral mucosa at the site of injection (Streitfeld and Zinner, 1958; Davis, 1961; Winther and Khan, 1971).

LAs are frequently administered topically before samples for microbiological evaluation are obtained from certain sensitive area of the body. These areas include the cornea, the upper respiratory tract including the sinuses, and areas of the urinogenital tracts. In such cases, it is

essential that the LA administered should not interfere with the growth of organisms present in the sample.

That LAs possess antibacterial properties was first suggested by Jonnesco (1909), though it was not until Schlegel and Swan (1954), described the inhibition of growth of two organisms by 0.2% benoxinate that more specific information began to be available. Over the next few years it became apparent that LAs may interfere with the growth of microorganisms in clinical specimens (Murphy et al., 1955; Erlich, 1961; Conte and Laforet, 1962) and that antimicrobial effectiveness related to both LA and cell type (Hughes and Stewart, 1957).

It has been commented that the antituberculous effects of amethocaine may be related to its chemical similarity to p-aminosalicylic acid (PAS) and p-aminobenzoic acid (PAB) (Conte and Laforet, 1962). However, no solid data is available to document such a suggestion.

Kleinfeld and Ellis (1966) showed that amethocaine, benoxinate, and cocaine displayed inhibitory activity against Staphylococcus albus, P.aeruginosa and Candida albicans. They also observed that proparacaine had only fungicidal action against C.albicans. These authors warned against the use of such topical anaesthetics before obtaining material for culture from ophthalmologic infections (Kleinfeld and Ellis, 1967). They also suggested that certain factors could influence the growth inhibitory effect of anaesthetic. These were, notably, LA dilution by tears or other body fluids, contact time, the number and type of organisms present, and the presence of viscous secretion which could protect the organism from contact with the anaesthetic.

Schmidt and Rosenkranz (1970) showed that both lignocaine and procaine inhibited the growth of most pathogenic bacterial and fungal isolates. Their results showed that Gram-negative bacteria were especially sensitive to the drugs, while species of C. albicans were resistant to concentration of drugs used topically (MIC > 2%). Since neither lignocaine nor procaine selectively inhibited the synthesis of DNA, RNA or protein, they suggested that the basis of action of these agents may involve the cell wall or cytoplasmic membrane.

They also mentioned that the use of LAs prior to the obtaining material for culture may account for frequent failure to recover etiologic agent. This is supported by evidence of the inhibition of two species of Pseudomonas by a fluoresein-anaesthetic solution, (Stewart, 1972).

El.Nakeeb and Farouk (1973) studied the effect of six LAs on the growth of Staph. aureus. Their results showed that amethocaine (tetracaine), cinchocaine (dibucaine) and benzocaine have the highest activity followed by butacaine, lignocaine and procaine which were practically inactive in the concentration used. Both amethocaine and cinchocaine were considered to be bactericidal and benzocaine bacteriostatic. Later (1975, 1976), the same authors studied the activity of an admixture of these drugs with some preservatives and found that LAs has synergistic, additive, or antagonistic combinations when evaluated against Staph. aureus, B. megaterium, E.coli, P.aeruginosa, and Sacharomyces cerevisiae. However, incubation of lignocaine (2%) with seven different antibiotics resulted in no change in antibacterial activity when measured by agar diffusion bioassay (Barza et al., 1974).

Of the agents used by Weinstein et al (1975), amethocaine and hexylcaine displayed the highest level of broad spectrum antimicrobial activity. Benoxinate showed almost no activity and procaine only limited antifungal activity.

Leung and Rawal (1977) investigated the mechanism of action of amethocaine against P.aeruginosa. They speculated that amethocaine acts on the phospholipids of the cell envelope possibly by inhibiting the binding of divalent cations to the anionic sites on the phospholipids. Such a speculation was supported by their observation that Mg^{++} antoganizes the lytic and bactericidal action of amethocaine on P. aeruginosa.

Observation of cellular lysis, leakage of intracellular material (260 nm absorbing materials), dehydrogenase activity, and a higher sensitivity of spheroplasts than of whole cells to amethocaine led them to the conclusion that amethocaine acts by damaging the cell membrane. They also found that the lytic action of lysozyme was potentiated by amethocaine, and they related this to the enhance permeability of the cell envelope.

Zaidi and Healy (1977) confirmed that amethocaine is bactericidal for some common hospital pathogens, and suggested its use for extradural and caudal anaesthesia, and in other situations where there is a risk of infection.

Cizmarik and Trupl (1978) studied the antimicrobial effect of heptacaine and two key synthetic intermediary products. Their results indicated that heptacaine and one of the intermediate (2-heptyloxyaniline) have antimicrobial activity. They concluded that, some microorganisms seem

to split heptacaine enzymatically to the intermediate which is the actual antimicrobial agent.

Silva et al (1979) described the effects that some LAs induced on Gram-positive bacterial cells. These effects included growth inhibition, reduction in the number of viable cells, lysis of protoplasts, permeability changes, characteristic ultrastructural alterations, and inhibition of membrane-bond enzymic activities. The quick and extensive K^+ efflux induced in treated bacteria led them to the conclusion that the cell membrane is directly affected by the anaesthetics and that membrane permeability is primarily disturbed. They also found a correlation between the activity of four drugs (chlorpromazine, nupercaine, amethocaine and procaine) and their lipid solubilities as indicated by octanol - water partition coefficients.

Salt and Traynor (1979) showed that LAs induce increases in the turbidity of non-growing bacterial cultures, and suggested a similarity between their mode of action and that of quaternary ammonium compounds. They found a close correlation between the deduced critical micelle concentration (CMC) for amethocaine and that concentration in excess of which large induced increases in culture turbidity were detected. They concluded that, the uptake of amethocaine by E.coli may occur preferentially when the molecules are in the micellar state.

1.2.4 Aim of the Project

The aim of this project was to review the current information on antimicrobial activity of local anaesthetics,

to evaluate their antibacterial effectiveness and their probable site(s) of antibacterial action.

Four commonly used local anaesthetics were chosen for evaluation and comparison, to represent the two major LA types. Amethocaine and Procaine represent the ester linked compounds and Cinchocaine and Lignocaine the amides.

CHAPTER 2

MATERIALS AND METHODS

Contents

SECTION ONE

MATERIALS

2.1.1 Organisms

The organisms used during investigation in this project were obtained from either The National Collection of Type Cultures (NCTC), London or The National Collection of Industrial Bacteriology (NCIB), Aberdeen. These were:

Escherichia coli	(E.coli)	NCTC 9001
Pseudomonas aeruginosa	(P.aeruginosa)	NCTC 6749
Bacillus megaterium	(B.megaterium)	NCIB 8291
Klebsiella aerogenes	(K.aerogenes)	NCIB 8267

2.1.2 Media and Associated Solutions

Preliminary shaken culture experiments were performed to determine the composition of most suitable synthetic growth medium for the range of organisms used. Also the pH of the buffer and its capacity was chosen in such a way to be suitable for both bacteria and local anaesthetics. From these, the growth medium (2.1.2 vi) was selected.

(i) Trace elements solution (Cruickshank, 1970)

$Fe\ SO_4.7H_2O$	0.5g
$Zn\ SO_4.7H_2O$	0.5g
$Mn\ SO_4.3H_2O$	0.5g
$H_2\ SO_4$ 0.1N	10 ml

Distilled water to 1000 ml

Sterilized by filtration.

(ii) Vitamin-free casamino acid solution 10g.100ml^{-1}

sterilized by autoclave.

(iii) Vitamin solution:

Nicotinic acid	1.0g
Thiamine hydrochloride	1.0g

Distilled water to 1000 ml

sterilized by filtration.

(iv) Carbohydrate solutions (aqueous)

(a) D-Glucose 20% ($^{w}/_{v}$) (unless otherwise stated)

(b) D-Glucose and D-Galactose 3% $^{w}/_{v}$)

sterilized by autoclave.

(v) Mineral salts solution

NH_4Cl	3.00g	
$Mg\ Cl_2.6H_2O$	0.20g	
$Na_2\ SO_4$	0.20g	
KH_2PO_4	9.25g	0.2 M phosphate buffer pH 6.4 ~ 6.5
K_2HPO_4	5.57g	

Distilled water to 1000ml

sterilized by autoclave.

(vi) Growth medium

Mineral salts solution	100ml
Trace elements solution	0.5ml
Vitamin-free casamino acid solution	0.2ml
Vitamin solution	0.1ml
D-Glucose (20% solution)	1.5ml

(vii) Carbohydrate free medium (CFM)

Growth medium from which the carbohydrate solution had been omitted.

(viii) Low phosphate medium (LPM)

Growth medium in which phosphate buffer had been replaced by:

Potassium chloride	$0.074g \cdot l^{-1}$	
Sodium citrate	$12.57g \cdot l^{-1}$	citrate buffer pH 6.2
Citric acid	$1.51g \cdot l^{-1}$	

and contained very low phosphate as impurities.

(ix) Medium of variable magnesium content

Growth medium with or without added carbohydrate in which the amount of magnesium chloride was varied.

(x) Maintenance (solid) medium

Growth medium solidified by the addition of agar (Oxoid No.3) 1.5% $^{w}/_{v}$.

(xi) Tryptone soya agar

Tryptone soya broth	30g
Agar No.3 (Oxoid)	15g
Distilled water to 1000ml	

sterilized by autoclave.

(xii) Acetate veronal buffer (AVB)

Basic mixture	5.0ml
HCl ($\frac{N}{10}$)	7.0ml
distilled water	13.0ml
$CaCl_2$ (1M)	0.25ml
$Mg\ Cl_2$ (1M)	0.25ml
pH adjucted to 6.1	

Basic mixture consists of:

Sodium acetate	19.43g
Sodium barbitone	29.43g
Sodium chloride	34.0g
distilled water to	1000ml

2.1.3 Reagents

(i) Dye solubilization

Sundan Black B — B.D.H. Chemicals Ltd. Poole, England.

(ii) Pentose estimation (Mejbaum, 1939)

$FeCl_3$	0.10g
Orcinol	0.30g
Conc. HCl to	100ml

(iii) Voltammetric determination of inorganic phosphate (Fogg & Bsebsu, 1981)

Acidic molybdate solution, 2% $^{w}/_{v}$: 35 ml of analytical-reagent grade concentrated sulphuric acid was added to 200 ml of water. 5.0g of ammonium molybdate was dissolved in the resulting solution, and diluted to 250 ml with water when cooled.

(iv) Dehydrogenase activity

Triphenyltetrazolium chloride (TTC)
Fisons, Loughborough, England.

2.1.4 Local Anaesthetics

(i) Amethocaine hydrochloride (B.P.). Tetracaine hydrochloride (Eur.P., U.S.P.), 2-Dimethylaminoethyl p-N-butylaminobenzoate hydrochloride. M.wt = 300.8

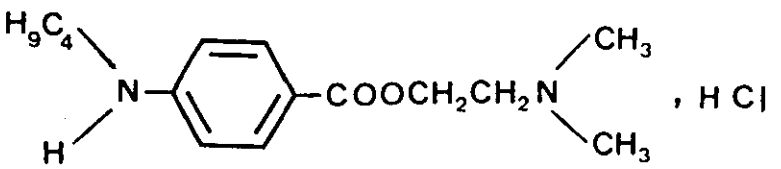


Fig 2.1a

A gift from Glaxo Group Research, Ware Ltd and purchased from Sigma Chemicals Co. Ltd. London, England.

(ii) Cinchocaine hydrochloride (B.P.). Dibucaine hydrochloride (U.S.N.F.); 2-Butoxyquinoline-4-carboxylic acid diethylaminoethylamide hydrochloride. M.wt. = 379.9

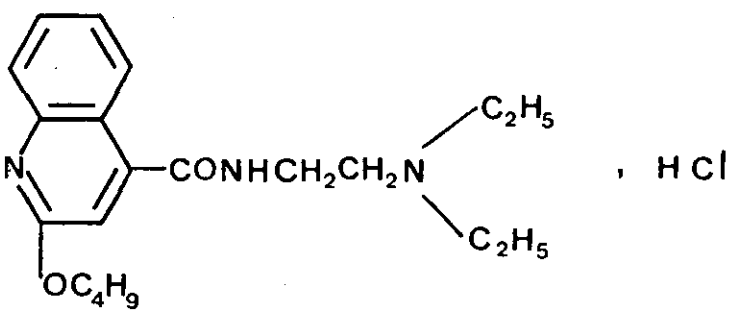


Fig.2.1b

A gift from Glaxo Group Research, Ware Ltd. Samples were also purchased from Sigma Chemicals Co. Ltd. London, England.

(iii) Lignocaine hydrochloride (B.P.). Lidocaine hydrochloride (Eur.P., U.S.P.), α-Diethylamino-2 , 6 -dimethylacetanilide hydrochloride.

M.wt. = 270.8

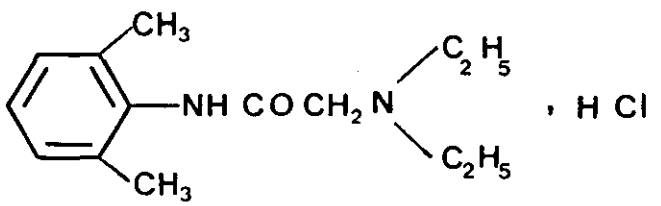


Fig. 2.1c

Gift from Johnson and Johnson, Slough, Bucks; Boots Co. Ltd. Nottingham, England and Astra Södertälje, Sweden.

(iv) Procaine hydrochloride (B.P., Eur. P.) .2-Diethylaminoethyl p-aminobenzoate hydrochloride. M.wt. = 272.8

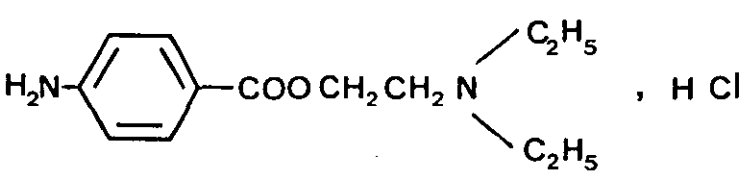


Fig.2.1d

Purchased from Sigma Chemicals Co. Ltd. London, England, or a gift from Boots Co. Ltd. Nottingham, England.

Stock solutions of LAs were prepared with sterile minimal salts solution or appropriate medium.

2.1.5 Antimicrobial Agents

Ampicillin B.P.	Beecham Research Labs.
Chloramphenicol	Sigma Chemicals Co.Ltd.
Puromycin	Sigma Chemicals Co.Ltd.
Cetylpyridinium bromide	B.D.H. Chemicals Ltd.

Stock solutions of antimicrobial agents were prepared with sterile minimal salts solution, or appropriate medium.

2.1.6 Miscellaneous

(i) General chemicals were of Analar grade, unless otherwise stated.

(ii) Radiotracers and associated materials:
^{32}P -orthophosphate (Radiochemical Centre, Amersham, Buckinghamshire, England).

Liquid scintillator KL 372 (Koch-Light Laboratories, Ltd. Colnbroack, Bucks., England).

(iii) Materials for electron microscopy:

Glutaraldehyde (E.M. Grade) (B.D.H. Chemicals Ltd., Poole, England).

Silver dag (Agar Aids)

Round glass cover-slips, 13mm (Chance Propper Ltd.).

Aluminium stubs , 15mm (Agar Aids)

Copper grids, 200 and 400 mesh (Agar Aids)

Gold palladium wire (Agar Aids)

Embedding medium:

Araldite (Polaron Equip. Ltd. Watford)

	GY 212	27ml
Hardener (Polaron)	HY 964	23ml
Accelerator (N-Benzyl-dimethylamine-Polaron)		1ml

(iv) Materials for coulter counter:

Polystyrene Beads (0.8 μm), Dow Chemical Co. Michigan, U.S.A.

Isoton II, Azide free electrolyte solution. Coulter Electronics Ltd., Luton, England.

Formaldehyde (Formalin) Fisons, Loughborough, England.

(v) Solvent for extraction:

Chloroform (Spectroscopic grade) Fisons, Loughborough, England.

SECTION TWO

GENERAL PROCEDURES

2.2.1 Sterilization of Solutions and Equipment

All solutions were sterilized either by standard autoclaving (at 121°C and 15 lb.in^{-2} for 15 min), or by membrane filtration using Nuflow 0.45 Millipore filters.

In most cases, equipment was sterilized by standard autoclaving. In some instances, however, equipment (e.g. pipettes) was sterilized in a dry air oven at 150°C for 2 hr.

2.2.2 Cultivation of Organisms: All cultures were available as freeze dried speciments from standard collections.

(i) Maintenance of cultures: Master cultures on growth medium slopes were stored at 2-4°C and subcultured at 3 month intervals. Cell sub-masters were stored in the dark at 15-20°C on growth medium agar slopes. These were subcultured at 7-14 day intervals. Cultures were subjected to monthly routine microscopic and streak-plating examinations.

(ii) Cultivation and preparation of cell suspensions: Daily slope subcultures from sub-master slopes were prepared, and after overnight incubation at 37°C, the cells washed from the surface with growth medium to cleated conical flasks (1 litre) containing approximately 200ml sterilized growth medium. Such cultures were incubated in a Gallenkamp orbital incubator at 37°C with a rate of ca

170 rev. min^{-1}. When an optical density of 0.6-0.7 (at 650nm, 1cm path) using a Pye Unicam SP 500 series II UV and visible spectrophotometer, was reached, the cells were harvested by centrifugation at 5000 R.P.M. (4500g) in a MSE Mistral 6L centrifuge for 15 minutes. The supernatant fluid was decanted off and the cells washed with, and then resuspended in, fresh carbohydrate free medium (CFM). The optical density of this suspension was adjusted to the required value (usually 0.600 at 650nm, 1cm path) by the addition of medium.

Alternatively, if dense cell suspensions were required, cells were grown on the surface of solidified medium in 1 litre-Roux bottles, containing approximately 200ml sterilized solid growth medium at 37°C, for 12-15 hr. Cells were then harvested into a sample of the appropriate medium and agar fragments removed by low speed centrifuging (130g; 10 min). The cell suspension was carefully decanted and subjected to a further centrifuging process (4500g; 15 min). The supernatant was discarded and the cells washed with and finally resuspended in the required medium. The final optical density (650nm; 1cm) of such a suspension was typically adjusted to give a cell concentration, as required, in the range $7.6 \times 10^{9\sim10}$ cells.ml^{-1}.

2.2.3 Determination of Dry Weight

Membrane filters(0.45 μm porosity) were washed with distilled water and dried at 95°C for 10 min. The filters were stored until use in a calcium chloride desiccator. 100ml volumes of bacterial suspensions were filtered through weighed membranes. The cells were washed on the membrane,

which were then dried to constant weight. The cell weight was found by difference.

2.2.4 Determination of Total Cell Numbers

The actual number of individual bacteria per ml of a suspension was determined by means of a Hawksley counting chamber (depth o.1 mm, $\frac{1}{400}$ mm^2). One loop of a suspension of diluted bacteria was transfered to the platform of the Hawksley slide and covered with a coverslip. The liquid was then spread over the platform. The slide was allowed to stand for 5min,after which it was examined under a microscope with a total magnification of X400. The number of organism in 50-100 small squares were counted (between 2 and 10), and the mean number of organisms per small square was calculated. From these the count per ml of the original suspension was determined.

SECTION THREE

SPECIFIC EXPERIMENTAL METHODS

2.3.1 Assessment of Growth Inhibition

(i) Tube dilution method

Equal volumes (5ml) of bacterial cell suspensions in CFM or appropriate medium and drug solutions of different concentration were mixed in sterile capped test tubes and maintained at 22°C for 10 min, after which 0.15ml 20% glucose solution was added to each tube. The tubes were incubated at 37°C for 36-48 hr and the optical density (650nm; 1cm) measured.

(ii) Direct assessment method

Suspensions (20ml) containing the test compound and bacterial cells in CFM were incubated at 37°C in 100ml conical flasks in a Gallenkamp shaking water bath. After 10 min contact time, sufficient glucose (0.30ml) was added to promote active cell growth. The optical density of samples of each culture was measured (650nm; 1cm) at 10 or 15 min intervals after which the samples were returned to the appropriate flask. Optical density measurement was continued for approximately 2 hr.

(iii) The effect of local anaesthetics on the growth and size distribution of division inhibited cell suspensions

The method based on that of Rye and Wiesman (1968)

was used. Equal volumes of cell suspension in CFM and of solution of the drug were mixed and shaken at 37°C in a water bath. After 10 min contact time, glucose (2mg.ml^{-1}) and sufficient ampicillin to produce a final concentration of 2.0 μg.ml^{-1} were added. (This concentration of ampicillin was determined by prior experimentation, and suppresses cell division without drastically affecting the growth rate). Shake incubation was continued and growth of the cells was followed spectrophotometrically.

At set time intervals 0.1ml samples were removed from each culture and diluted with Isoton diluent, containing 0.2% formaldehyde, to facilitate counting and sizing with a Coulter electronic particle counter, model ZB, (Coulter Electronics Ltd. Harpenden, Herts., England). The counter was fitted with a 30 μ orifice tube and was calibrated with polystyrene beads of 0.8 μm mean diameter. The size distributions were obtained by making counts with a two threshold unit window and with lower threshold setting at two unit intervals.

(iv) Reversibility of growth inhibition

20ml volumes of drug-cell mixtures were incubated for approximately 1 hr as described under 'Direct assessment method (2.3.1. ii). The cultures were then subjected to one of the following: (a) the cells were centrifuged down (4500g, 10 min, 4°C) washed with and then resuspended in 20ml fresh growth medium at 37°C or (b) diluted 50-50 with fresh growth medium at 37°C. In each case shake incubation was continued and cultures optical density monitored for a further 2 hr.

(v) Diauxic growth curves in the presence of antimicrobial agents

Two different techniques were employed:-

(a) Drug added before the carbohydrates.

(b) Drug added just before diauxic lag phase.

(a) To each 10 ml of cells suspended in CFM, an equal volume of antimicrobial drug solution was added to give the required final concentration. One flask was left without drug, so as to act as a control. The flasks were incubated in a shaking water bath at 37°C and after 10 min, 0.10ml of glucose-galactose (3% w/v) solution, was added to promote active cell growth. Samples were removed at about 10 min intervals from the shaking flasks and the optical density read at 650nm (1cm).

(b) To each flasks containing bacterial cell suspensions in CFM, 0.10ml of a mixture of glucose-galactose (3% w/v) was added to promote active cell growth. The exact time of onset of the diauxic lag had been determined by prior experimentation and at this time, equal volumes of antibacterial drug solutions were added to give the required concentrations. Samples were removed at about 10 min intervals throughout the continuance of the experiment. Optical density were measured at 650nm (1cm).

2.3.2 Viable Counting

A set of sterile capped test tubes containing equal volumes (5ml) of different concentration of the test solution were prepared. 5ml of bacterial cells suspended in CFM (optical density 0.60 at 650nm) were added to each tube. After 10 min contact time at 22°C, cell suspensions were serially diluted, to obtain a countable colonies. From the last three sets of tubes, duplicate 0.2ml samples were added separately to three pairs of petri dishes. This was repeated for each different concentration of the drug. To each petri dish, 10-15ml tryptone soya agar (previously melted and cooled to ca. 50°C) was added and the samples were incubated at 37°C. After 24 hr the colonies were counted.

The experiment was repeated varying the medium composition , the number of bacterial cells initially present and the contact time.

Preliminary experiments showed that use of tryptone soya broth or Ringer solution, with or without Tween 80 as a diluent, for the inactivation of LAs did not alter significantly the number of colonies counted, therefore, minimal salts solution was used as a diluent.

2.3.3 Leakage of Intracellular Materials

(i) Effect of LAs on ^{32}P labelled bacterial cells

A method similar to that described by Rye and Wiseman (1966) was used. ^{32}P labelled bacterial cells were prepared by addition of 1 μCi of ^{32}P orthophosphate to washed growing cells briefly resuspended in LPM at 37°C, followed 90 seconds later by addition of 5ml of 0.1M

potassium dihydrogen orthophosphate. After 1.0 min the labelled cell suspension was centrifuged (5 min, 7000g) washed with and then suspended in CFM. The optical density was adjusted to 0.60 with more CFM.

Equal volumes (2.5 ml) of suitable concentration of drug in CFM and of labelled cell suspension were mixed and maintained at 22°C. After 10 min contact time, the cells were removed by centrifuging at 4500g for 10 min and 1ml of the supernatant (duplicate) was added to 10 ml of liquid scintilator. The ^{32}P content of the supernatant of these samples was determined by counting for 10 min, using a liquid scintilation counter (LKB Wallac 1215 Back Beta).

^{32}P content of the cold trichloroacetic acid soluble fraction (metabolic pool) was determined by mixing equal volumes of bacterial cell suspensions with 10% trichloroacetic acid and maintaining the reaction mixture at 4°C for 30 min. The radioactivity in the supernatant was measured as above.

(ii) Voltammetric determination of inorganic phosphate

Orthophosphate can be determined voltammetrically at a glassy carbon electrode in dilute sulphuric acid solution (pH2) as 12-molybdophosphate or molybdenum blue, which give anodic waves at +0.14 V or+0.30 V vs. S.C.E. (saturated calomel electrode) respectively (Fogg and Bsebsu, 1981).

Equal quantities (2.5ml) of cell suspensions ($7.6 \times 10^9 .ml^{-1}$) and LA solutions in LPM were mixed and maintained at 22°C for 10 min. Cells were then removed by centrifuging at 7000g for 15 min (MSE Mistral-super speed)

and the supernatants collected for assay.

Aliquots of standard orthophosphate solution (10^{-4} – 10^{-5}M) or samples of supernatant were transfered to a series of 50 ml volumetric flasks. To each flask 5ml of acidic molybdate solution (2% $^{w}/_{v}$) was added and the volume of each made up to approximately 20ml with distilled water. The solutions were left to stand for 15 min, then diluted to 50ml with more distilled water. Each solution was transfered to a votammetric cell and deoxygenated for 5 min with nitrogen gas. A clean dried glassy carbon electrode was then placed in the solution for 10 seconds, after which the cell circuit was closed. The initial potential was set at OV and a differential pulse mode was selected. The circuit was left closed for 20 seconds to stabilise the current and a differential pulse voltammogram between OV and + 0.3V was obtained.

The electrode was cleaned between scans by washing with 1M solution of sodium hydroxide and then with distilled water before drying.

Conditions set up for the polarographic analyser (PAR 174, Princeton Applied Research): a sweep rate of 5 mVs^{-1} and a pulse height of 50 mV and a pulse frequency of 0.5 s were used.

(iii) Estimation of pentoses

When pentoses are heated with concentrated HCl, furfural is formed which condenses with orcinol in the presence of ferric ions to give a blue green colour (Mejbaum, 1939)

Equal volumes (2.5ml) of the LA solutions and of

bacterial cells suspended in CFM were mixed to give final cell concentrations of 3.8×10^9 cells.ml^{-1}. After 15 min or after other set time intervals at 22°C or 1°C the cells were removed by centrifuging at 7000g for 15 min at 4°C and 1.0ml samples of the supernatants were added to 1.0ml of reagent for pentose estimation in test tubes. The tubes were heated in a boiling water bath for 20 min. The contents of each tube were then diluted with an equal volume of distilled water and the optical density measured at 675nm. D-ribose was used as the standard for a calibration curve.

(iv) Release of 260nm absorbing material

Equal volumes (5ml) of LA solutions and of cell suspensions were mixed to give the final cell concentration of 3.8×10^9 cells.ml^{-1}. After 30 min contact time at 22°C, the cells were removed by centrifugation (7000g, 15 min, 4°C). As the supernatant contained both the non-adsorbed LA (λ_{max} = 220 - 320 nm) and the leaked material (λ_{max} = 260 nm), the direct measurement of 260nm absorbing material would not have realistically reflected the concentration of leaked cellular constituents. To facilitate the assessment of 260nm absorbing materials leaked from the cells, the liquid/liquid extraction procedure of Beckett, Patki and Robinson (1959) was employed. The supernatant solution was shaken with portions of a water immiscible solvent, (chloroform of spectroscopic grade) to remove all the LA from the aqueous phase. The optical density (260nm) of aqueous phase after complete extraction was measured.

2.3.4 Uptake of Local Anaesthetics by Bacterial Cells

Preliminary experiments determined that a suspension of at least 3.8×10^{10} E.coli per ml was required to get measurable uptake.

10 ml of double strength LA was prepared and equal volumes (5ml) was added to two sets of tubes; one containing equal volumes of CFM and the other bacterial cell suspension. Both sets were allowed to stand at 37°C with occasional shaking for a period of 30 min.

After centrifugation of the set containing bacteria at 7000g (4°C for 15 min), the supernatant of each tube was divided into two portions. The optical density of one portion was measured at the appropriate λ_{max} of the LA. This gave the optical density of the mixture of LA remaining after uptake, plus any leaked material. The second portion was extracted as described earlier (2.3.3 iv) and the optical density of this layer was measured at the appropriate λ_{max}. The difference between the two measurement corresponds to the optical density of LA remaining in the supernatant after uptake has taken place by bacteria (c'). The difference between the optical density of set containing no bacteria (c) and (c'), after correction for the cell volume (see 3.4) will give uptake of LA by bacteria.

2.3.5 Determination of the Critical Micelle Concentration (CMC)

This was achieved using two different methods:

(i) Dye solubilization

The solubilization of Sudan Black B by solution of LA

in CFM and doubled distilled water was studied by the equilibrating with the solutions an excess of solid dye (10mg dye in 10ml solution) while they were shaking for 1 hr at 22°C. The mixture was centrifuged at 4500g for 40 min to remove the undissolved dye. The maximum absorption wave length of one of the coloured supernatant was assessed using an SP 800 Ultraviolet Spectrophotometer (300 - 700 nm) and then the optical density (absorbance) of all supernatants was measured at that maximum wavelength.

Preliminary experiments showed that the use of Sudan Black instead of Victoria Blue (Salt and Traynor, 1979) gave a sharper end point, probably due to the slight water solubility of the latter dye.

(ii) Surface tension measurement

The surface tension of solutions containing different concentrations of LAs in CFM or doubled distilled water was measured using a Du Nouy Tensiometer, ring method (Harkins and Jordan, 1930).

2.3.6 LAS' Partition Coefficients ($K^{o/w}$)

Technique used was based on the method described by Lang and Rye (1972). Equal volumes (20ml) of known concentrations of the LAs in CFM and either chloroform, n-heptane or octanol were mixed and continuously shaken at 22°C for 6 hr. Each container was then allowed to stand for ½ hr. In each cases two phases were separated and the concentration of LA remaining in the aqueous phase determined by measuring the optical density at the wavelength of maximum absorbance of the particular drug. From these

measurements the partition coefficient of the drug between these organic solvents and the aqueous medium were calculated.

2.3.7 Measurement of Turbidity Changes

(i) Standard technique for non-growing bacterial cells

To a set of test tubes containing equal volumes (5ml) of different concentrations of local anaesthetics in the appropriate medium, equal volumes (5ml) of bacterial cell suspensions were added. After 10 min contact time or at other set times at 22°C, the optical density of samples of each suspension was measured at 650nm (1 cm).

(ii) Effect of LAs on the turbidity of isolated cell envelope and cytoplasmic constituent preparations

a) Preparation of isolated cell envelope and cytoplasmic constituents:

Cell suspensions of E. coli in CFM were sonicated (Salton, 1960), for about 8 min in an ice-cooled vessel at a rate of 9 microns (peak to peak) in a MSE Ultrasonic disintegrator (Model 60W). Intact cells were removed from the "sonicated" by centrifugation (2000g for 10 min). The supernatant liquid was decanted and recentrifuged (10,000g for 15 min), a force sufficient to bring down isolated cell walls (Rogers, 1963). The pellet, consisting of isolated cell envelopes , was washed three times with 1% w/v sodium chloride as recommended by Salton and Horne (1951) to remove adhering cytoplasmic contents and suspended in CFM. The supernatant fluid obtained in this instance was a suspension of intracellular material. It was diluted with CFM such that the cytoplasmic constituents contained in 1ml of solution

were derived from about 0.24 mg dry weight of cells.

b) Effect of LAs: To a set of test tubes containing 5ml of different concentrations of LA, equal volumes of either cell envelope preparation or cytoplasmic constituents were added. The mixtures were maintained at 22°C for 10 min after which the optical density of samples from each tube was measured at 650nm (1cm).

(iii) Effect of LAs on the turbidity of lipid depleted cells and cell free lipid dispersions

a) Preparation of lipid extracted cells and isolation of total lipids:

Large volumes of E.coli cells which were grown in liquid growth medium were centrifuged (4500g, 15 min) to isolate the cells which were then subjected to an extraction procedure based on the method of Shaw and Dinglinger (1969) for the extraction of total lipid. The cells were first freeze dried (Chem Lab Instruments Ltd) and then suspended in chloroform:methanol (2:1, v/v) and shaken for 48 hr at 22°C. After filtration, the pellet (lipid extracted cells) was washed with and then suspended in CFM to give required density. The bulk of the solvent, containing extracted lipids was removed under vacuum using rotatory evaporator (Rotavapor-R Büchi Switzerland) and the extracted lipids were dried over P_2O_5.

b) Effect of LAs: Two sets of test tubes contain equal volume of different concentrations of LA in CFM were prepared. To the first set, equal volumes of lipid extracted cells in CFM (OD = 0.6, 650nm) were added and to the second set equal volumes of the lipid emulsion

(ultrasonicated) in CFM. After 10 min at 22°C the optical density of samples for each set was assessed at 650nm.

2.3.8 Effect of Local Anaesthetics on Respiration

(i) The use of triphenyltetrazolium chloride (TTC)

The optical density of a dense E.coli cell suspension in either 0.2M phosphate buffer (pH 6.4) or CFM was adjusted so that when added to the reaction mixture, a dry weight of ca 1.20mg ml^{-1} (3.8×10^9 cells ml^{-1}) was achieved.

The dehydrogenase activity of cell suspensions was established under aerobic conditions, by the triphenyltetrazolium chloride method of Hugo (1954). Into a series of sterile universal bottles, 2.5ml of phosphate buffer or CFM containing different concentrations of LA, 1.0ml of TTC (0.1% $^{w}/_{v}$), 0.5ml of cell suspension and 1.0ml of 0.02M substrate (D-glucose, sodium succinate, sodium malate or sodium lactate) were added. Several sets were prepared to facilitate sampling. Also bottles containing neither LA nor substrate were prepared to be used as blanks. All the bottles were shaked in a water bath at 37°C. At various time intervals one control (no LA), one blank and one of the concentrations of LA were removed. The reaction mixture was acidified with 5ml of glacial acetic acid and the triphenylformazan produced by the reduction of TTC by dehydrogenases, Fig. 2.2, was extracted using several 5ml quantities of toluene (Fahmy and Walsh, 1952). The cells were removed by centrifugation and the optical density of toluene solution containing the formazan determined spectrophotometrically at 490nm (1 cm). Values obtained were corrected for the actual

volumes of toluene used.

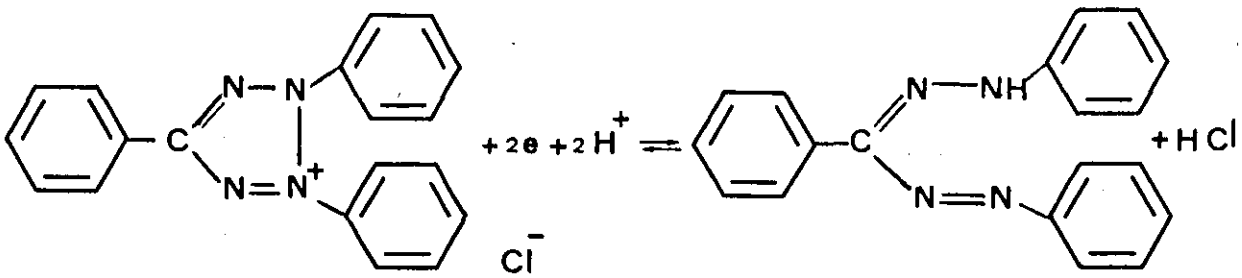


Fig 2.2 Formation of triphenylformazan (red) from TTC (colourless)

(ii) Measurement of oxygen uptake (Warburg Manometry)

Studies on respiration were performed using standard manometric techniques (Umbreit, Burris and Stauffer, 1964) in an atmosphere of air at 100 oscillations/min (Warburg Apparatus, B.Braun Melsungen, made in W. Germany).

Experiments were carried out using cells prepared as earlier described (2.2.2 ii) and employing the following procedure:

(1) The following materials were placed in the main compartment of clean, dry, Warburg flasks equipped with a centre well: 1.5ml LA solution at required concentration in CFM and 0.5ml of 0.03M substrate (either D-glucose or sodium succinate).

(2) 0.2ml of 20% KoH was placed into the centre well, with a fluted filter strip.

(3) 0.5ml of cell suspension was put into the side arm (final concentration, 3.8×10^8 organisms. ml^{-1}).

(4) After the attachment joint on the manometer and the side arm plug were greased and the plug inserted into the side arm, the flask was attached to the manometer.

(5) The flask was placed in a constant temperature bath ($37^{o}C$) and the contents of the flask were allowed to equilibrate with shaking.

(6) Manometer fluid was adjusted to the reference point on closed side of manometer with stop-cock open.

(7) Contents of the side arm were added to the main compartment.

(8) The stop-cock was closed and manometer readings taken every 10-15 min for 2 hr.

The flask constant was found using the nomogram of Dixon (1951).

2.3.9 Electron Microscopy

(i) Scanning electron microscopy (SEM)

Cells of E.coli were shake-incubated at $37^{o}C$ in the presence of different concentrations of LA for 15 min (Initial cell concentration approximately 3.8×10^{8} cells ml^{-1}). Glutaraldehyde (E.M. grade) was then added to cell culture to give final concentration of 1.5% ($^{v}/_{v}$). After 2 min, the cells were removed by centrifugation at 3000g for 15 min at $4^{o}C$, then resuspended in 2ml of glutaraldehyde (5%$^{v}/_{v}$) for 16 hrat $4^{o}C$. The cells were removed by centrifugation, washed 3 times with and finally resuspended in distilled water to give the required density (faint opalescence). One drop from each of these suspensions was allowed to air dry on a 13mm round microscope cover slips and then dehydrated over $Ca\ Cl_2$ under partial vacuum for 12-18 hr. The cover slips were fixed to 15mm aluminium stubs using quick drying silver cement (DAG) and coated in

an Edwards high vacuum unit, with gold palladium to give a coating film of ca 10nm thickness. The samples were examined in an ISI-Alpha-9 Scanning Electron Microscope with a beam angle of 45° and a voltage of 15KV. Magnifications ranged from X30 to X30,000. Images of typical cell groups were produced on the screen of a cathode ray tube and photographed on 35mm film (ILFORD FP4 or HP5).

(ii) Transmission electron microscopy (TEM)

Suspensions of E.coli in CFM were left at 22°C in the presence of different concentrations of LA for 15 min (final cell concentration about 3.8×10^8 cells.ml^{-1}). Cells were prepared for electron microscopy as follows:

(1) The cells were fixed by adding glutaraldehyde (E.M. grade) to give final concentration of 1.5% ($^{v}/_{v}$) for 2 min.

(2) The cells were removed by centrifugation (4°C, 3000g, 15 min)

(3) The pellet obtained was resuspended in 2ml glutaraldehyde (5% $^{v}/_{v}$) for 16 hr at 4°C.

(4) The cells were harvested by centrifugation, and washed 3 times with acetate veronal buffer.

(5) The pellets were suspended in molten (45-50°C) 3% purified agar and when set the agar was cut into small cubes (1mm).

(6) The agar cubes were refixed in glutaraldehyde (1.5% $^{v}/_{v}$) for 1 hr.

(7) The cubes were washed with A.V.B. for 15 min and stained in uranyl acetate (2% $^{v}/_{v}$ in AVB) for 25 min.

(8) To stain the agar, the agar cubes were left in

methylene blue for 2 min.

(9) The agar cubes were left in series of ethanol concentration range of 25, 50, 75 and 100% for 15, 20, 30 and 60 min respectively, in order to be dehydrated.

(10) To clear the alcohol, the cubes were left in propylene oxide - ethanol in the ratio of 50:50 and then 100:0 for 15 and 2 x 20 min respectively.

(11) To embed the cells in resin, the agar cubes were left in mixtures of propylene oxide - embedding agent of the ratio 1:1, 1:3 and 0:1 respectively at room temperature, each one for about 12 hr.

(12) The agar cubes in embedding mixture were left at 60°C for 1 hr (to get rid of any propylene oxide residue).

(13) The agar cubes were then embedded in capsules (Taab Laboratories Reading) and kept at 60°C for 48 hr.

(14) Section were cut using a Reichert - Jung ultra-microtome equipped with glass knives (angle 55°) and the sections (80-100nm) collected on copper grids (200 or 400 mesh).

(15) The sections were observed under the AEI, EM6B Electron microscope (Associated Electrical Industries Ltd., Manchester, England), using an accelerating voltage of 60KV. The microscope was fitted with a 70mm roll film camera and electron micrographs were recorded directly on Agfa Rapidoline Ortho FO 71P film.

CHAPTER 3

RESULTS

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RESULTS

3.1 Growth Inhibition

(i) Tube dilution and direct assessment techniques

The minimum inhibitory concentrations (MICs) of amethocaine, procaine, cinchocaine and lignocaine determined for the four different bacteria using the tube dilution method are shown in Table 3.1. All bacteria were more sensitive to the inhibitory action of amethocaine and cinchocaine than to that of procaine and lignocaine. MIC values for the various LAs against E.coli, K.aerogenes and P.aeruginosa (Gram-negative) are essentially of the same order, though K.aerogenes was particularly resistant to procaine. B.megaterium (Gram-positive), however, was more sensitive to the LAs than were the other organisms.

Table 3.2 shows the influence of inoculum size (E.coli) on the MIC of amethocaine. The MIC is virtually unchanged for increases in cell number from 2.66×10^8 to 0.89×10^9 cells.ml^{-1} and increases only slightly as the E.coli cell concentrations ranged from 0.89×10^9 to 4.05×10^9 cells.ml^{-1}.

Table 3.3 shows the effect of Mg Cl_2 concentration on the MIC of amethocaine against E.coli. The MIC remains constant up to about $\frac{M}{1000}$, after which increases in the Mg Cl_2 content of the medium, up to $\frac{M}{100}$, increase the MIC value.

Figs 3.1 to 3.16 inclusive are growth curves (optical density vs time) for E.coli (Figs. 3.1 to 3.4); K.aerogenes (Figs 3.5 to 3.8); P. aeruginosa (Figs 3.9 to 3.12) and B.megaterium (Figs 3.13 to 3.16) growing in the absence or presence of different concentrations of amethocaine, procaine,

Table 3.1 Minimum inhibitory concentrations (MICs) in $mg.ml^{-1}$ of different LAs for different bacteria.

Drug conc ($mg.ml^{-1}$) \ Bacteria	E.coli	K.aerogenes	P.aeruginosa	B.megaterium
Amethocaine	0.93 ± 0.08	1.26 ± 009	1.1 ± 0.2	0.56 ± 0.05
Procaine	28.3 ± 1.25	51 ± 2.94	30 ± 1.5	15 ± 2
Cinchocaine	0.8 ± 0.13	0.9 ± 0.15	0.9 ± 0.10	0.3 ± 0.1
Lignocaine	18.6 ± 0.94	26.3 ± 1.24	25 ± 1	8.6 ± 0.94

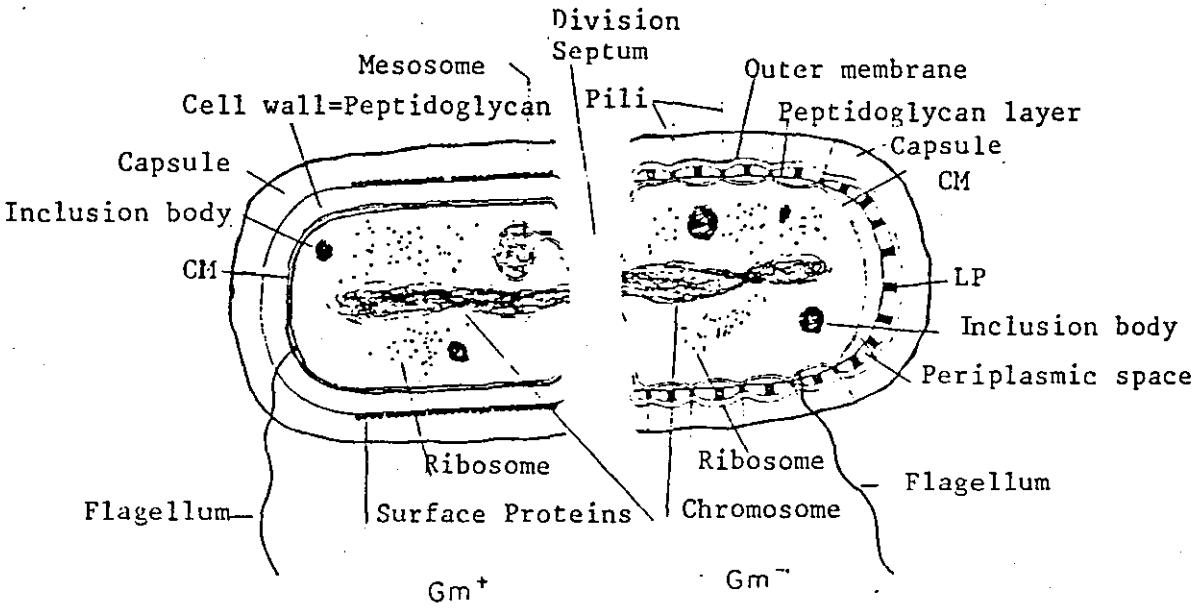
Table 3.2 Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of amethocaine ($mg.ml^{-1}$) for E.coli cells when different cell concentration was used

Cell No. ml^{-1}	2.66×10^8	5.2×10^8	0.89×10^9	2.01×10^9	4.05×10^9
MIC ($mg.ml^{-1}$)	0.93 ± 0.08	0.93 ± 0.08	0.93 ± 0.08	1.1 ± 0.06	1.1 ± 0.08
MBC ($mg.ml^{-1}$)	7.5	7.5	7.5	7.5	7.5

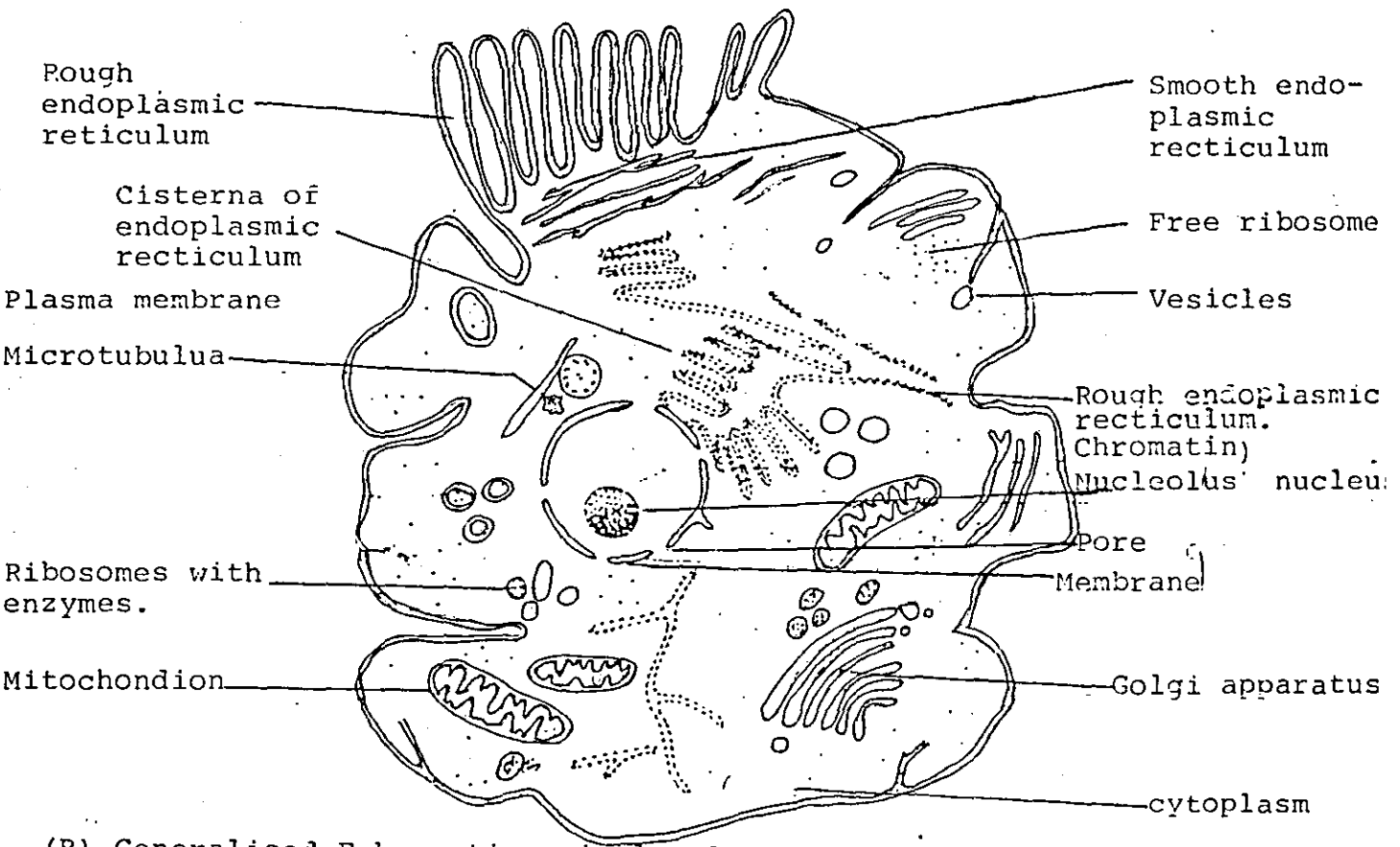
Table 3.3 MICs and MBCs of amethocaine ($mg.ml^{-1}$) for E.coli cells suspended in media containing different concentrations of $Mg\ Cl_2$

Mg^{++}	$\frac{M}{10,000}$	$\frac{M}{2,000}$	$\frac{M}{1000}$	$\frac{M}{200}$	$\frac{M}{100}$
MIC ($mg.ml^{-1}$)	0.93	0.93	0.93	1.1	1.3
MBC ($mg.ml^{-1}$)	7.5	7.5	7.5	7.8	7.8

Fig. 1.10



(A) Diagram of "Earliest form of organism" that from which others are developed. Gram-positive (Gm^+) cell on left and Gram-negative cell (Gm^-) on right, showing similarities and cell envelope differences. CM, cytoplasm membrane, LP, lipoprotein. (After Joklik, 1984).



(B) Generalised Eukaryotic animal cell (After Wilson, 1977).

cinchocaine or lignocaine.

Essentially all curves show similar patterns. The graphs for untreated and treated suspensions with LA concentrations lower than MIC (see Table 3.1) are linear. The rate of increase in OD in these cultures is exponential at a rate dependent on LA concentration, i.e. increasing the concentration of LA causes a reduction in growth rate, the extent of reduction produced by equal increments in LA concentration becoming progressively larger at higher concentrations. For example, growth rate of E.coli in the presence of amethocaine concentrations ranging from 0.25 $mg.ml^{-1}$ to 1.0 $mg.ml^{-1}$ (Fig 3.1) decreases gradually. In each case increasing the LA concentration, above the MIC (Table 3.1), causes a decrease with time in the turbidity of cultures. For example, concentration of 1.25 $mg.ml^{-1}$ amethocaine acting on E.coli (Fig 3.1), causes a reduction in the OD, the extent of which increases with time. Higher concentration (1.5 $mg.ml^{-1}$ amethocaine) causes a further decrease in the turbidity of bacterial cultures. However, there is an exception with cinchocaine concentrations higher than MIC (Table 3.1). Such concentrations still reduce culture turbidity, but the extent of turbidity change is less than that caused by lower concentrations. For example, a culture treated with 1.5 $mg.ml^{-1}$ cinchocaine (Fig 3.3) shows less decrease in turbidity than those treated with 0.5 or 0.6 $mg.ml^{-1}$ cinchocaine, although both cause marked decreases in the OD compared with the control.

Comparing MICs of LAs acting on different bacteria, using two different methods, tube dilution (Table 3.1) and direct assessment (Figs 3.1 to 3.16), it can be deduced that

except with cinchocaine, all the LAs have either higher or equal MICs using the shaking method than with the tube dilution method. For example, MICs of amethocaine for E.coli are 0.93 ± 0.08 $mg.ml^{-1}$ (Table 3.1) and 1.25 $mg.ml^{-1}$ (Fig 3.1), using tube dilution method and direct assessment respectively. In the case of cinchocaine the direct assessment gives lower MIC (0.5 $mg.ml^{-1}$) than tube dilution (0.8 ± 0.13 $mg.ml^{-1}$).

Fig 3.17 shows the effect of LA concentration on the percentage inhibition of growth of E.coli, K.aerogenes, P.aeruginosa and B.megaterium, 70 min after addition of glucose. It is based on data derived from the appropriate growth curves, assuming the increase in the OD of the control after 70 min to be 100%. Concentrations of LAs which cause 50% growth inhibition for the organisms are given in Table 3.4. From that the order of LAs concentration which cause 50% growth inhibition for the organisms are:

Cinchocaine < Amethocaine < Lignocaine < Procaine

The growth rate constants of exponentially growing cultures of E.coli in the presence of LAs (Figs 3.1 to 3.4) were calculated (Stanier et al., 1980) and are shown in Fig 3.18 as a function of LAs concentration. Increasing the concentrations of LAs causes a reduction in the growth rate, the extent of the reduction produced by equal increments in LA concentration becoming progressively larger at higher concentrations. At very low concentrations (e.g. 0.1 $mg.ml^{-1}$) amethocaine and cinchocaine have nearly the same effects on E.coli, though at higher concentrations cinchocaine is more effective in inhibiting growth than equivalent concentrations of amethocaine. Lignocaine, at all concentrations, is more

Table 3.4 Concentrations of LAs which cause 50% inhibition of growth of different organisms, 70 min after the addition of glucose

LA ($mg.ml^{-1}$) / Organism	Amethocaine	Procaine	Cinchocaine	Lignocaine
E.coli	0.66	28	0.37	14.0
K.aerogenes	1.0	28	0.39	12.5
P.aeruginosa	1.0	18	0.45	10.0
B.megaterium	0.39	13	0.17	2.5

Table 3.5 Concentrations ($mg.ml^{-1}$) of local anaesthetic causing an 80% reduction in cell viability in bacteria suspended in CFM at $22^{o}C$. Initial cell optical density 0.30, contact time 10 min.

LA / Organism	Amethocaine	Procaine	Cinchocaine	Lignocaine
E.coli	5.5	> 100	2	> 50
K.aerogenes	4	> 100	2	> 50
P.aeruginosa	5	> 50	2	> 50
B.megaterium	0.64	40	0.25	15

effective in inhibiting growth than procaine.

(ii) Growth inhibition of division-inhibited cells

Fig 3.19 shows the changes in the log OD which occurred after the addition of glucose (2 $mg.ml^{-1}$) and ampicillin (2.0 $\mu g.ml^{-1}$) to resting suspensions of E.coli containing different concentrations of amethocaine in CFM at 37°C. Growth in the presence of concentration of amethocaine up to about 1.0 $mg.ml^{-1}$ was exponential but occurred at a slower rate than the control suspension. This preliminary experiment was performed to determine the concentrations of amethocaine required to partially inhibit the growth of E.coli in the presence of 2.0 $\mu g.ml^{-1}$ ampicillin which prevent cellular division without having significant effect upon the rates of increase in the optical density.

Fig. 3.20 compares the size distributions (threshold units) of amethocaine treated E.coli cells (0.5 $mg.ml^{-1}$) and those of control (untreated) suspension before and at intervals following the addition of glucose and ampicillin to the cells and incubation at 37°C. These results show that the size distribution patterns for amethocaine treated and untreated cells at different times are similar, except that as the bacterial growth continued, the size distribution curves shifted to the higher size range accompanied with broadening of the peak. For example after about 60 min, in both cultures very few cells were left in the smallest size ranges.

(iii) Reversibility of growth inhibition

Evidence that the inhibition of growth of E.coli

caused by amethocaine, procaine, cinchocaine and lignocaine in concentrations up to the relevant MICs is reversible is shown in Figs 3.21 to 3.24 respectively. In each case the cultures were initially treated with sufficient concentrations of LA to significantly alter the growth rate. Resuspension and incubation of the cells in fresh growth medium, resulted in cultures that exhibited growth curves that are linear over much of their range and having approximately the same slope as the control, or which are initially convex with time becoming linear. The latter case was most notable with cultures initially treated with concentration of LA that were about the MIC.

The reversibility of the growth inhibitory properties of amethocainewas also investigated by diluting cultures inhibited partially or totally by this agent with an equal volume of fresh complete growth medium. The results (Fig 3.25) show that amethocaine inhibited cultures up to about 0.75 $mg.ml^{-1}$, on dilution immediately resume growth at a rate characteristic of the new amethocaine concentration. The growth rate for cultures initially treated with higher concentrations of the agent either increases gradually (1.25 $mg.ml^{-1}$ on dilution 0.625 $mg.ml^{-1}$) or show no growth (1.5 $mg.ml^{-1}$ on dilution 0.75 $mg.ml^{-1}$).

(iv) Diauxic growth

Figs 3.26 to 3.32 show the effect of amethocaine, procaine, cinchocaine, lignocaine, chloramphenicol, ampicillin and cetylpyridinium bromide respectively, on the growth of E.coli under diauxic conditions. The carbohydrates present were glucose (0.15 $mg.ml^{-1}$) and galactose

(0.15 $mg.ml^{-1}$). In each case the control showed the anticipated growth curve with a diauxic lag indicating cessation of glucose utilization and the onset of the use of galactose. All growth curves obtained in the presence of LAs are essentially similar (Figs 3.26 to 3.29), and following the diauxic lag phase, the cells treated with subinhibitory concentrations of each LA continued to grow in the presence of secondary carbon source (galactose), though the growth rate depended on LA concentration. As the concentration of LA was increased, the length of the diauxic lag phase was increased. In the presence of LA concentrations which approximate to the MIC, as determined previously (Table 3.1), the characteristic diauxic curves were disrupted. For example, amethocaine treated cells of E.coli growing on glucose as the sole carbohydrate during shake-incubation at 37°C (Fig 3.1) showed some growth up to amethocaine concentrations of 1.0 $mg.ml^{-1}$. When glucose and galactose were present, amethocaine at 0.75 $mg.ml^{-1}$ permitted only a short period of cellular growth, after which a decrease in culture turbidity was observed. However, with concentration of LAs more than MICs (Table 3.1) the growth was completely inhibited and in fact a decrease in culture turbidity was observed.

In the presence of subinhibitory concentrations of chloramphenicol (Fig 3.30), the length of the diauxic lag phase was considerably increased, the extent of the increased lag depending on chloramphenicol concentration.

Fig. 3.31 shows the effect of ampicillin on the diauxic growth of E.coli. Concentrations up to about 7.5 $\mu g.ml^{-1}$ gave rise to curves with a shorter diauxic lag than

the control, but nearly the same length as each other. The cells continued to grow after the diauxic lag, though with concentrations of 2.5 $\mu g.ml^{-1}$ or more the optical density decreased within a few min of commencement of utilization of galactose.

When cetylpyridinium bromide (CPB) was present (Fig 3.32), the effect on growth was markedly dependent on CPB concentration. Concentration of more than 6 $\mu g.ml^{-1}$ completely inhibited growth and in fact caused a decrease in culture turbidity; with 6 $\mu g.ml^{-1}$ CPB the growth was inhibited for about 70 min, after which the culture turbidity increased. Sub-inhibitory concentrations permitted growth on both glucose and galactose with a very slight increase in the diauxic lag phase with increasing the CPB concentration.

Figs 3.33 to 3.39 show the effect of the addition of the various antimicrobial agents (amethocaine, procaine, cinchocaine, chloramphenicol, puromycin, ampicillin and cetylpyridinium bromide respectively) to E.coli cells already growing under the diauxic conditions. In each case the drug was added after approximately 28 min incubation in the presence of both glucose and galactose. In comparison with results from experiments on diauxic performed with the drug present from the onset of incubation (Figs 3.26 to 3.32) effects on the diauxic lag are essentially similar and relatively easier to observe.

When the LAs were present (Figs 3.33 to 3.35) the length of diauxic lag phase increased as the concentration increased. This increase in lag phase was greater, when cells were growing in the presence of chloramphenicol (Fig. 3.37) or puromycin (Fig 3.38), and with these drugs no

decrease in culture turbidity was observed even at high concentration (25 $\mu g.ml^{-1}$ chloramphenicol and 600 $\mu g.ml^{-1}$ puromycin).

Ampicillin (Fig 3.38), up to about 10.0 $\mu g.ml^{-1}$ decreased the length of the diauxic lag; concentrations between 5.0 and 10.0 $\mu g.ml^{-1}$ permitted only brief growth at a rate similar to the lower concentrations (1.0 and 2.5 $\mu g.ml^{-1}$), followed by decreases in culture turbidity. Even higher concentrations of ampicillin (15-20 $\mu g.ml^{-1}$) inhibited growth and eventually caused decreases in culture turbidity.

Cetylpyridinium bromide (Fig 3.39) gave curves qualitatively very similar to those for the local anaesthetics (particularly amethocaine and cinchocaine) and basically as described previously.

3.2 Cell Viability

Figs 3.40 to 3.43 illustrate the effect of procaine, lignocaine, amethocaine and cinchocaine on the percentage viability of cells of *E.coli* (Fig 3.40), *K.aerogenes* (Fig 3.41), *P.aeruginosa* (Fig 3.42) and *B.megaterium* (Fig 3.43) suspended in CFM, after 10 min contact time at 22°C.

As a group, all curves are essentially similar, though *B.megaterium* (Fig 3.43) was more sensitive to procaine and lignocaine than were the Gram-negative organisms. All curves in which losses of viability were eventually significant, exhibit thresholds below which little or no change in percentage viability is apparent (e.g. in Fig 3.40, 2.0 and 1.0 $mg.ml^{-1}$ of amethocaine and cinchocaine

respectively for E.coli). Above this value, small increases in LA concentration, produces large reductions in cell viability. This reduction is generally greater per unit concentration for cinchocaine comparing with amethocaine.

There is no or very little decrease in cell viability of E.coli and K.aerogenes treated with procaine over the wide concentration range used (Figs 3.40-3.41). However this is not the case when P.aeruginosa or B.megaterium (Figs 3.42-3.43) were treated with procaine. With B.megaterium there is threshold of about 15 $mg.ml^{-1}$ procaine below which little or no change in cell viability occurs, and above which it decreases considerably, falling to 20% at about 40 $mg.ml^{-1}$ procaine. With P.aeruginosa, however, the length of this threshold region is longer (up to 20 $mg.ml^{-1}$ procaine) and after that percentage cell viability decreases, reaching about 85% at 50 $mg.ml^{-1}$ procaine.

In the case of lignocaine, (Figs 3.40 to 3.43) there are thresholds of about 30, 30, 20 and 8 $mg.ml^{-1}$ for E.coli, K.aerogenes, P.aeruginosa and B.megaterium respectively, above which increases in lignocaine concentration cause decreases in percentage cell viability. With B.megaterium % viability drops to zero at 20 $mg.ml^{-1}$ lignocaine. Other organisms were not so sensitive and at a concentration of 50 $mg.ml^{-1}$ lignocaine, typically about 70% of the cells are viable.

For amethocaine, however, effecting on E.coli, K.aerogenes, P.aeruginosa and B.megaterium (Figs 3.40 to 3.43), the minimum bactericidal concentrations (MBCs) are 7.5, 6.0, 7.5 and 1.5 $mg.ml^{-1}$ respectively. The MBCs of cinchocaine for these organisms are 2.5, 2.5, 4.0 and 0.60

$mg.ml^{-1}$ respectively. (Figs 3.40 to 3.43).

Generally all organisms were sensitive to the local anaesthetics in the order: cinchocaine > amethocaine > lignocaine > procaine. Concentrations causing 80% reduction in cell viability are summarized in Table 3.5.

Table 3.2 shows the effect of inoculum level (E.coli) on the MBC of amethocaine. Variations in cell number from 2.66×10^8 to 4.05×10^9 $cells.ml^{-1}$ did not give any detectable difference in MBC.

Similarly Table 3.3 suggests that increasing the $Mg\ Cl_2$ content of cell suspending medium from $\frac{M}{10000}$ to $\frac{M}{1000}$ did not have detectable change on the MBC of amethocaine for E.coli. Higher $Mg\ Cl_2$ concentrations, however, did cause a small increase in the MBC.

Table 3.6 shows the effect of contact time on the influence of amethocaine and cinchocaine on the viability of E.coli suspended in CFM. Increasing the contact time of amethocaine ($5.0\ mg.ml^{-1}$) on E.coli from 5 to 25 min reduced the cell viability from 55% to 0%, but with $7.5\ mg.ml^{-1}$ amethocaine even after 5 min contact time 100% reduction in cell viability was observed. Similarly with cinchocaine ($1.0\ mg.ml^{-1}$), increase in contact time from 10 to 25 min, reduced the cell viability from about 100% to about 56%. However, $2.5\ mg.ml^{-1}$ was bactericidal even after 5 min contact time.

3.3 Leakage of Intracellular Materials

(i) Effect of LAs on ^{32}P labelled bacterial cells

Fig 3.44 shows the effect of various concentrations of LAs (10 min) on the release of ^{32}P labelled components

Table 3.6: Effect of contact time on the percentage cell viability of E.coli treated with LAs in CFM

LA	%viability Time (min) / Con. ($mg.ml^{-1}$)	0	5	10	15	20	25
Amethocaine	5.0	100	55±5	36±3	9±1	2±0.2	0
Amethocaine	7.5	100	0	0	0	0	0
Cinchocaine	1.0	100	100	100	80±8	65±5	56±6
Cinchocaine	2.5	100	0	0	0	0	0

Table 3.7 The partition coefficients of LAs between chloroform, n-heptane, octanol and CFM (pH 6.4) Temp. 22°C

Solvent / LA	Chloroform	n-Heptane	Octanol
Amethocaine	79	0.34	17
Procaine	1	0.015	0.18
Cinchocaine	100	1.2	100
Lignocaine	10.90	0.16	5.23

from cells of E.coli pulse labelled with ^{32}P and suspended in CFM at 22°C.

While procaine and lignocaine, in the concentration range used, had no effect on ^{32}P release, amethocaine and cinchocaine caused the leakage of ^{32}P and showed a similar pattern of activity. In initial part of the curve there is either a small decrease or no change in ^{32}P release as the LA concentration is slightly increased. This is followed by a phase of increased ^{32}P release with increasing LA concentration. This approaches a maximum release at about 10 $mg.ml^{-1}$ amethocaine and is approximately equal to 90% of the ^{32}P content released by cold trichloroacetic acid (TCA). With cinchocaine the maximum release occurred at about 7.5 $mg.ml^{-1}$, and was approximately equal to the amount of ^{32}P labelled compounds released by cold TCA.

(ii) Voltammetric determination of inorganic phosphate

The loss of inorganic phosphate from E.coli cells suspended in LPM containing different concentrations of LA at 22°C for 10 min is shown in Fig. 3.45.

Procaine and lignocaine did not initiate the release of inorganic phosphate from the cells. Amethocaine and cinchocaine, however, caused a linear increase in the amount of phosphate released up to about 8 $mg.ml^{-1}$ amethocaine and 5 $mg.ml^{-1}$ cinchocaine after which the release reached a maximum level. The maximum inorganic phosphate release from cinchocaine treated cells was higher than that due to amethocaine.

(iii) Estimation of pentoses

The leakage of pentoses from E.coli cells treated with

LAs is illustrated in Fig. 3.46.

Procaine and lignocaine in the concentration range used, did not cause the release of pentose from E.coli with amethocaine and cinchocaine, however, leakage of pentose occurred linearly as LA concentration was increased up to 7.5 $mg.ml^{-1}$ and 5.0 $mg.ml^{-1}$ respectively. Higher concentrations of amethocaine (up to 10 $mg.ml^{-1}$) caused slight increase in leakage. Further increase in concentration caused slight reduction in the total amount of pentose released. becoming constant at about 20 $mg.ml^{-1}$. Concentrations in excess of 5.0 $mg.ml^{-1}$ cinchocaine caused nearly the same (maximum) release of pentose. The maximum pentose released for amethocaine and cinchocaine was equivalent to ca 8.0 and 7.4 µg ribose per mg dry weight of bacteria.

These results are also illustrated in plates 3.1 to 3.4 for amethocaine, procaine, cinchocaine and lignocaine respectively. Increasing pentose concentrations cause colour changes from yellow to green. With procaine and lignocaine there is no colour change (all solutions are yellow, i.e. no pentose released). Both sets of tubes for amethocaine and cinchocaine show colour changes from yellow (nopentose) to green (maximum pentose) and to yellowish-green (less pentose).

Fig. 3.47 shows the release of pentose from E.coli cells after treatment with LA for different time intervals at 22°C.

Procaine (50 $mg.ml^{-1}$) and lignocaine (40 $mg.ml^{-1}$) did not cause detectable leakage of pentose up to about 3 hr. After that, however, increasing the contact time caused minor release. Amethocaine and cinchocaine caused the release of pentose at rates that dependent on the concentration

of LA used. Relatively low concentration of either drug (10 $mg.ml^{-1}$ amethocaine or 5 $mg.ml^{-1}$ cinchocaine) caused an initial rapid release of pentose followed by a slower secondary phase that continued for up to 5 hr. Higher concentrations, however, (30 $mg.ml^{-1}$ amethocaine or 20 $mg.ml^{-1}$ cinchocaine) only showed the rapid primary release. This was followed up to 5 hr contact time. Fig 3.48 shows the release of pentose from E.coli cells treated with LA for different time intervals at 1°C. After about 10 min contact time amethocaine and cinchocaine caused considerable release of pentose from E.coli cells at rates depending on LA concentration. However, increasing the contact time did not increase the amount of leaked material.

(iv) Leakage of 260 nm absorbing substances

Fig. 3.49 shows the leakage of 260 nm absorbing material at 22°C from E.coli cells suspended in CFM containing different concentrations of amethocaine, procaine, cinchocaine or lignocaine.

Procaine and lignocaine did not cause any leakage of 260 nm absorbing material in the concentration range used. With both amethocaine and cinchocaine the leakage/concentration curve is diphasic, an approximately linear increase in leakage occurs up to about 7.5 and 6.0 $mg.ml^{-1}$ of amethocaine and cinchocaine respectively. With higher concentrations, however, the total amount of material released is slightly reduced.

3.4 Uptake of LAs by Bacterial Cells

The uptake isotherms for amethocaine, procaine,

cinchocaine and lignocaine (corrected for leakage of 260 nm absorbing material and also the influence of cell volume) after 30 min contact by cultures of E.coli at 37°C are shown in Figs 3.50 to 3.52.

For the correction because of the influence of bacterial cell volume, the method of Lang and Rye (1972) was followed. The LA uptake was obtained by calculating K, the ratio of the concentration of drug within the cells to the concentration in the suspending medium. K may be derived as follows:

(1) $$K = \frac{\text{conc. in cells}}{\text{conc. in supernatant}}$$

(2) $$\text{Conc. in cells} = \frac{\text{Wt. in cells}}{\text{cell volume}} = \frac{\text{Wt. in control} - \text{Wt. in supernatant}}{\text{cell volume}}$$

(3) $$K = \frac{\text{Wt. in control} - \text{Wt. in supernatant}}{\text{Cell volume} \times \text{Con. in supernatant}}$$

(4) $$K = \frac{VC - C'(V-v)}{v \times C'}$$

(5) $$K = 1 + \frac{V}{v}\left\{\frac{C - C'}{C'}\right\}$$

Where:

V = total volume of reaction mixture.

v = volume occupied by the cells

C = drug concentration in the supernatant liquids from the control mixture

C' = drug concentration in the supernatant liquids from the treated cell suspension.

Using calibration curve, the optical density measurements were related to the concentrations, and from that and the other data, the uptake isotherms were constructed.

The average intracellular: extracellular concentration ratio (K) were 0.8, 1.2, 6.4 and 13.6 for procaine, lignocaine,

amethocaine and cinchocaine respectively.

As the K values or the uptake isotherms show, while the uptake of procaine (Fig 3.50) and lignocaine (Fig 3.52) with E.coli cells is very low, the amount of amethocaine (Figs 3.50 and 3.51) or cinchocaine (Fig 3.52) taken up by the cells is considerably greater. Throughout the concentration ranges studied the cellular uptake was directly proportional to the concentration of LA remaining in the suspending medium. With amethocaine, however, the uptake isotherm eventually flattens out at an uptake of approximately 200 $mg.ml^{-1}$ amethocaine and an equilibrium concentration of about 80 $mg.ml^{-1}$ amethocaine.

3.5 Determination of Critical Micelle Concentration(CMC)

(i) Dye solubilization

Fig 3.53 shows the extent of solubilization of Sudan Black B by solutions of LAs in CFM and distilled water. From these graphs, values for the CMC of amethocaine in the two systems were estimated as 10.5 $mg.ml^{-1}$ (CFM) and 31.5 $mg.ml^{-1}$ (water). In the case of cinchocaine these values were 7.5 $mg.ml^{-1}$ (CFM) and 23.5 $mg.ml^{-1}$ (water). Procaine and lignocaine did not show any coloured solutions in the presence of Sudan Black B up to about 40 $mg.ml^{-1}$ in CFM and in water.

(ii) Surface tension measurement

Figure 3.54 shows the surface tension changes occuring in relation to LA concentration in both CFM and doubled distilled water. From these curves, value for the CMC of amethocaine was found to be 10 and 30 $mg.ml^{-1}$ in CFM and

doubled distilled water respectively. Procaine did not cause any significant change in surface tension measurement at air-water interface.

3.6 LAS' Partition Coefficients

The partition coefficients of LAs used between chloroform or n-heptane or octanol and CFM at 22°C are shown in Table 3.7.

The order of LA solubility in these organic solvents is: Cinchocaine > Amethocaine > Lignocaine > Procaine

3.7 Effect of LAs on the Changes in the Turbidity of Non-Growing Cultures

Figs 3.55 to 3.58 inclusive show the effect of different LAs (amethocaine, procaine, cinchocaine and lignocaine) on the turbidity (650 nm, 1 cm) of non-growing cells of E.coli (Fig 3.55), K.aerogenes (Fig 3.56), P.aeruginosa (Fig 3.57) and B.megaterium (Fig 3.58) suspended in CFM at 22°C, after 10 min contact.

In all Gram-negative cell suspensions, procaine and lignocaine produced little or no change in turbidity over the concentrations range studied. With B.megaterium, however, increasing lignocaine concentration from about 25 $mg.ml^{-1}$, caused a minor increase in turbidity. For amethocaine and cinchocaine, a general pattern of activities emerges. Curves show three main regions. A primary region in which little or no variation in turbidity is detected as LA concentration is increased, and a secondary region in which small concentration changes induce large increase in turbidity. This phase of activity terminates either in a

plateau (Fig 3.58, B.megaterium) or reaches a maximum after which turbidity decreases (Figs 3.55 to 3.57, Gram-negative bacteria). However, the maximum OD value induced by amethocaine was not as high as that induced by cinchocaine in the case of all bacterial cultures used. Maximum OD was reached at about 20 and 10 $mg.ml^{-1}$ for amethocaine and cinchocaine respectively for all the organisms used. The extent of the first region depends on both the bacteria and the LA. In the case of the Gram-negative bacteria used in this project (Figs. 3.55 to 3.57) the primary region ends for amethocaine and cinchocaine, at about 5 and 2.5 $mg.ml^{-1}$ respectively. For B.megaterium (Fig 3.58) this region is only detected as an initial 'S' shape to the curves.

Fig 3.59 shows the influence of contact time on the induced turbidity changes in non-growing cells of E.coli suspended in CFM containing different LAs.

Procaine (50 $mg.ml^{-1}$) and lignocaine (40 $mg.ml^{-1}$) which did not normally induce marked turbidity changes over the normal short contact time (10 min), by extending the contact time for up to 5 hr caused a slight decrease in turbidity. With both amethocaine (10 $mg.ml^{-1}$) and cinchocaine (5 $mg.ml^{-1}$) the increase in culture turbidity is very rapid, being complete after approximately 10 min. Longer contact time resulted in a slight fall in turbidity in both cases. Higher concentrations showed little variation in turbidity after the initial large and rapid increase.

Figs 3.60 to 3.62 show the effect of LAs (10 min contact) on the turbidity of E.coli cells suspended in various media.

When the cells were suspended in distilled water

containing various concentrations of amethocaine, and cinchocaine (Fig 3.60), at low concentrations a large increase in turbidity was observed. Then it approached a maximum with concentration in excess of approximately 5 $mg.ml^{-1}$ for both LAs. Moreover, the maximum OD value induced by amethocaine was not as high as that induced by cinchocaine.

Fig 3.61 shows the effect of LA concentration on the turbidity of E.coli cells suspended in 0.2 M phosphate buffer (pH 6.4). The shape of curves is essentially similar to that reported earlier for the E.coli/CFM/LA system (Fig 3.55), except that the extent of first region is much shorter.

Fig 3.62 shows the effect of increase in amethocaine concentration on the turbidity of E.coli cells suspended in CFM of increased $Mg\ Cl_2$ concentration. The shape of the curves is basically similar to that reported earlier for E.coli cells suspended in CFM (Fig 3.55). However, the first region of the curve is extended, the maximum slope of each curve and the height of the final plateau is reduced as the $Mg\ Cl_2$ content of the medium is increased.

Changes in the OD (650nm) of the soluble intracellular material released from sonicated E.coli cells and of the remaining cell "ghosts" in the CFM containing different concentrations of amethocaine, procaine and cinchocaine, lignocaine are shown in Figs 3.63 and 3.64 respectively.

Procaine and lignocaine did not have significant effect on OD of either cell envelope or intracellular material in CFM, though at higher concentrations (30 $mg.ml^{-1}$ lignocaine) the OD of intracellular material showed minor increase.

Amethocaine and cinchocaine induced turbidity increases in both cell envelope and cell contents fractions, the effect being most marked with cell contents. The changes can be divided

into three regions: A short initial phase of nearly no change in optical density, followed by large changes as the concentration of LA is increased and finally a plateau after which the OD remains virtually constant with further increases in LA concentration. The initial short part of the curves relating to cell contents ends at approximately 5 $mg.ml^{-1}$ and 2.5 $mg.ml^{-1}$ for both amethocaine and cinchocaine respectively. The second phase ends at higher concentration for amethocaine (25 $mg.ml^{-1}$) comparing with cinchocaine (10 $mg.ml^{-1}$). The maximum OD value induced with cinchocaine was higher than that with amethocaine.

Amethocaine and cinchocaine showed a slight increase in the OD of the cell envelope preparations with concentration in excess of 5 $mg.ml^{-1}$ and 2.5 $mg.ml^{-1}$ respectively. This increase reached a maximum at 20 $mg.ml^{-1}$ amethocaine and 10 $mg.ml^{-1}$ cinchocaine.

Figs 3.65 and 3.66 show, respectively, the effect of 10 min contact with different LAs on the optical density (650 nm) of both lipid depleted cells of E.coli and the cell free lipids dispersed in CFM at 22°C.

With procaine and lignocaine no large change in turbidity of lipid depleted cells of E.coli was detected, although a small increase was noted with concentrations of lignocaine in excess of 20 $mg.ml^{-1}$ (Fig 3.65). For amethocaine and cinchocaine, however, increases in concentration establish curves similar to that reported earlier for E.coli cells/CFM/LA system (Fig 3.55). The primary phase ends at about 1.25 $mg.ml^{-1}$ for both amethocaine and cinchocaine and the plateau commences with approximately 20 $mg.ml^{-1}$ amethocaine and 15 $mg.ml^{-1}$ cinchocaine.

Fig 3.66 shows that amethocaine and cinchocaine have similar pattern of activity on the dispersed lipid extracts. The curves consist of several regions. The initial part, in which turbidity increases markedly with increase in LA concentration, followed by a maximum or plateau region. The OD then decreases to zero as the LA concentration is further increased (ca 30 $mg.ml^{-1}$ amethocaine, ca 15 $mg.ml^{-1}$ cinchocaine). No further changes were observed and the optical density remains at zero as the LA concentration is increased.

Procaine did not increase the turbidity of lipids extracted from E.coli cells up to about 25 $mg.ml^{-1}$ (Fig 3.66). Further increase in concentration caused a slight increase in turbidity. With lignocaine, however, the curve consists of three phases (Fig 3.66). The initial part which extends to about 7.5 $mg.ml^{-1}$ shows no change in OD. The second part in which increases in lignocaine concentration, causes an increase in the OD and a final plateau which commences at about 20 $mg.ml^{-1}$.

3.8 Effect of LAs on Respiration

(i) Reduction of triphenyltetrazolium chloride (TTC)

Figs 3.67 to 3.71 show the effect of amethocaine, procaine, cinchocaine and lignocaine concentration on the rate of reduction of TTC (changes in OD at 490nm) by E.coli cells, suspended either in CFM (Fig 3.67) or in phosphate buffer (Figs 3.68 to 3.71). Substrates were either glucose (Figs 3.67 and 3.68), succinate (Fig 3.69), malate (Fig 3.70) or lactate (Fig 3.71).

There is a general common pattern of variation in

reduction rates dependent on concentration of LA. With glucose as a substrate, however, very low LA concentration often stimulated the rate of reduction of TTC by bacterial cells, at least for the first 30 min (e.g. 0.25 $mg.ml^{-1}$ amethocaine, 4 and 10 $mg.ml^{-1}$ procaine, 0.10 and 0.25 $mg.ml^{-1}$ cinchocaine, 2 and 10 $mg.ml^{-1}$ lignocaine, Fig 3.67). However, with all different substrates, as the concentration of LA increased, the rate of TTC reduction decreased by an amount dependent on the LA concentration. Concentrations of amethocaine and cinchocaine approximately equal to the MBC virtually inhibited TTC reduction (7.5 $mg.ml^{-1}$ amethocaine and 2.5 $mg.ml^{-1}$ cinchocaine, Fig 3.67), while with 50 $mg.ml^{-1}$ procaine or 20 $mg.ml^{-1}$ lignocaine formazan production continued. However, succinate, malate or lactate utilization was particularly sensitive (Figs 3.69 to 3.71) the rate of reduction of TTC decreasing more for the same increase in LA concentration than when glucose (Figs 3.67 and 3.68) was used as substrate.

(ii) Measurement of oxygen uptake

The rate of oxygen uptake by cells of E.coli in the presence of different concentration of amethocaine, procaine, cinchocaine and lignocaine is shown in Fig 3.72 (glucose as substrate) or Fig 3.73 (succinate as substrate).

Overall the curves are similar. Very low concentrations of LA stimulated or had no effect on the rate of oxygen comsumption (e.g. 0.25 and 0.5 $mg.ml^{-1}$ amethocaine, 10 $mg.ml^{-1}$ procaine, 0.1 and 0.25 $mg.ml^{-1}$ cinchocaine, 2 and 10 $mg.ml^{-1}$ lignocaine, in Fig 3.72). Further increases in the LA concentration caused a reduction in the rate of oxygen uptake,

the extent of which depends on the LA concentration, until at higher concentration it reached to the level of endogenous respiration (e.g. 6.5 mg.ml amethocaine, 2.0 $mg.ml^{-1}$ cinchocaine in Figs 3.72 and 3.73). However, complete inhibition of respiration was not observed in the presence of procaine or lignocaine. Comparing Fig 3.72 and 3.73, it can be seen that, for approximately the same decrease in the rate of oxygen comsumption, a higher concentration of LA is required with glucose as substrate than with succinate. For example, 1.5 $mg.ml^{-1}$ amethocaine caused about 56% reduction in oxygen consumption after 1 hr when glucose was the substrate (Fig 3.72), while this concentration of amethocaine caused,when succinate was the substrate, about 79% reduction in oxygen uptake (Fig 3.73).

3.9 Electron Microscopy

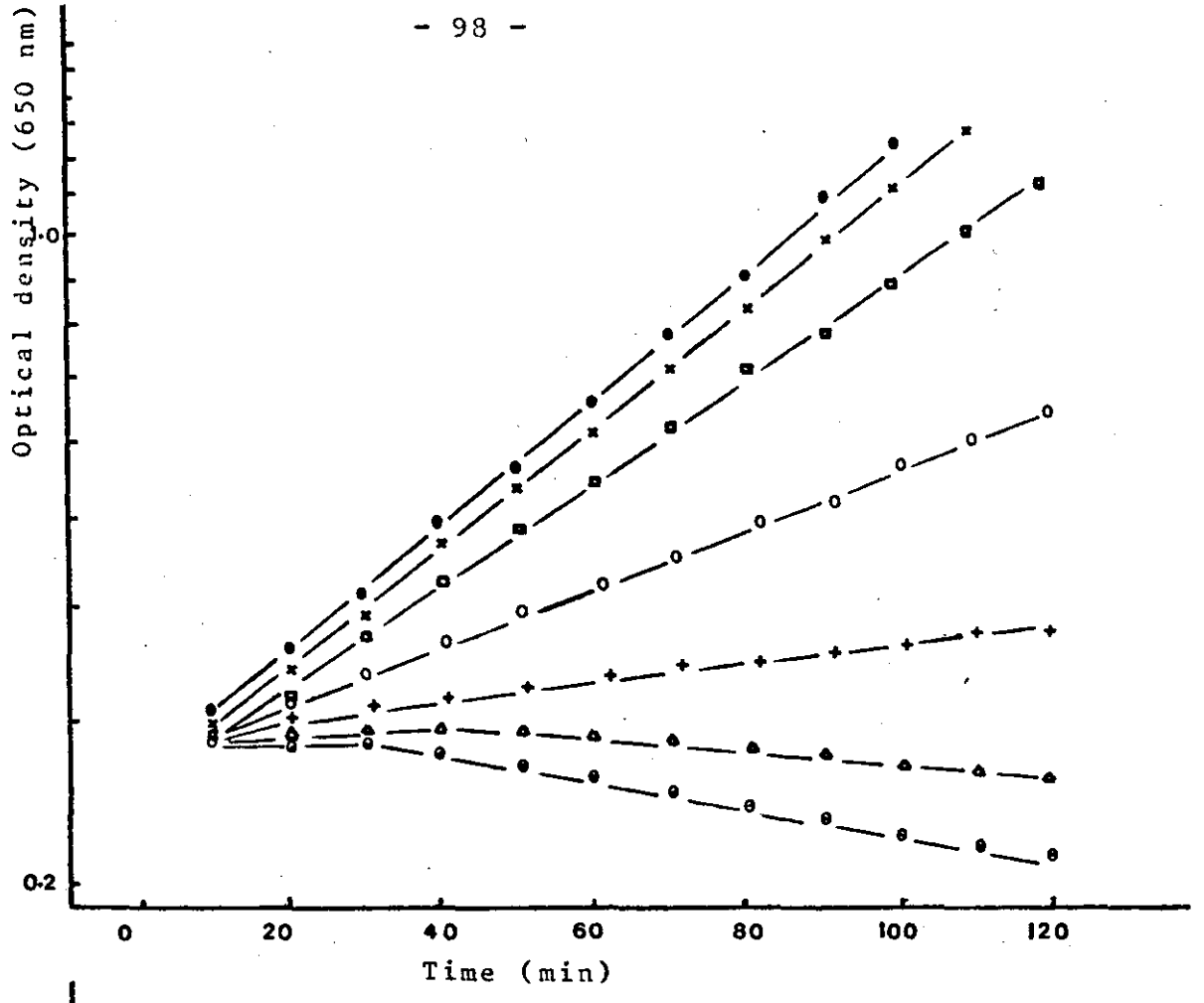
Plates (3.5 to 3.15) are scanning electron micrographs of untreated and LA treated cells of E.coli. Both treated and untreated cells appear rod-shaped. Some of the cells grown in the presence of low concentrations of LAs are elongated without having any apparent surface damage, i.e. the cell surface appears smooth (Plates 3.6, 3.10 3.14 and 3.15), and some appear as a clump (Plates 3.7 and 3.10). Higher concentrations (about MBC of LA) did not induce elongation, and some surface damage is apparent (Plates 3.8, 3.9 and 3.13).

Plates 3.16 to 3.19 show the electron micrographs of ultra thin sections of E.coli in the absence or presence of LAs. Sections had not been post stained prior to viewing. In plates 3.16 (control) and 3.17 (50 $mg.ml^{-1}$ procaine) the

cell envelope can be seen to be continuous and apparently undamaged, the intracellular material is stained faintly and uniformly. The white region in the section could be the nuclear region. In plates 3.18 (10 $mg.ml^{-1}$ amethocaine) and 3.19 (20 $mg.ml^{-1}$ amethocaine) the cytoplasm is heavily and non-uniformly stained. In some parts it is contracted away from the cell envelope and the outer membrane appears less well defined.

Fig 3.1 Changes with time in the optical density (650 nm) of suspension of E.coli in CFM containing different concentrations of amethocaine after the addition of glucose at zero time. Initial contact time 10 min. Initial cell concentration 3.8 x 10^8 cells.ml^{-1}. Incubation temp 37°C.
Amethocaine concentration (mg.ml^{-1}); ●——●, 0.0; x——x, 0.25; ▫——▫, 0.50; o——o, 0.75; +——+, 1.0; Δ——Δ, 1.25; ⊙——⊙, 1.5.

Fig 3.2 Changes with time in the optical density (650 nm) of suspensions of E.coli in CFM containing different concentrations of procaine after the addition of glucose at zero time. Initial contact time 10 min. Initial cell concentration 3.8 x 10^8 cells.ml^{-1}. Incubation temp 37°C.
Procaine concentration (mg.ml^{-1}); ●——●, 0.0; x——x, 10.0; ▫——▫, 20.0; o——o, 25.0; +——+, 30.0; Δ——Δ, 35.0; ⊙——⊙, 40.0; ▲——▲, 50.0.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0·2
0
20
40
60
80
100
120
Time (min)

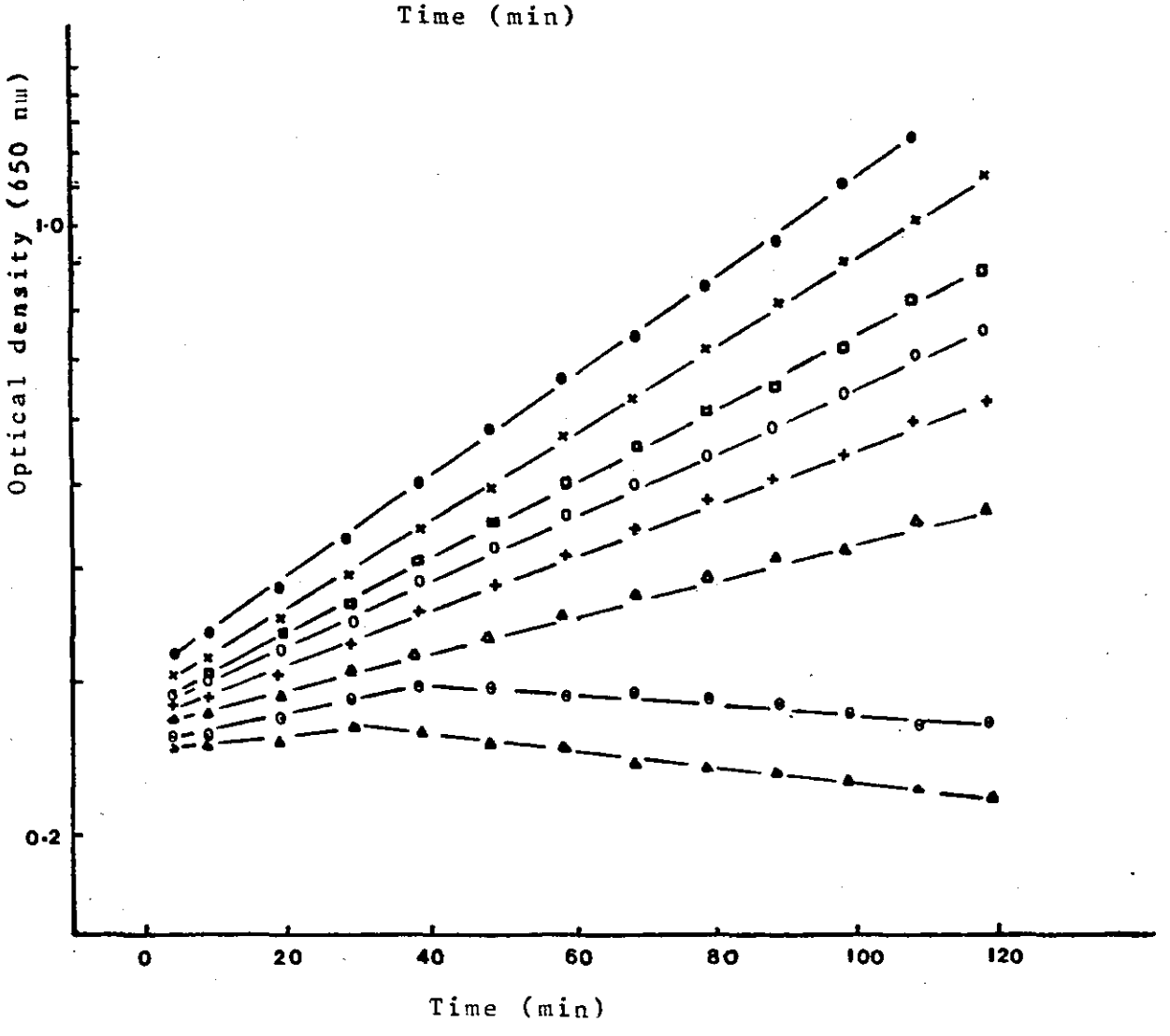
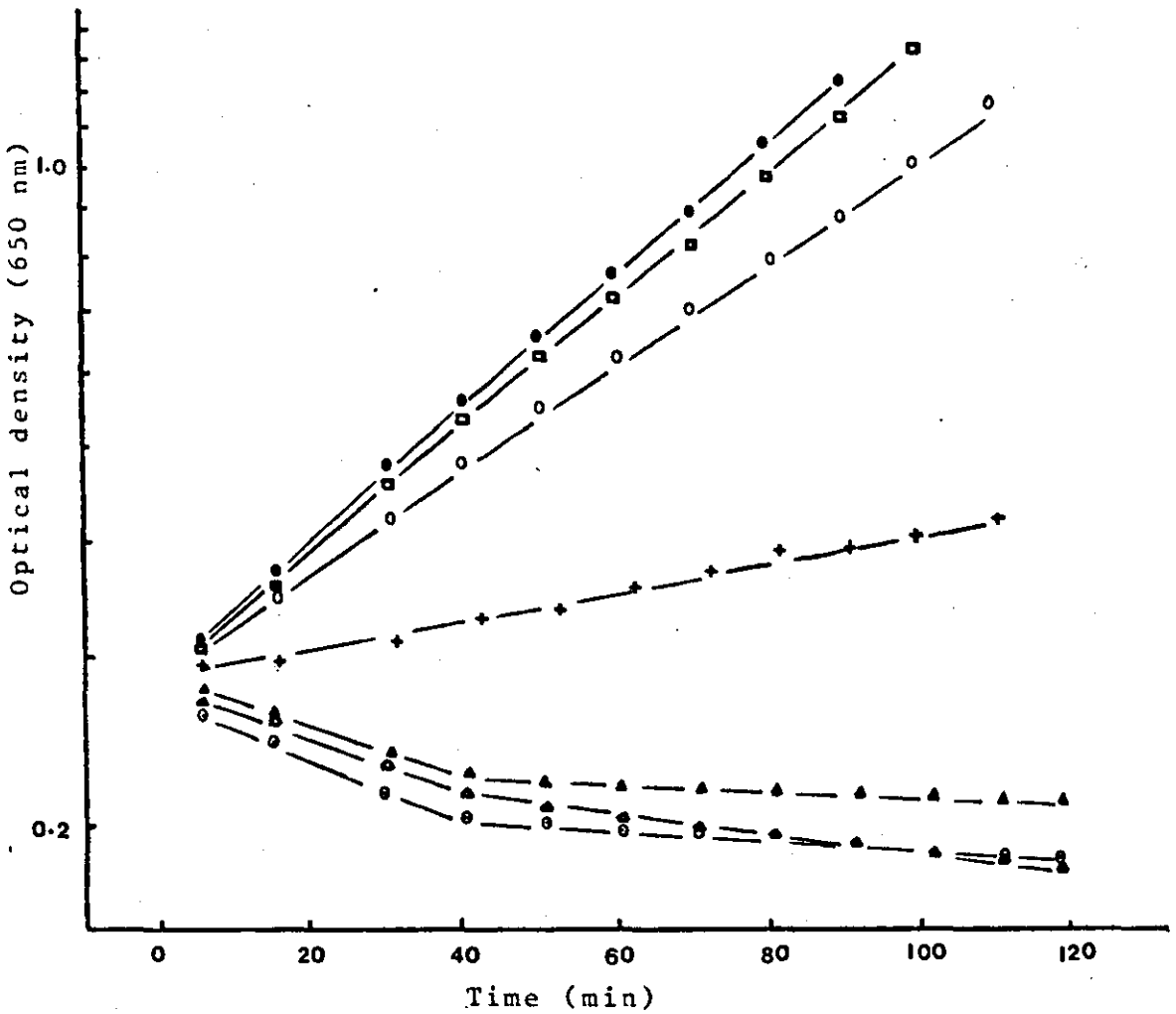

Optical density (650 nm)
1·0
0·2
0
20
40
60
80
100
120
Time (min)

Fig 3.3 Changes with time in the optical density (650 nm) of suspension of E.coli in CFM containing different concentrations of cinchocaine after the addition of glucose at zero time. Initial contact time 10 min. Initial cell concentration 3.8×10^8 cells. ml^{-1}. Incubation temp $37^{o}C$.
Cinchocaine concentration ($mg.ml^{-1}$); ●——●, 0.0 and 0.10; ▫——▫, 0.25; o——o, 0.30; +——+, 0.40; Δ——Δ, 0.50; ⊙——⊙, 0.60; ▲——▲, 1.50.

Fig 3.4 Changes with time in the optical density (650 nm) of suspension of E.coli in CFM containing different concentrations of cinchocaine after the addition of glucose at zero time. Initial contact time 10 min. Initial cell concentration 3.8×10^8 cells.ml^{-1}. Incubation temp $37^{o}C$.
Lignocaine concentration ($mg.ml^{-1}$); ●——●, 0.0; x——x, 10.0; ▫——▫, 15.0; o——o, 20.0; +——+, 25.0; Δ——Δ, 30.0; ⊙——⊙, 40.0.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0·2
0
20
40
60
80
100
120
Time (min)

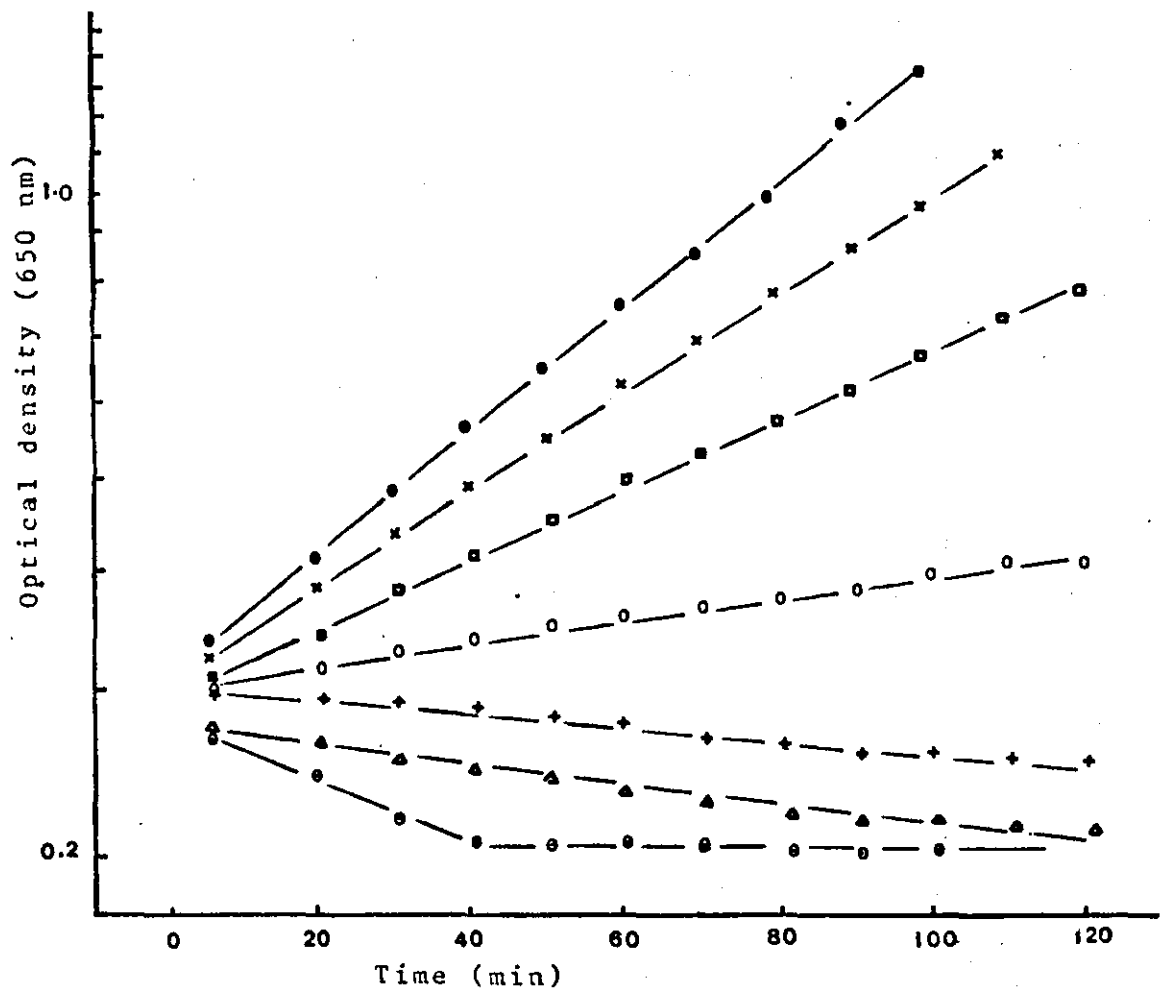
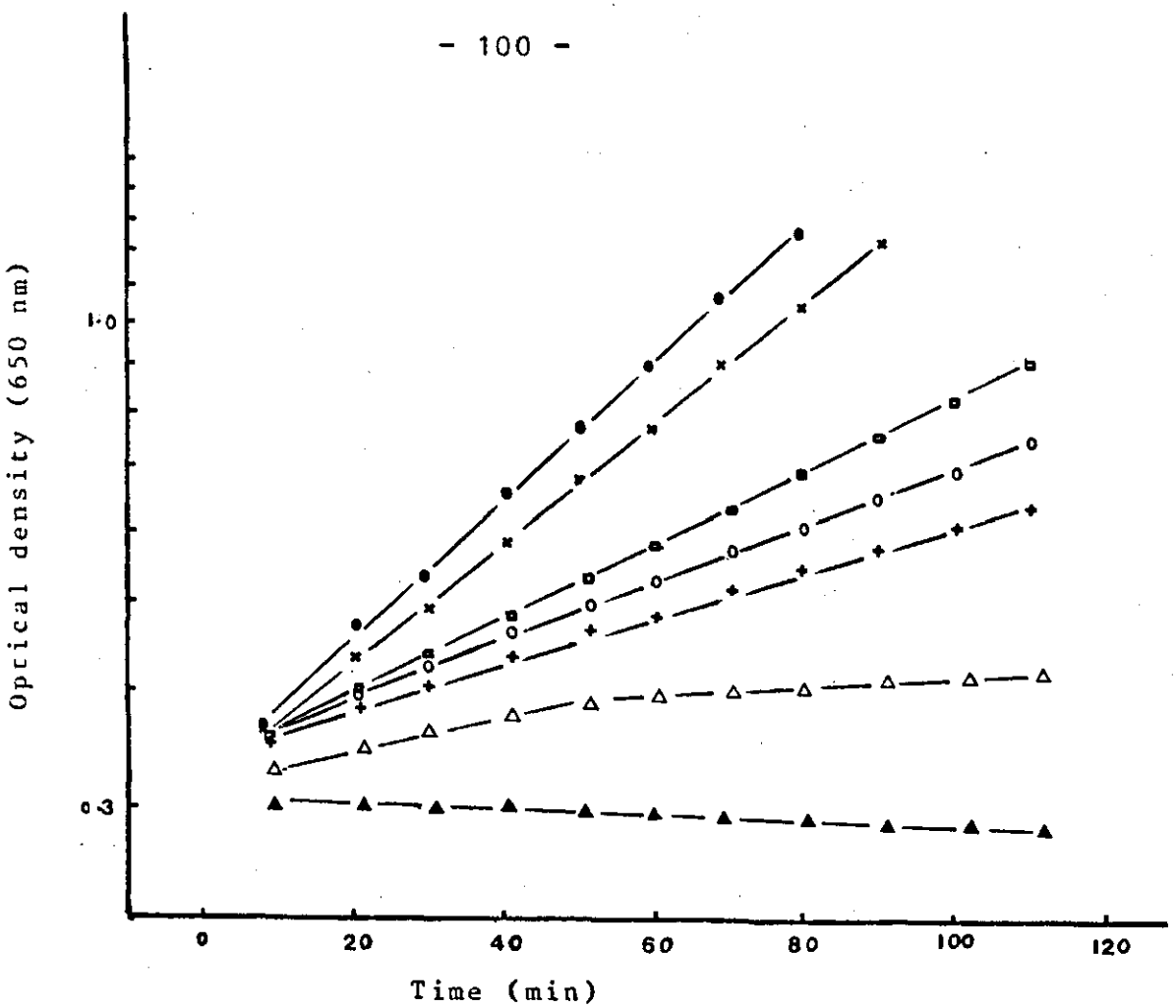

Optical density (650 nm)
1·0
0·2
0
20
40
60
80
100
120
Time (min)

Fig 3.5 Changes with time in the optical density (650 nm) of suspensions of K.aerogenes in CFM containing different concentrations of amethocaine after the addition of glucose at zero time. Initial contact time 10 min. Initial cell concentration 0.12 $mg.ml^{-1}$. Incubation temp $37^{o}C$.

Amethocaine concentration ($mg.ml^{-1}$): ●——●, 0.0; x——x, 0.50; ▫——▫, 1.00; o——o, 1.25; +——+, 1.50; Δ——Δ, 2.00; ▲——▲, 3.00.

Fig 3.6 Changes with time in the optical density (650 nm) of suspensions of K.aerogenes in CFM containing different concentrations of procaine after the addition of glucose at zero time. Initial contact time 10 min. Initial cell concentration 0.12 $mg.ml^{-1}$. Incubation temp $37^{o}C$.

Procaine concentration ($mg.ml^{-1}$); ●——●, 0.0; x——x, 10.0; ▫——▫, 20.0; o——o, 30.0; +——+, 40.0; Δ——Δ, 50.0; ▲——▲, 60.0.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0·3
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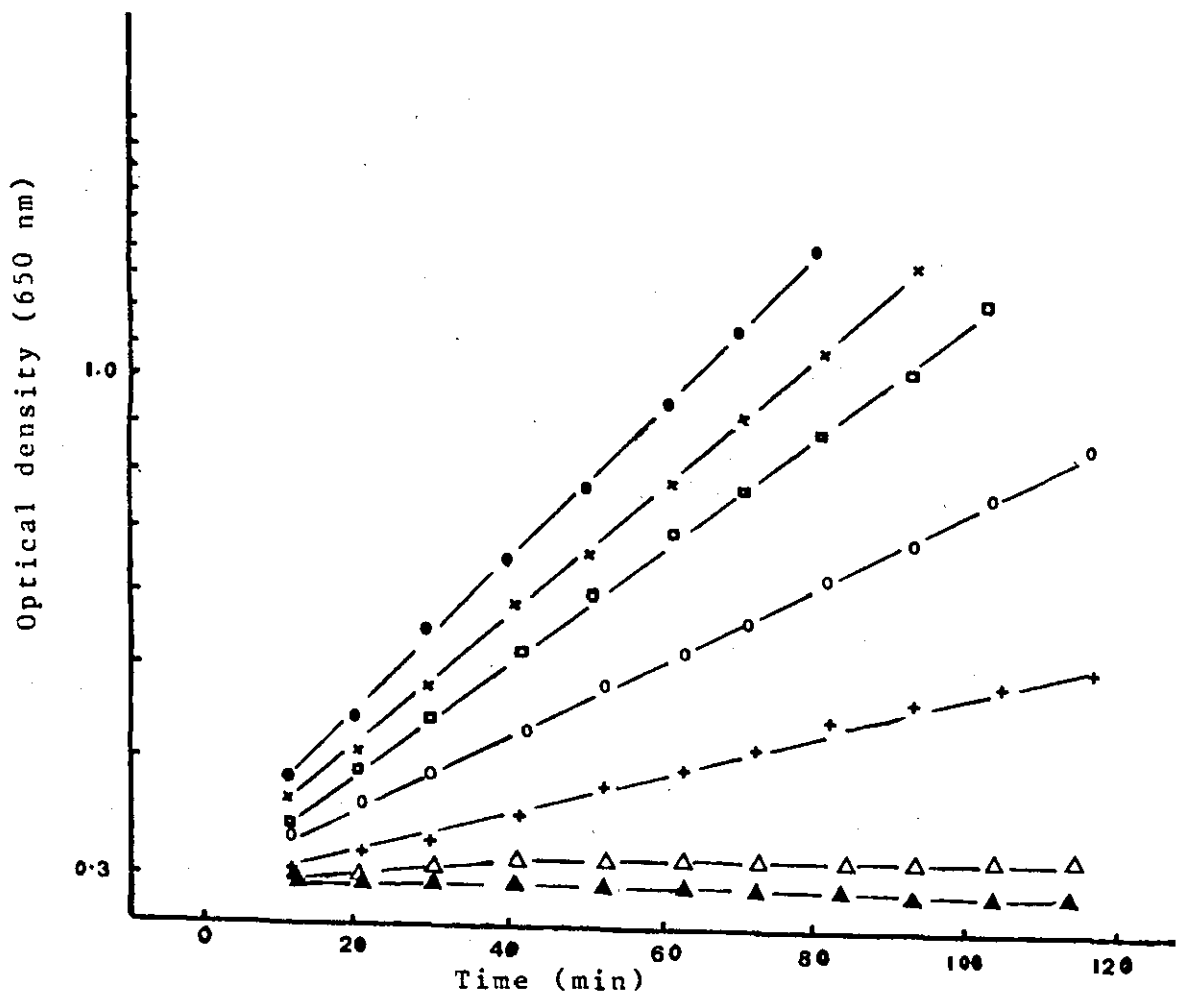
20
40
60
80
100
120
Time (min)


Optical density (650 nm)
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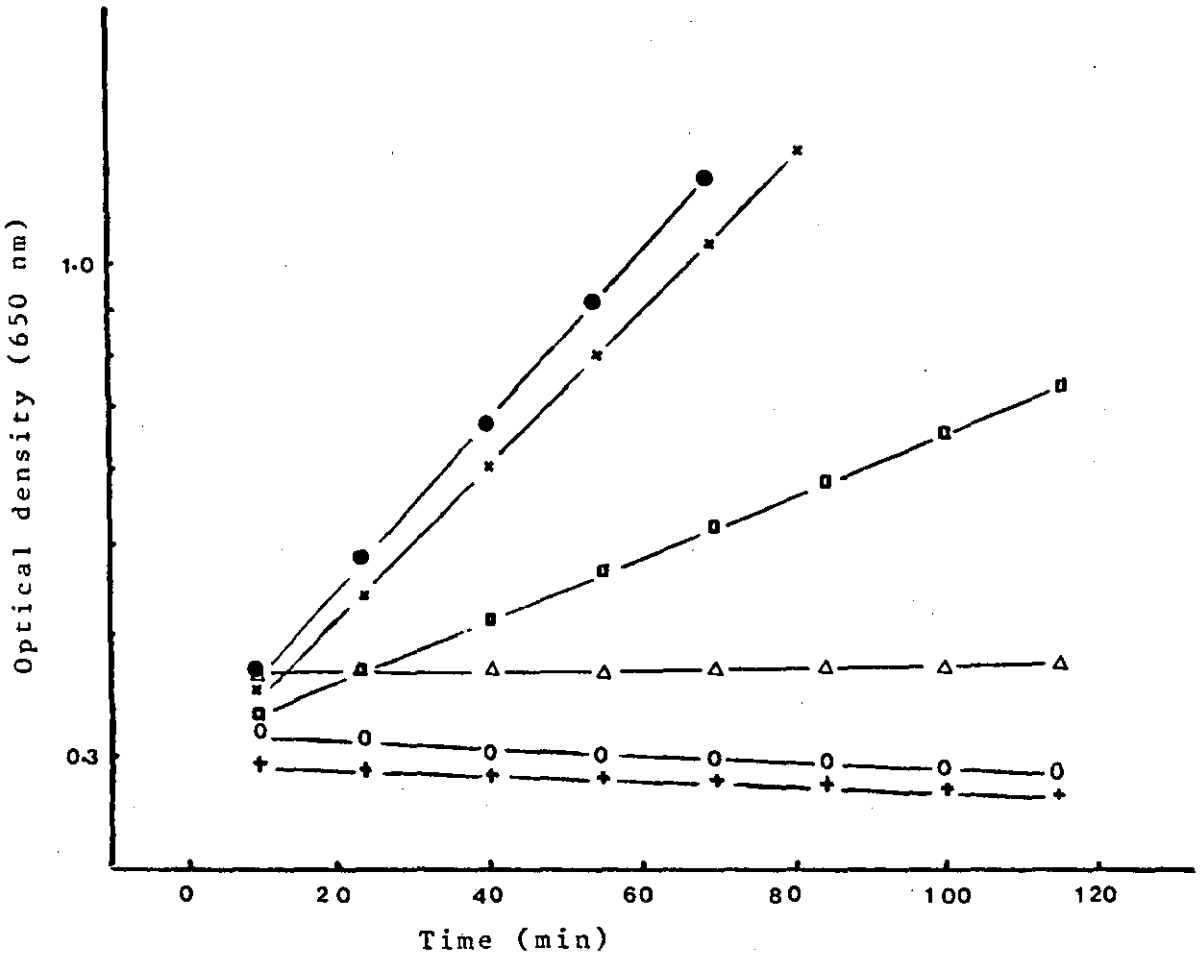
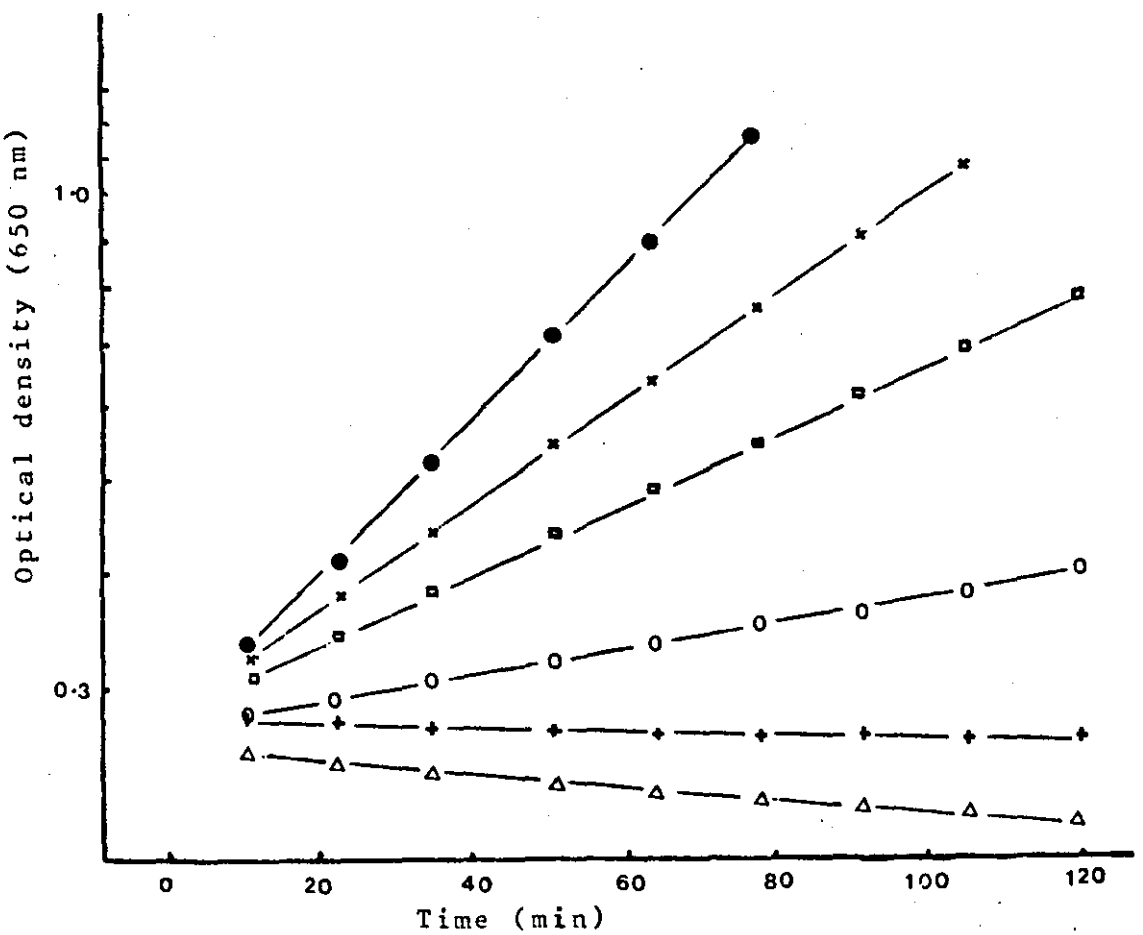
0·3
0
20
40
60
80
100
120
Time (min)

Fig 3.7 Changes with time in the optical density (650 nm) of suspension of K.aerogenes in CFM containing different concentrations of cinchocaine after the addition of glucose at zero time. Initial cell concentration 0.12 $mg.ml^{-1}$. Initial contact time 10 min. Incubation temp $37^{o}C$.
Cinchocaine concentration ($mg.ml^{-1}$) ●——●, 0.00; x——x, 0.25; □——□, 0.50; o——o, 0.75; +——+, 1.00; Δ——Δ, 2.00.

Fig. 3.8 Changes with time in the optical density (650 nm) of suspension of K.aerogenes in CFM containing different concentrations of lignocaine after the addition of glucose at zero time. Initial cell concentration 0.12 $mg.ml^{-1}$. Initial contact time 10 min. Incubation temp $37^{o}C$.
Lignocaine concentration ($mg.ml^{-1}$) ●——●, 0.0; x——x, 10.0; □——□, 15.0; o——o, 20.0; +——+, 25.0; Δ——Δ, 30.0.


Optical density (650 nm)
1·0
0·3
0
20
40
60
80
100
120
Time (min)


Optical density (650 nm)
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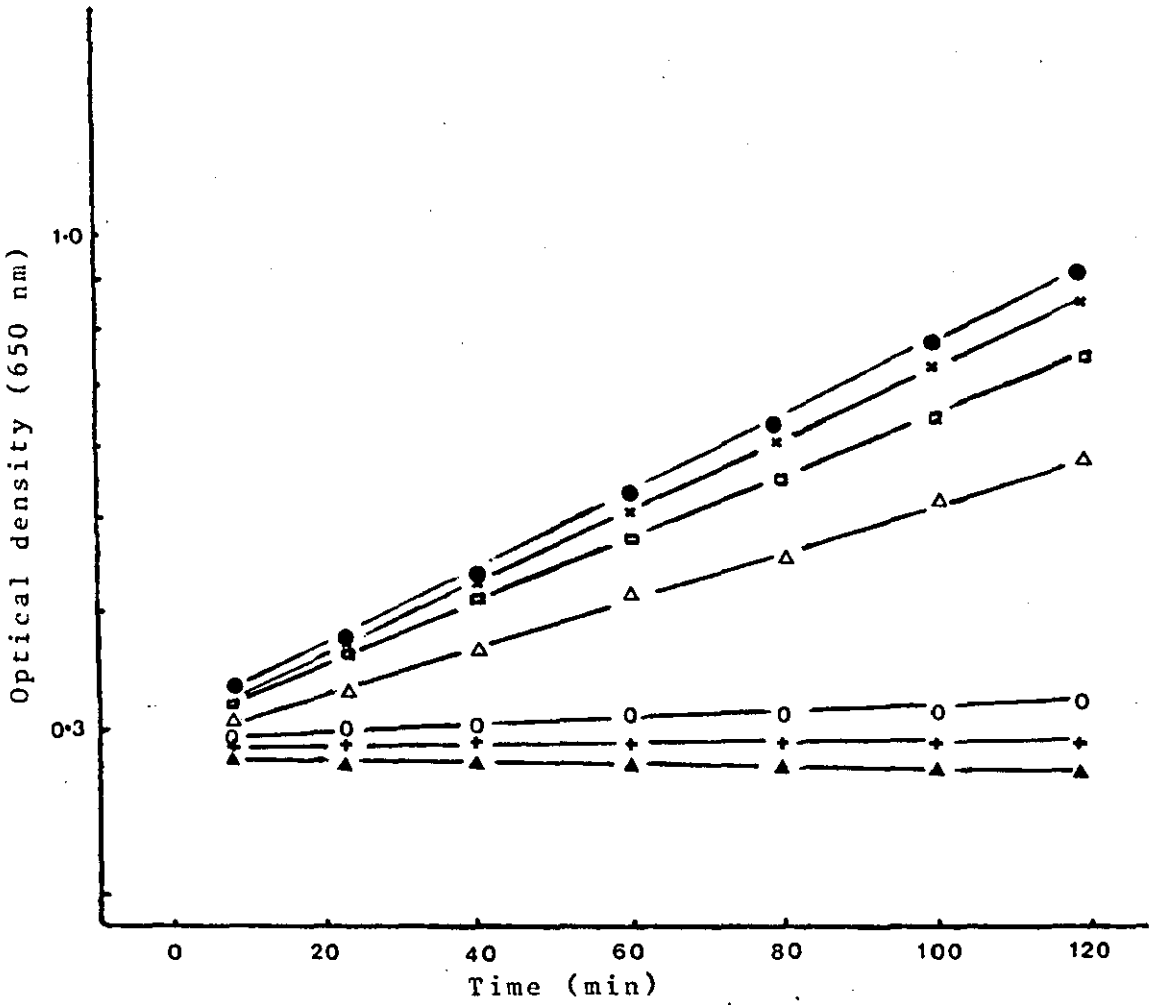
0·3
0
20
40
60
80
100
120
Time (min)

Fig 3.9 Changes with time in the optical density (650 nm) of suspension of P.aeruginosa in CFM containing different concentrations of amethocaine after the addition of glucose at zero time. Initial cell concentration 0.125 $mg.ml^{-1}$. Initial contact time 10 min. Incubation temp $37^{o}C$.
Amethocaine concentration ($mg.ml^{-1}$) ●——●, 0.0; x——x, 0.25; □——□, 0.75; Δ——Δ, 1.0; o——o, 1.5; +——+, 2.0; ▲——▲, 2.5.

Fig 3.10 Changes with time in the optical density (650 nm) of suspension of P.aeruginosa in CFM containing different concentrations of procaine after the addition of glucose at zero time. Initial cell concentration 0.125 $mg.ml^{-1}$. Initial contact time 10 min. Incubation temp $37^{o}C$.
Procaine concentration ($mg.ml^{-1}$) ●——●, 0.0; x——x, 10.0; □——□, 20.0; Δ——Δ, 30.0; o——o, 40.0; +——+, 50.0.


Optical density (650 nm)
1·0
0·3
0
20
40
60
80
100
120
Time (min)

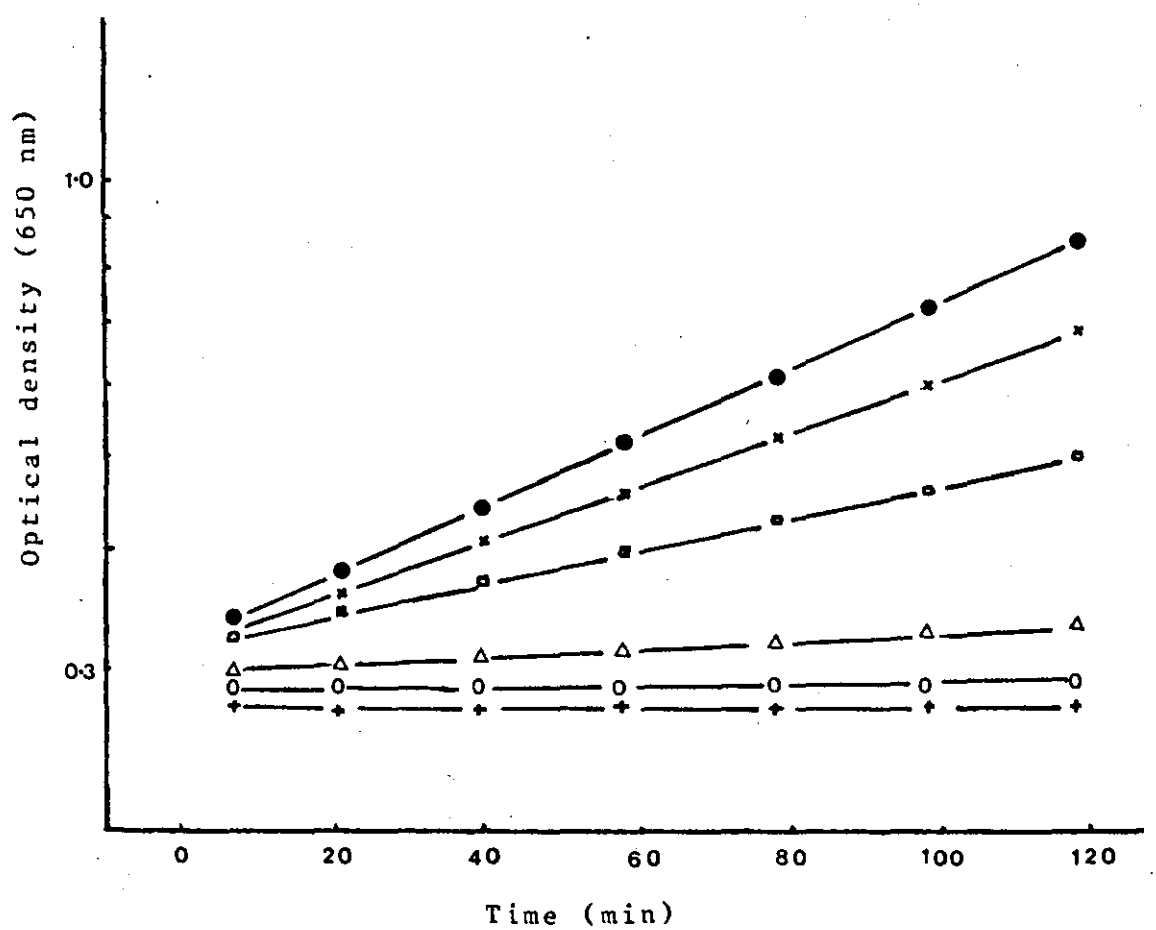
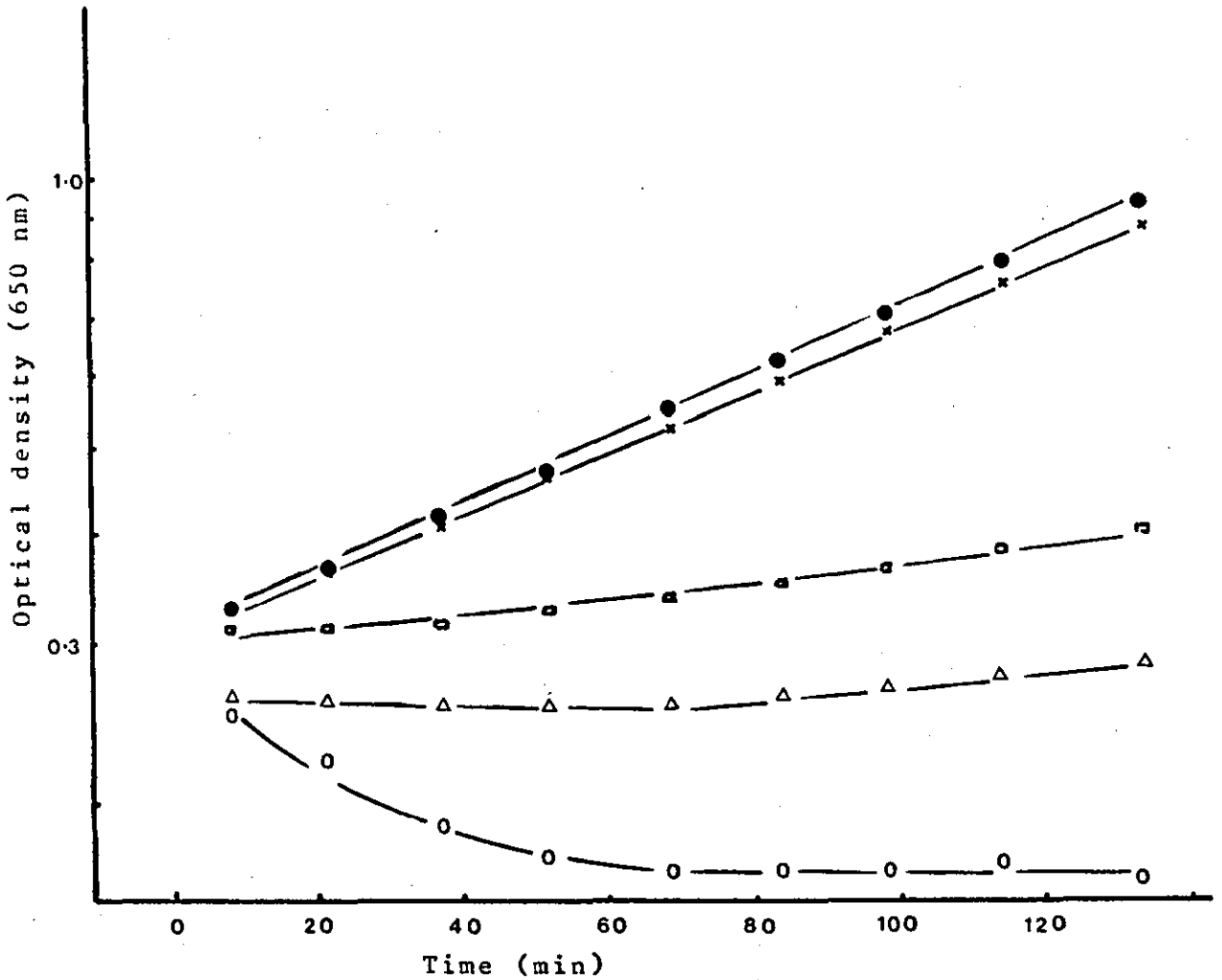

Optical density (650 nm)
1·0
0·3
0
20
40
60
80
100
120
Time (min)

Fig 3.11 Changes with time in the optical density (650 nm) of suspension of P.aeruginosa in CFM containing different concentrations of cinchocaine after the addition of glucose at zero time. Initial cell concentration 0.125 $mg.ml^{-1}$. Initial contact time 10 min. Incubation temp $37^{o}C$.
Cinchocaine concentration ($mg.ml^{-1}$), ●——●, 0.00; x——x, 0.25;□——□, 0.50; Δ——Δ, 0.75; o——o, 1.00;.

Fig 3.12 Changes with time in the optical density (650 nm) of suspension of P.aeruginosa in CFM containing different concentrations of lignocaine after the addition of glucose at zero time. Initial cell concentration 0.125 $mg.ml^{-1}$. Initial contact time 10 min. Incubation temp $37^{o}C$.
Lignocaine concentration ($mg.ml^{-1}$), ●——●, 0.0; x——x, 10.0;□——□, 15.0; Δ——Δ, 20.0; 0——0, 25.0; +——+, 30.0.


Optical density (650 nm)
1·0
0·3
0
20
40
60
80
100
120
Time (min)

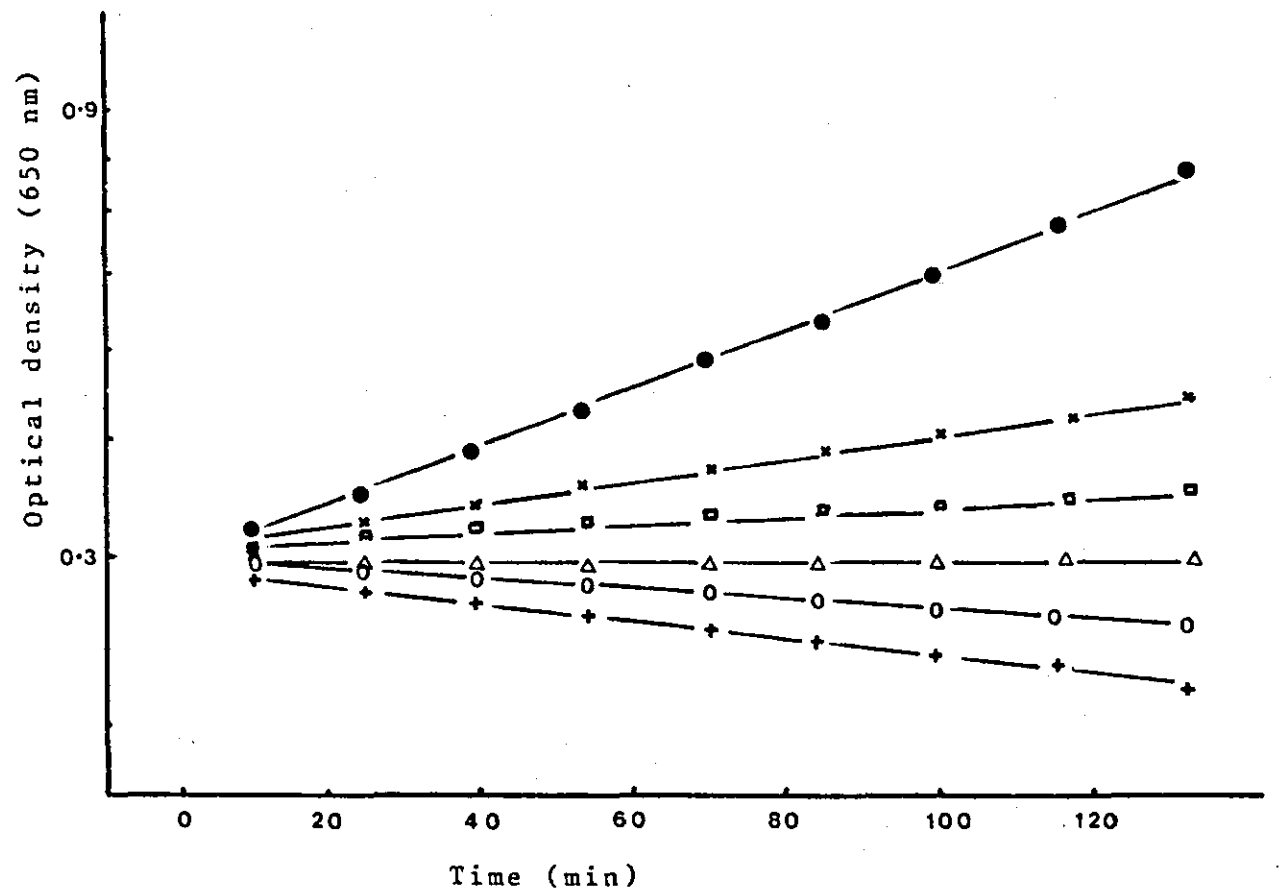
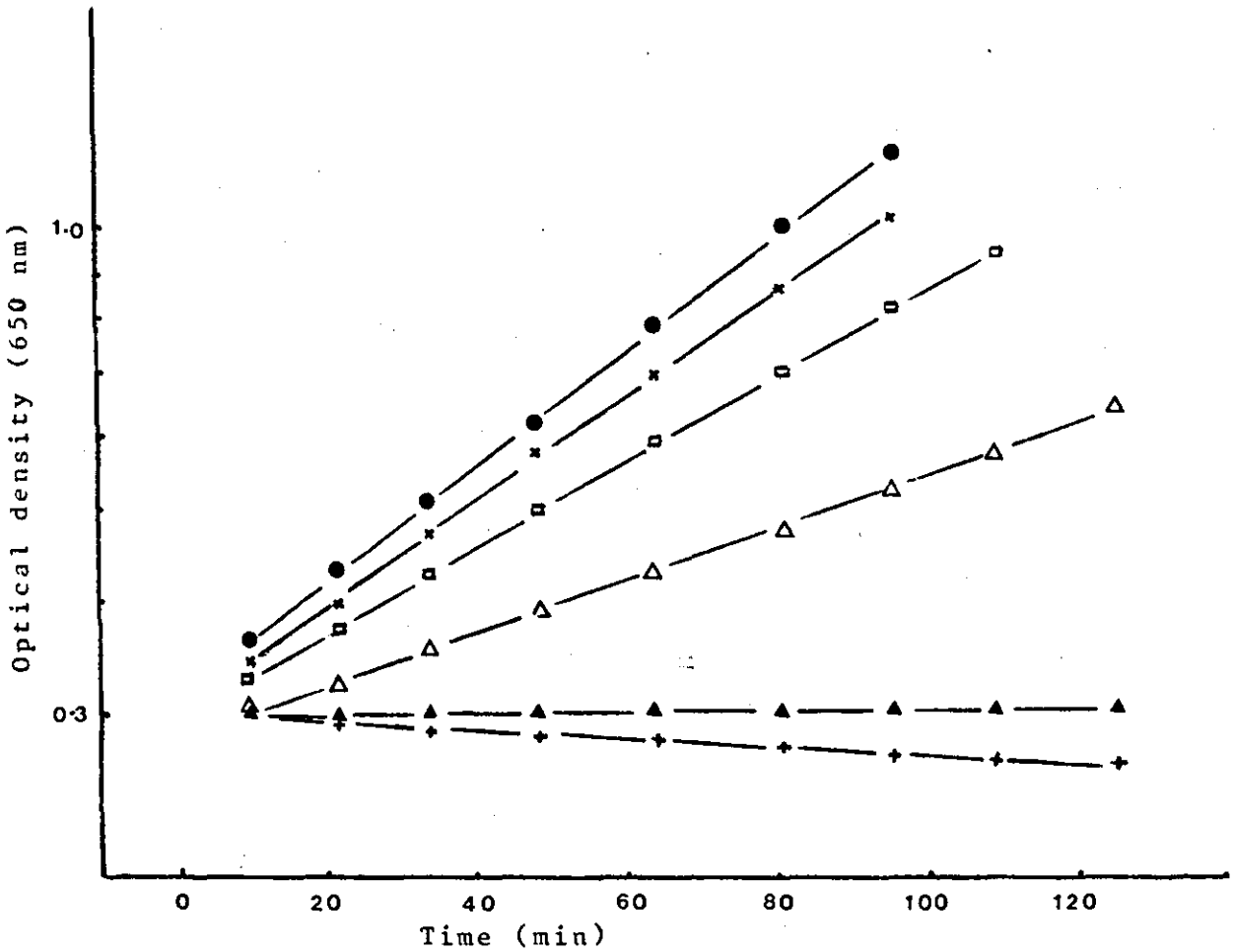

Optical density (650 nm)
0·9
0·3
0
20
40
60
80
100
120
Time (min)

Fig 3.13 Changes with time in the optical density (650 nm) of suspension of B.megaterium in CFM containing different concentrations of amethocaine after the addition of glucose at zero time. Initial cell concentration 0.070 $mg.ml^{-1}$. Initial contact time 10 min. Incubation temp $37^{o}C$.
Amethocaine concentration ($mg.ml^{-1}$): ●—●, 0.0; x—x, 0.25; ▫—▫, 0.30; Δ—Δ, 0.40; ▲—▲, 0.50; +—+, 1.0.

Fig 3.14 Changes with time in the optical density (650 nm) of suspension of B.megaterium in CFM containing different concentrations of procaine after the addition of glucose at zero time. Initial cell concentration 0.070 $mg.ml^{-1}$. Initial contact time 10 min. Incubation temp $37^{o}C$.
Procaine concentration ($mg.ml^{-1}$), ●—●, 0.0; x—x, 5.0; ▫—▫, 10.0; Δ—Δ, 15.0; ▲—▲, 20.0; +—+, 25.0.


Optical density (650 nm)
1·0
0·3
0
20
40
60
80
100
120
Time (min)

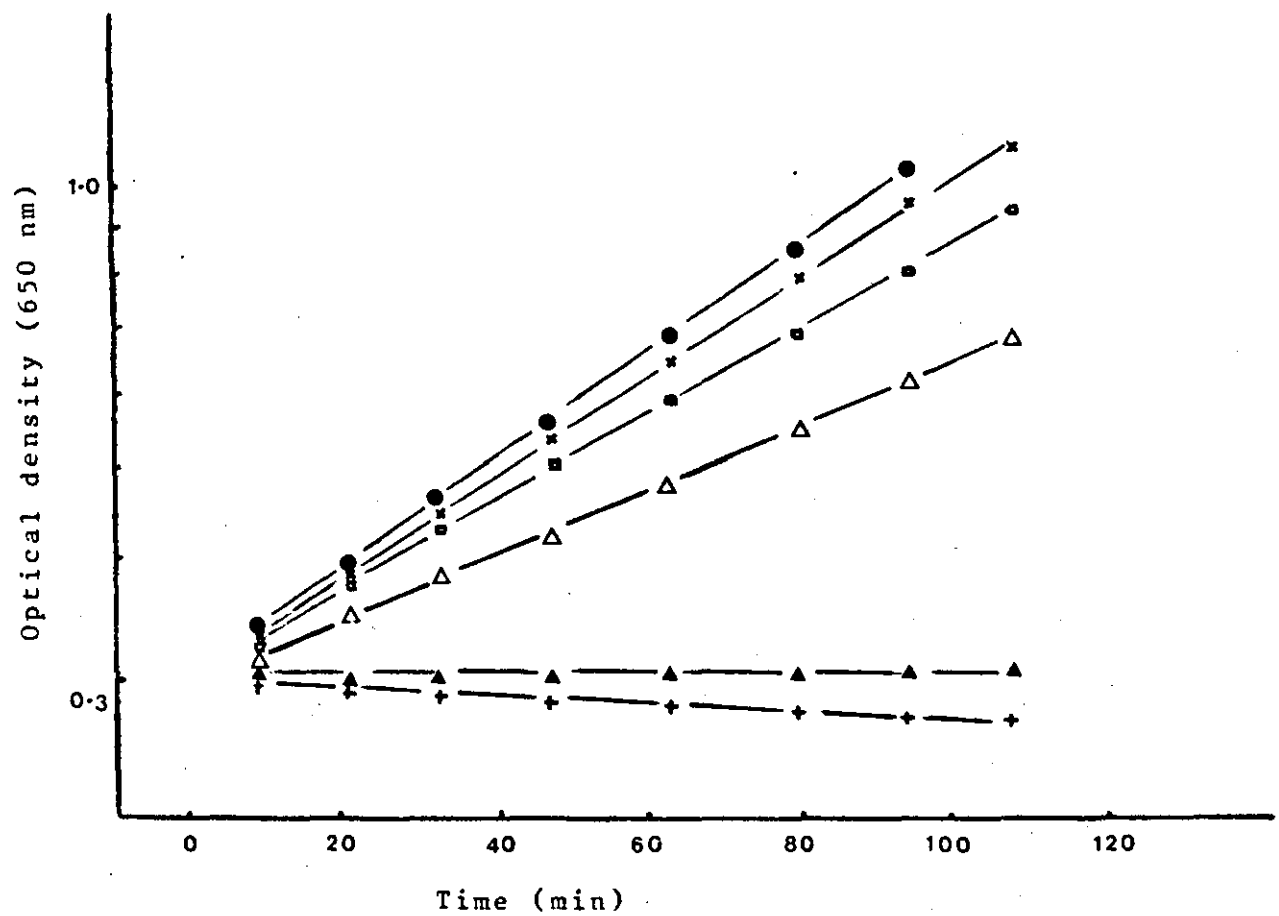
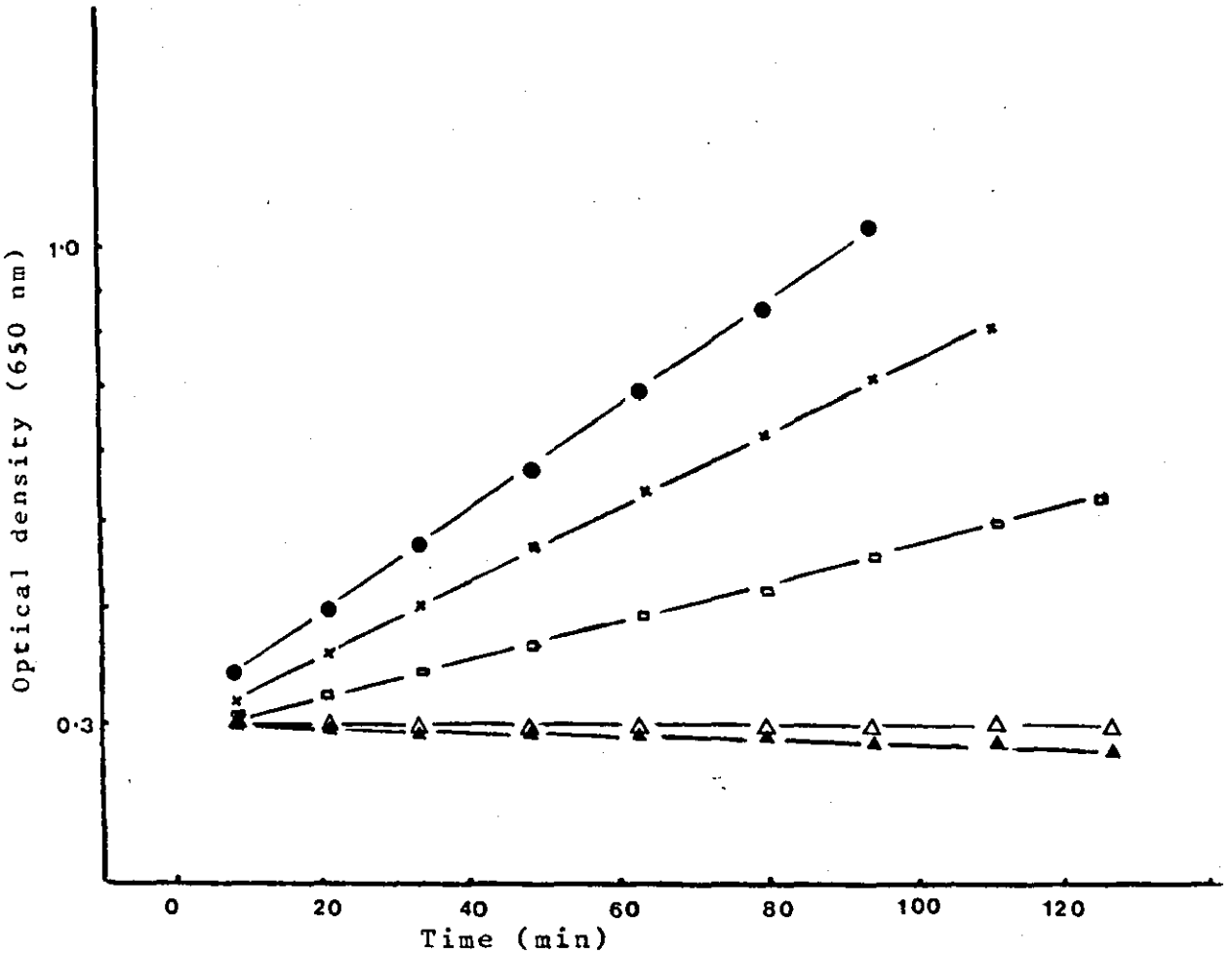

Optical density (650 nm)
1·0
0·3
0
20
40
60
80
100
120
Time (min)

Fig 3.15 Changes with time in the optical density (650 nm) of suspension of B.megaterium in CFM containing different concentrations of cinchocaine after the addition of glucose at zero time. Initial cell concentration 0.070 $mg.ml^{-1}$. Initial contact time 10 min. Incubation temp $37^{o}C$.
Cinchocaine concentration ($mg.ml^{-1}$), ●—●, 0.0; x—x, 0.15; □—□, 0.20; Δ—Δ, 0.25; ▲—▲, 0.50.

Fig 3.16 Changes with time in the optical density (650 nm) of suspension of B.megaterium in CFM containing different concentrations of lignocaine after the addition of glucose at zero time. Initial cell concentration 0.070 $mg.m.^{-1}$. Initial contact time 10 min. Incubation temp $37^{o}C$.
Lignocaine concentration($mg.ml^{-1}$), ●—●, 0.0; x—x, 2.0; □—□, 2.5; Δ—Δ, 4.0; ▲—▲, 8.0; +—+, 10.0.


Optical density (650 nm)
1·0
0·3
0
20
40
60
80
100
120
Time (min)

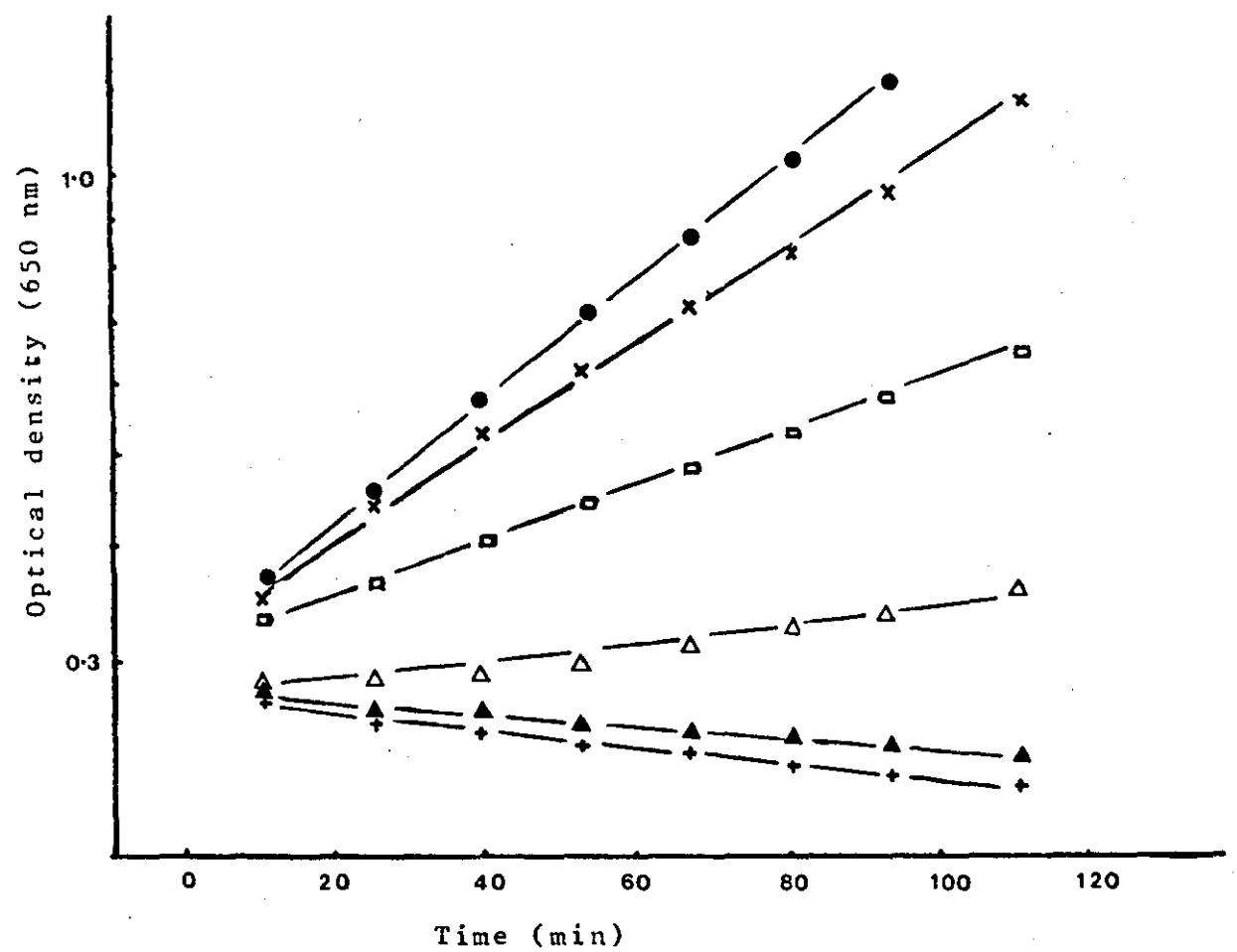
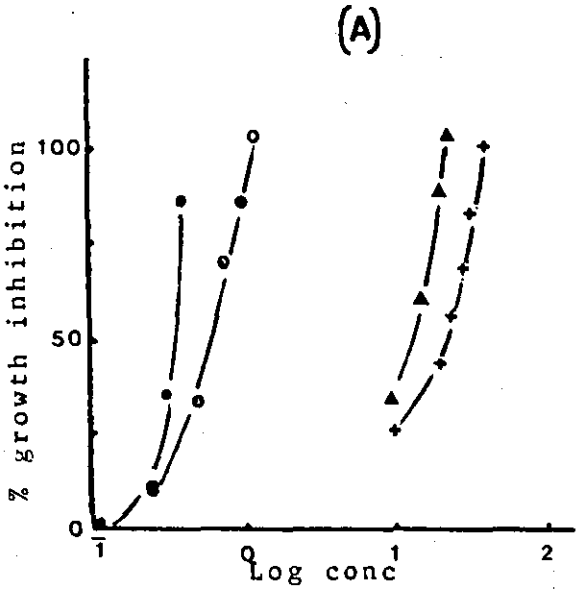
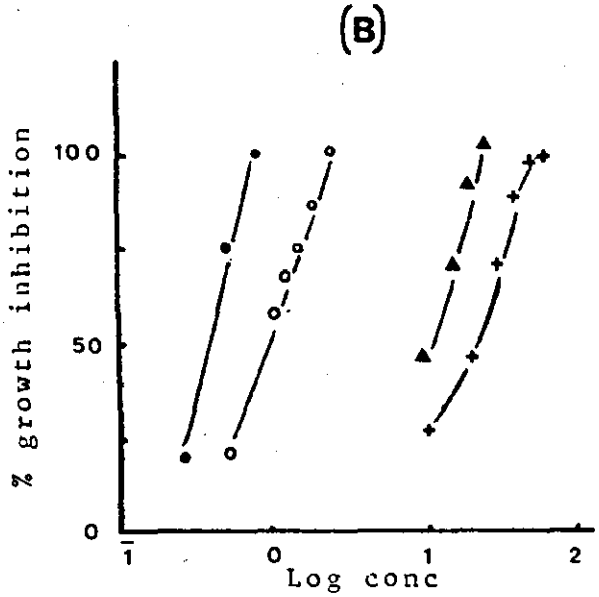
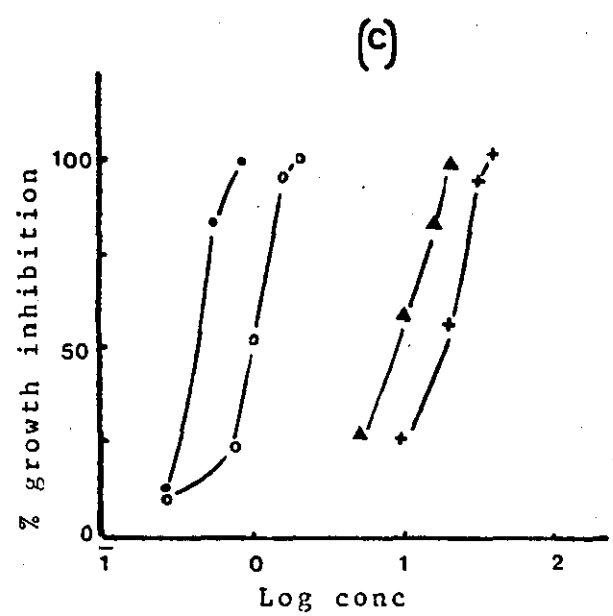

Optical density (650 nm)
1·0
0·3
0
20
40
60
80
100
120
Time (min)

Fig 3.17 Effect of LAs concentration on the % inhibition of growth of E.coli (A); K.aerogenes (B); P.aeruginosa (C) and B.megaterium (D) 70 min after the addition of glucose. Temp 37°C.
o——o amethocaine, +——+ procaine, ●——● cinchocaine, ▲——▲ lignocaine.


(A)
% growth inhibition
100
50
0
1̄
0
1
2
Log conc


(B)
% growth inhibition
100
50
0
1̄
0
1
2
Log conc


(C)
% growth inhibition
100
50
0
1̄
0
1
2
Log conc

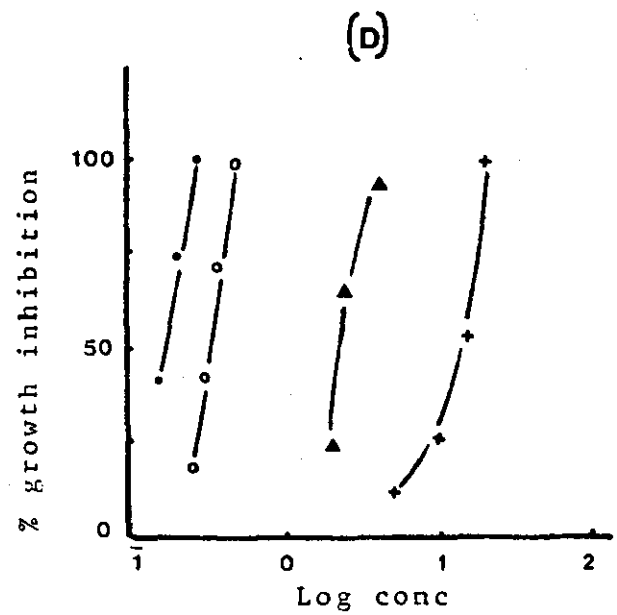
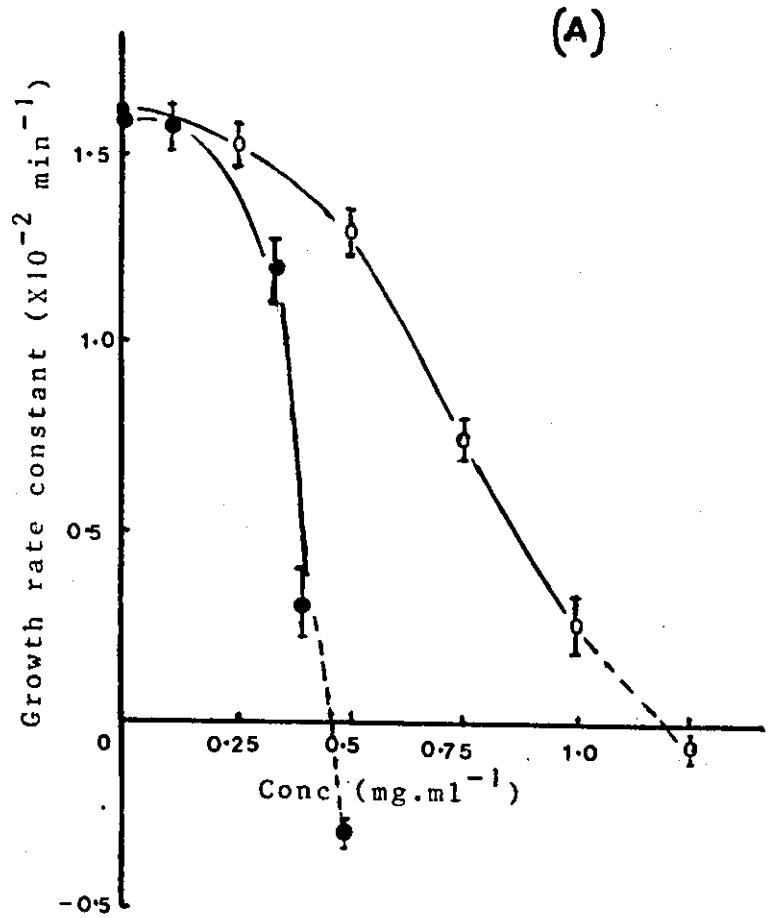

(D)
% growth inhibition
100
50
0
1̄
0
1
2
Log conc

Fig 3.18 Exponential growth rate constants of E.coli in cultures partially inhibited by (A) o—o amethocaine and ●—● cinchocaine; (B) +—+, procaine and ▲—▲, lignocaine.


(A)
Growth rate constant ($X10^{-2}$ min^{-1})
1·5
1·0
0·5
0
-0·5
0·25
0·5
0·75
1·0
Conc ($mg.ml^{-1}$)

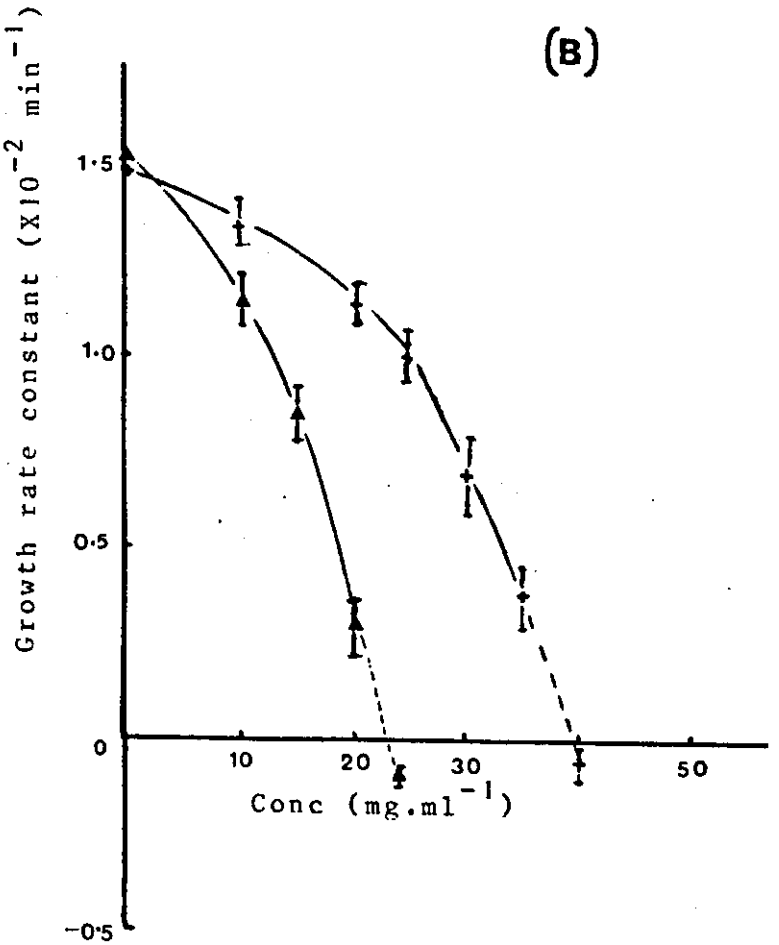

(B)
Growth rate constant ($X10^{-2}$ min^{-1})
1·5
1·0
0·5
0
-0·5
10
20
30
50
Conc ($mg.ml^{-1}$)

Fig 3.19 Changes in the optical density (650 nm) of E.coli cell suspension in CFM after the addition of glucose (2·0 mg.ml^{-1}) and ampicillin (2.0 µg.ml^{-1}) in the presence or absence of different concentrations of amethocaine. Temp 37^{o}C. Contact time 10 min. Cell concentration 1.3 x 10^{8} cells.ml^{-1}.
Amethocaine concentrations (mg.ml^{-1}) ●——●, 0.0; x——x, 0.20; ▫——▫, 0.40; o——o, 0.50; +——+, 0.60; Δ——Δ, 1.00; ⊙——⊙, 1.50.

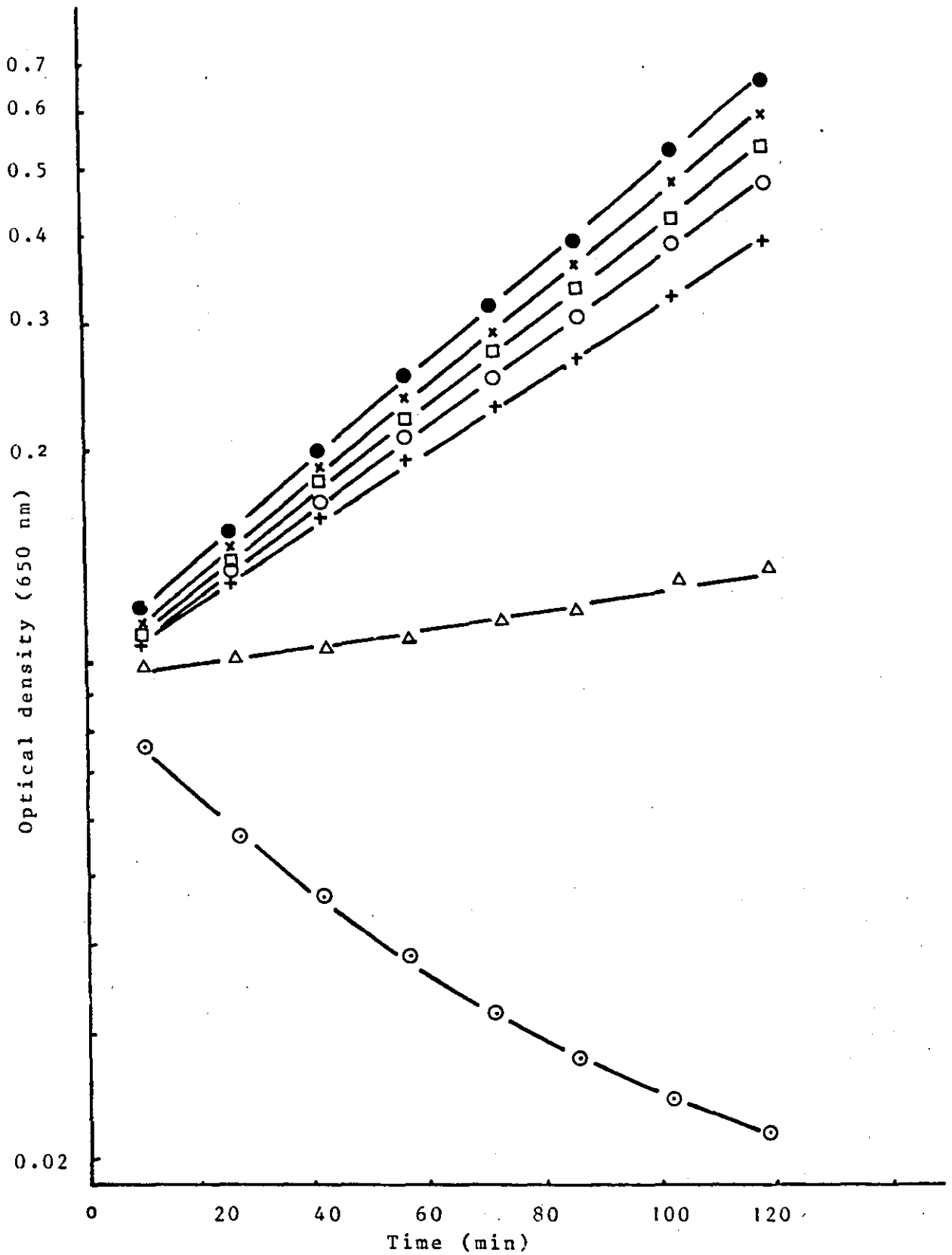

0.7
0.6
0.5
0.4
0.3
0.2
0.02
Optical density (650 nm)
0
20
40
60
80
100
120
Time (min)

Fig 3.20 The size distributions (threshold units) of untreated and amethocaine treated (0.50 $mg.ml^{-1}$) cells of E.coli growing in complete growth medium containing 2.0$\mu g.ml^{-1}$ ampicillin.
●——●, control at zero time; ▲——▲, control 60 min after the addition of glucose (2.0 $mg.ml^{-1}$) and ampicillin; o——o, amethocaine treated cells at zero time;▫——▫, amethocaine treated cells after 20 min growth; ⊙——⊙,amethocaine treated cells after 40 min growth; Δ——Δ, amethocaine treated cells after 60 min growth;◇——◇, amethocaine treated cells after 90 min growth.

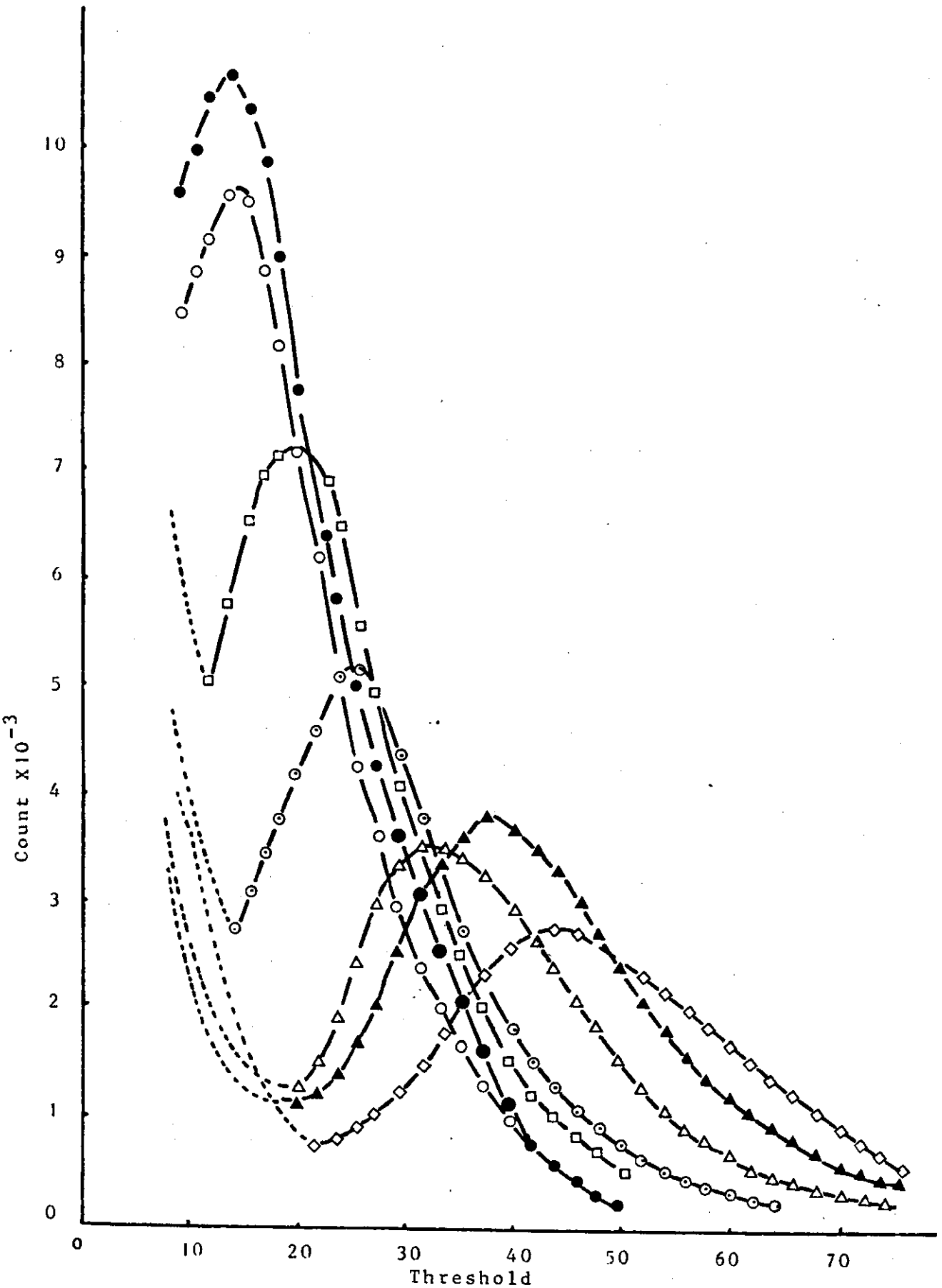

Count X10^{-3}
0
1
2
3
4
5
6
7
8
9
10
0
10
20
30
40
50
60
70
Threshold

Fig 3.21 Changes with time in the optical density (650 nm) of cultures of E.coli initially suspended in CFM containing different concentrations of amethocaine, after the addition of glucose (at zero time) and after resuspension in the same volume of the complete growth medium (at 80 min). Initial contact time (prior to addition of glucose) 10 min. Incubation temp 37°C. Cell concentration 3.8 x 10^8 cells.ml^{-1}.
Amethocaine concentration (mg.ml^{-1}): ●—●, ○—○, 0.0; x—x, +—+, 0.5; ■—■, □—□, 0.75; ▼—▼, ▽—▽, 1.0; ◆—◆, ◇—◇, 1.25; ▲—▲, △—△, 1.50 (the lower symbol represents changes measured after resuspension).

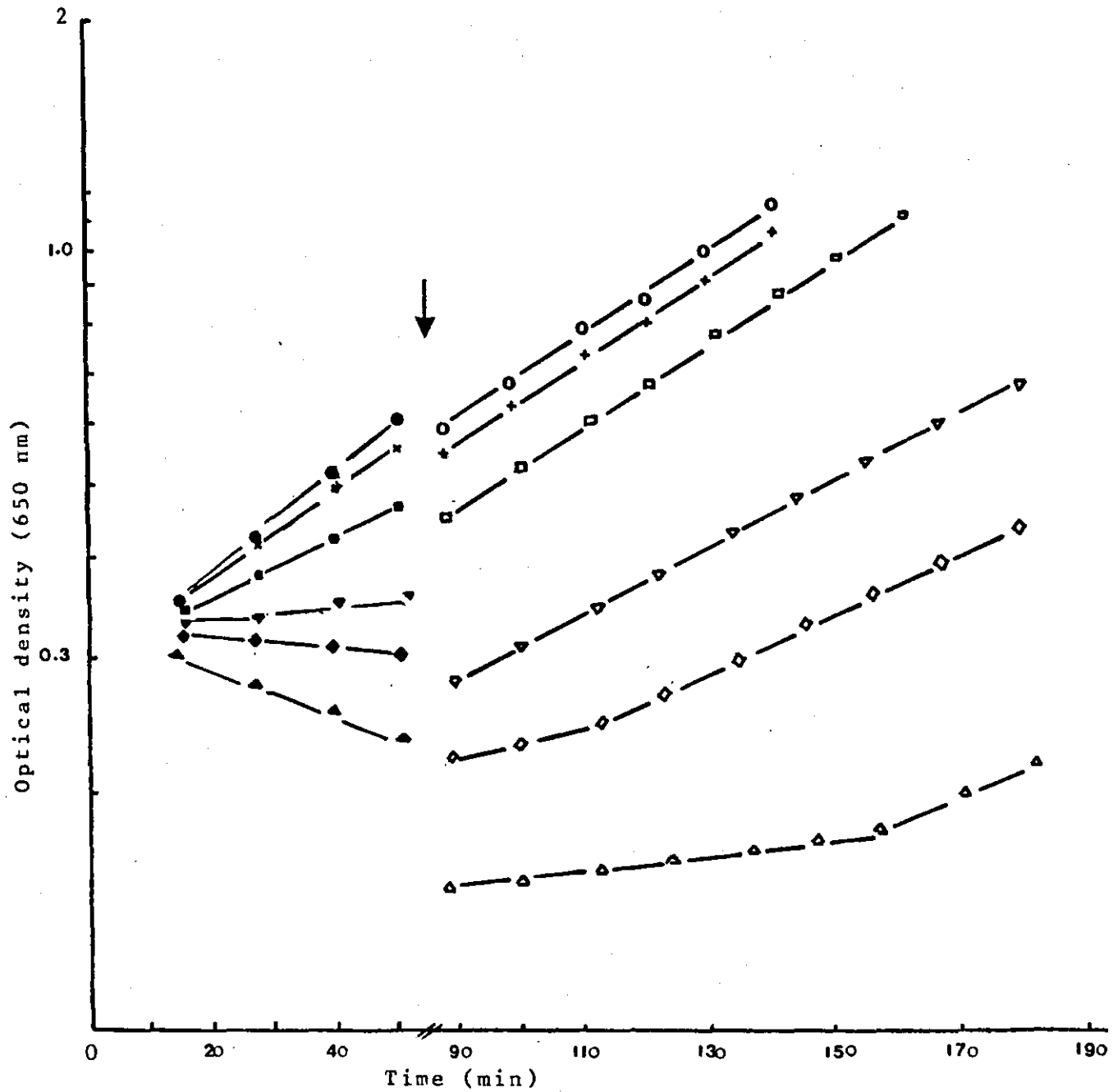

2
1.0
0.3
Optical density (650 nm)
0
20
40
90
110
130
150
170
190
Time (min)

Fig 3.22 Changes with time in the optical density (650 nm) of cultures of E.coli initially suspended in CFM containing different concentrations of procaine after the addition of glucose at zero time and after resuspension in the same volume of the complete growth medium (at 80 min). Initial contact time (prior to addition of glucose) 10 min. Incubation temp 37°C. Cell concentration 3.8 x 10^8 cells.ml^{-1}.
Procaine concentration (mg.ml^{-1}): ●—●, ○—○, 0.0; x—x, +—+, 10.0; ■—■, □—□, 20.0; ▼—▼, ▽—▽, 30.0; ◆—◆, ◇—◇, 40.0; ▲—▲, △—△, 50.0 (lower symbol represents changes measured after resuspension).

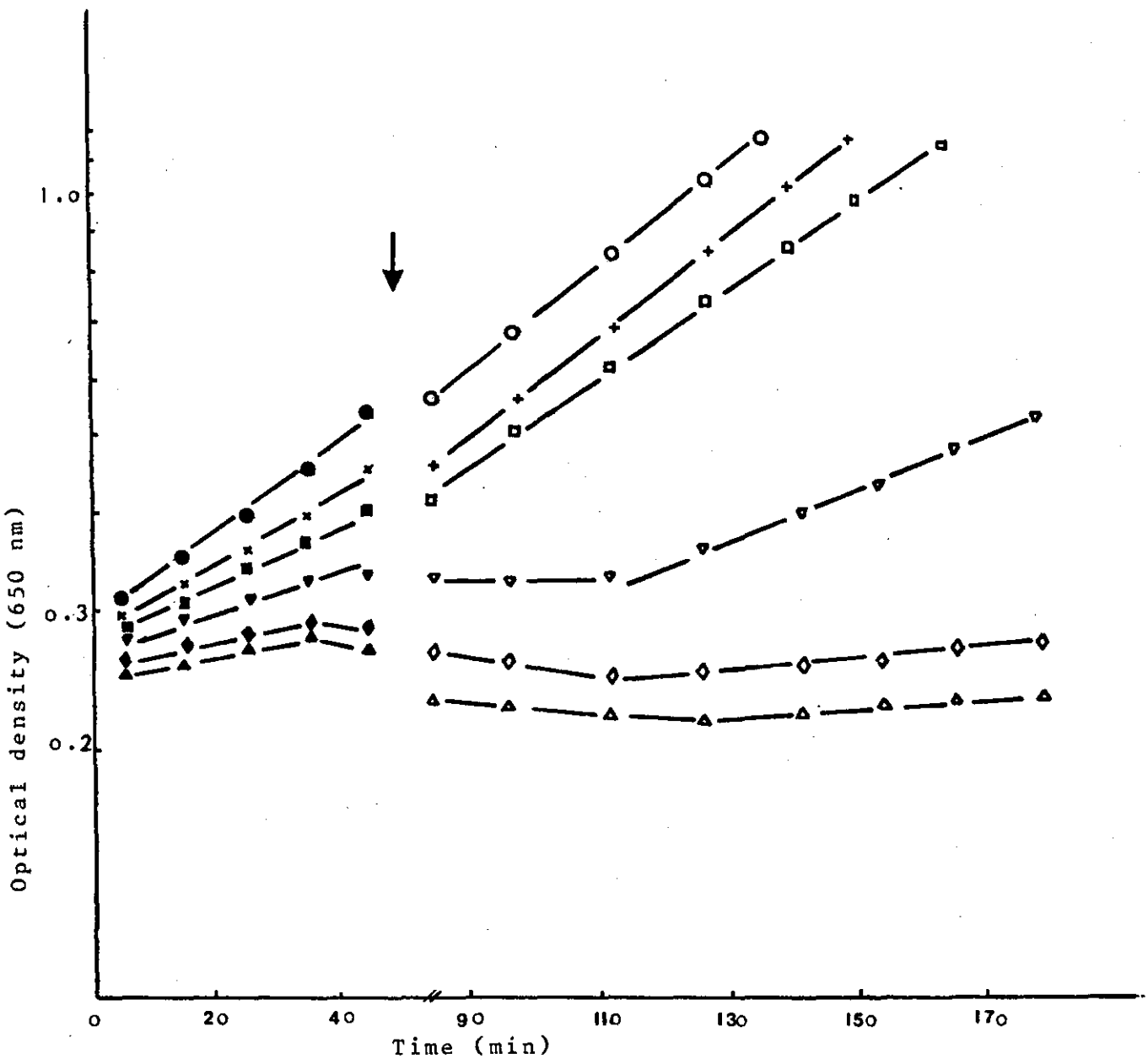

1.0
0.3
0.2
Optical density (650 nm)
0
20
40
90
110
130
150
170
Time (min)

Fig 3.23 Changes with time in the optical density (650 nm) of cultures of E.coli initially suspended in CFM containing different concentrations of cinchocaine after the addition of glucose at zero time and after resuspension in the same volume of the complete growth medium (at 80 min). Initial contact time (prior to addition of glucose) 10 min. Incubation temp 37°C. Cell concentration 3.8 x 10^8 cells.ml^{-1}.
Cinchocaine concentration (mg.ml^{-1}): ●—●, o—o, o.o; x—x, +—+, 0.20; ■—■, □—□, 0.30; ▼—▼, ▽—▽, 0.40; ◆—◆, ◇—◇, 0.60.
(lower symbol represents changes measured after resuspension).

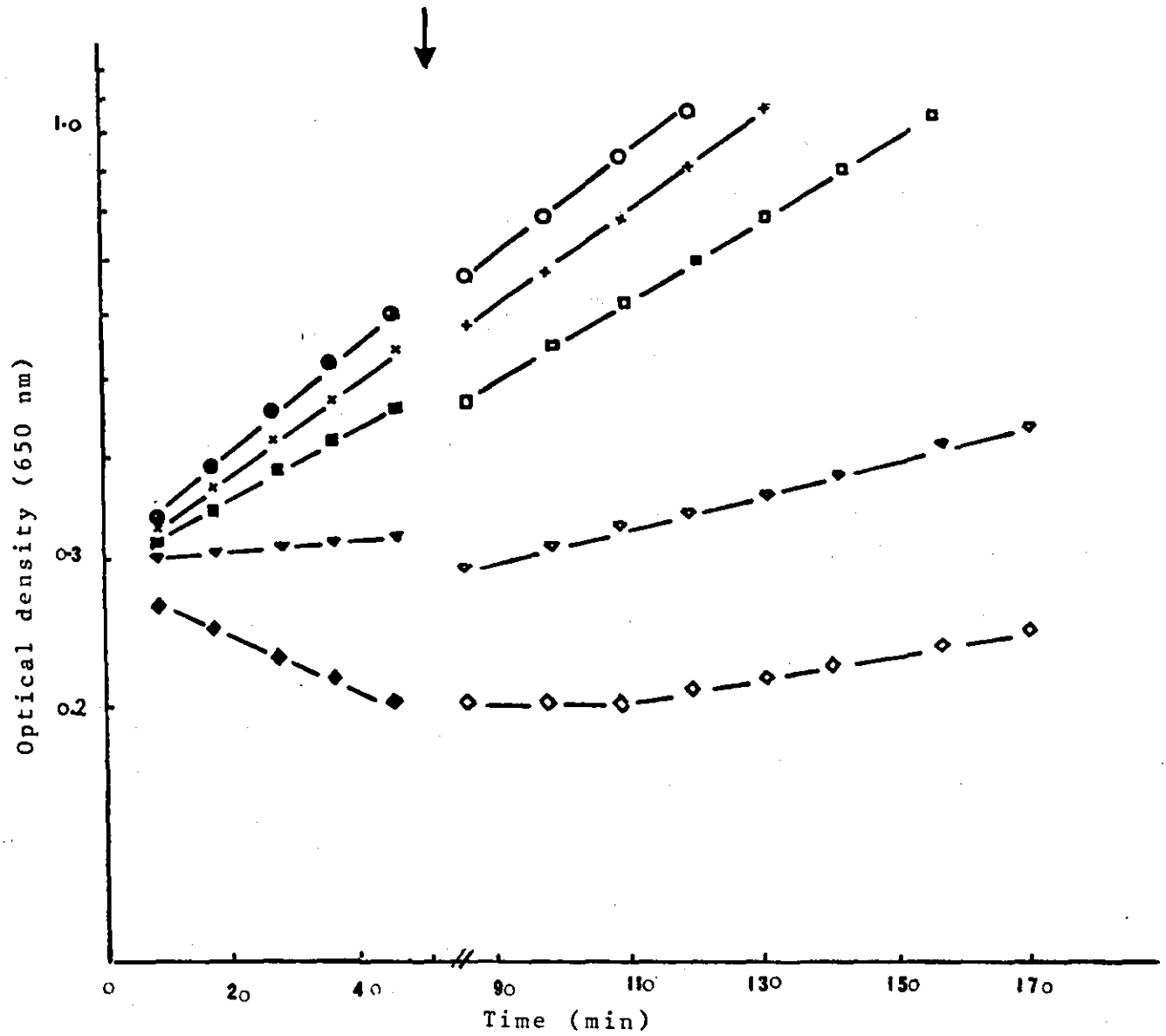

Optical density (650 nm)
1·0
0·3
0·2
0
2o
4o
9o
11o
13o
15o
17o
Time (min)

Fig 3.24 Changes with time in the optical density (650 nm) of cultures of E.coli initially suspended in CFM containing different concentrations of lignocaine after the addition of glucose at zero time and after resuspension in the same volume of the complete growth medium (at 80 min).

Initial contact time (prior to addition of glucose) 10 min. Incubation temp 37°C. Cell concentration 3.8×10^8 cells. ml^{-1}.

Lignocaine concentration ($mg.ml^{-1}$): ●—●, 0.0; x—x, o—o, +—+, 10.0; ■—■, □—□, 15.0; ▼—▼, ▽—▽, 20.0; ◆—◆, ◇—◇, 25.0.

(lower symbol represents changes measured after resuspension).

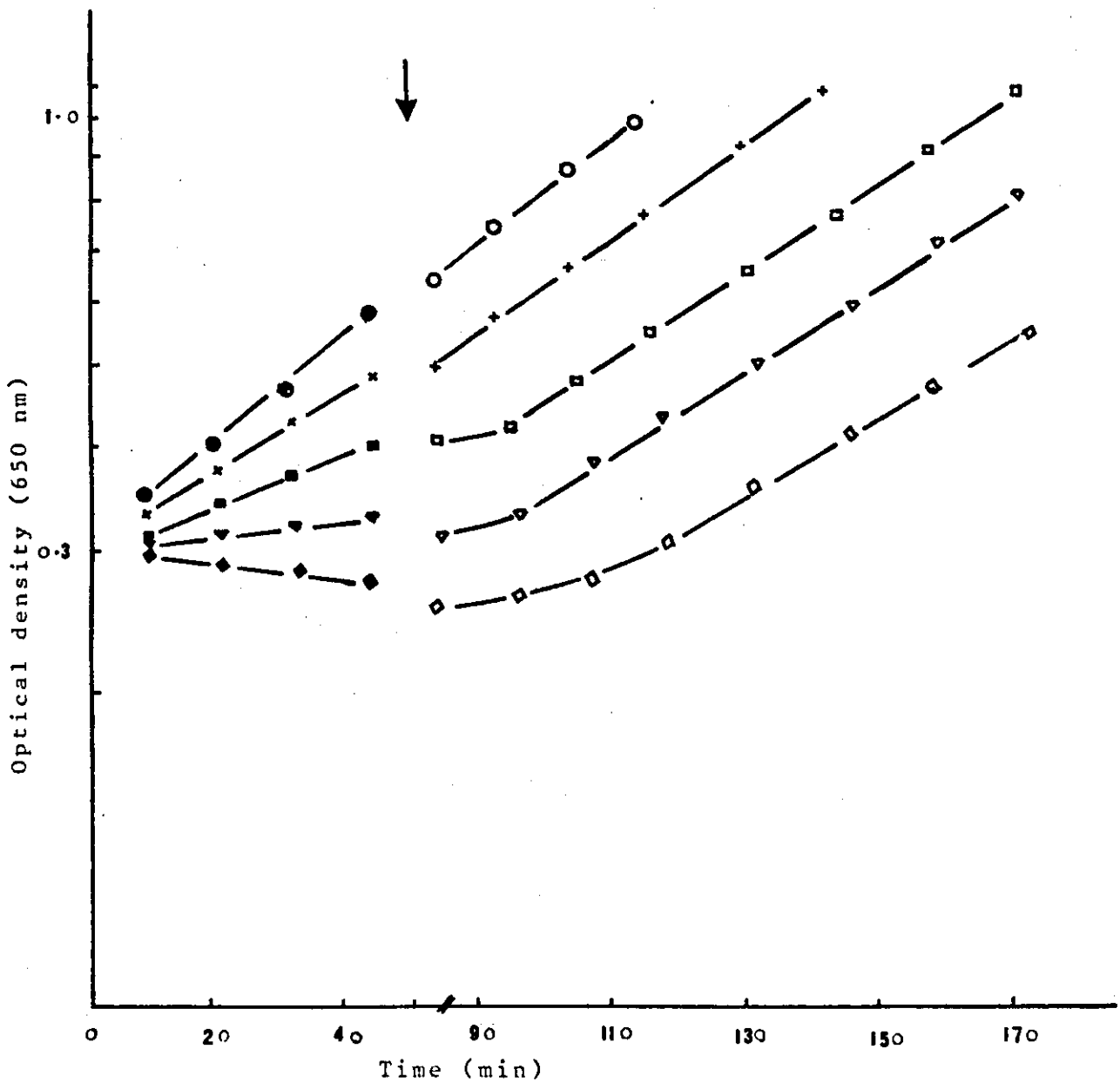

1·0
0·3
Optical density (650 nm)
0
20
40
90
110
130
150
170
Time (min)

Fig 3.25 Changes with time in the optical density (650 nm) of cultures of E.coli initially suspended in CFM containing different concentrations of amethocaine, after the addition of glucose (at zero time) and after the dilution with an equal volume of complete growth medium (at 55 min). Initial contact time (prior to addition of glucose) 10 min. Temp 37°C. Cell concentration 3.8×10^8 cells.ml^{-1}.

Initial amethocaine concentrations (mg.ml^{-1}): ●—●, 0.0; ×—×, 0.50; ■—■, 0.75; ▼—▼, 1.00; ◆—◆, 1.25; ▲—▲, 1.50 (lower symbol represents changes measured after dilution).

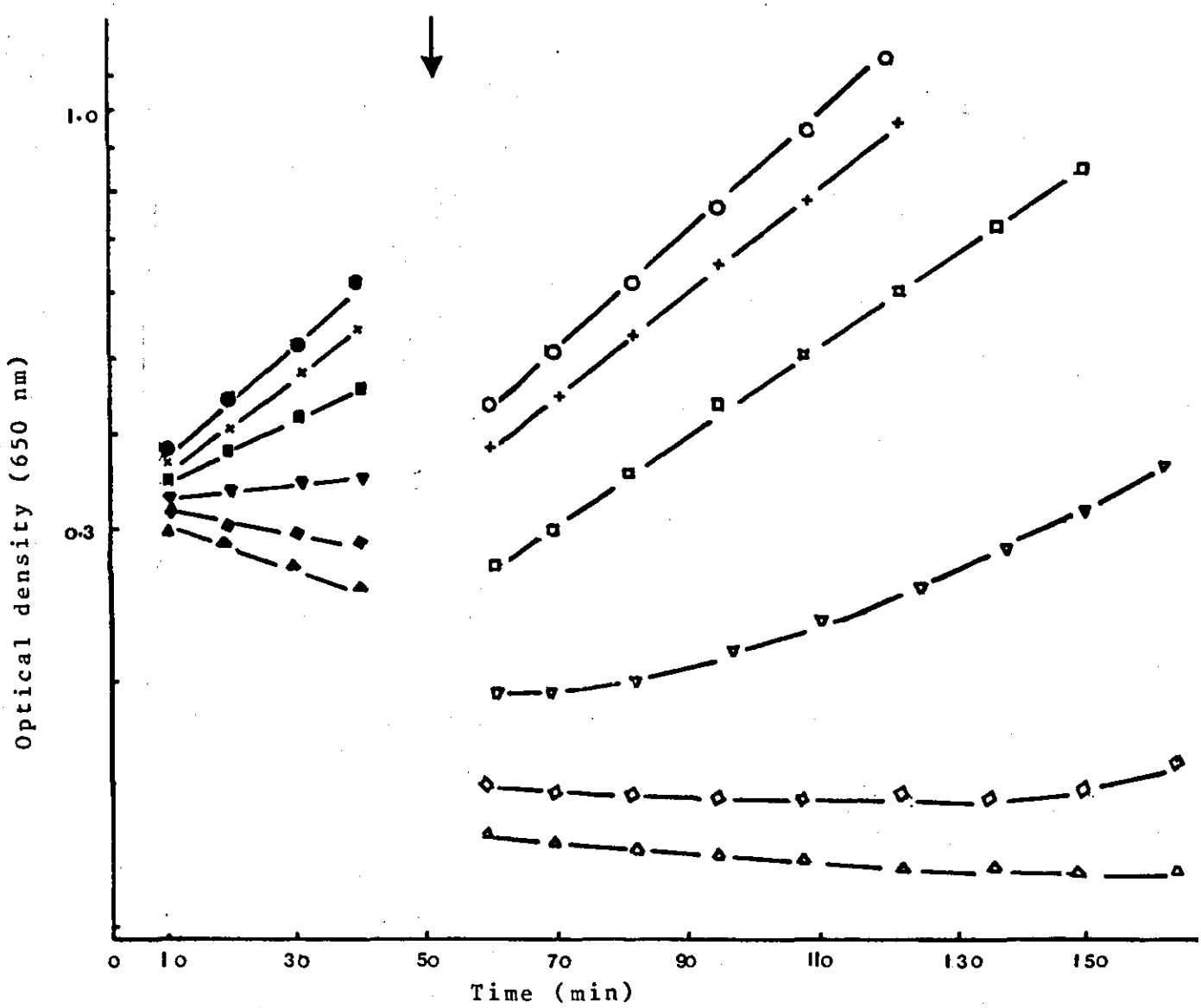

1.0
0.3
Optical density (650 nm)
0
10
30
50
70
90
110
130
150
Time (min)

Fig 3.26 Diauxic growth of E.coli in growth medium containing glucose-galactose (0.15 $mg.ml^{-1}$) with amethocaine added 10 min before the addition of carbohydrates. Initial cell concentration 3.8×10^8 $cells.ml^{-1}$. Temp $37^{o}C$.

Amethocaine concentration ($mg.ml^{-1}$) o——o, 0.0; x——x, 0.25; □——□, 0.50; Δ——Δ, 0.75; :——:, 1.00; +——+, 1.50.

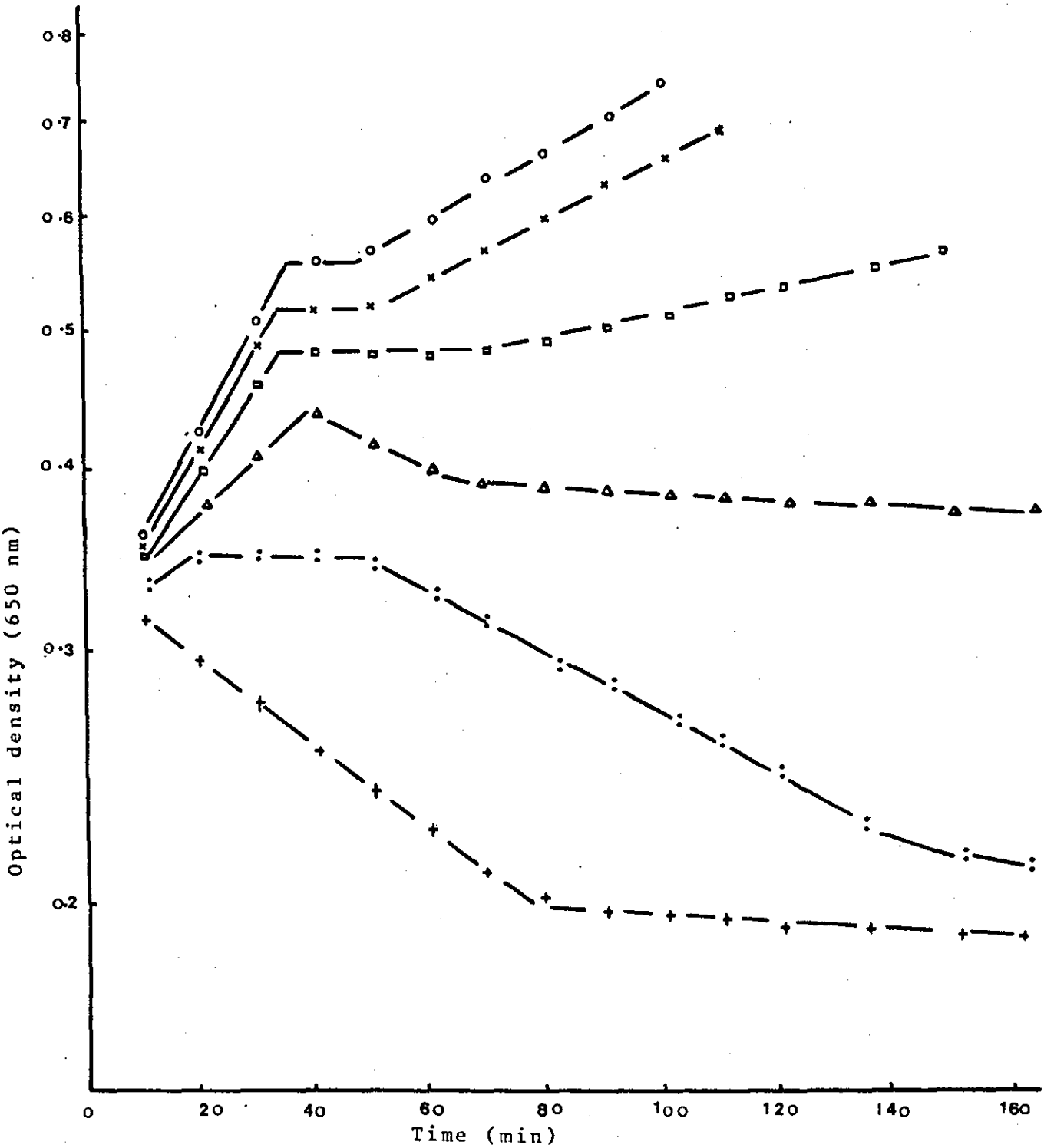

0·8
0·7
0·6
0·5
0·4
0·3
0·2
Optical density (650 nm)
0
20
40
60
80
100
120
140
160
Time (min)

Fig 3.27 Diauxic growth of E.coli in growth medium containing glucose-galactose (0.15 mg.ml^{-1}) with procaine added 10 min before the addition of carbohydrates. Initial cell concentration 3.8 x 10^8 cells.ml^{-1}. Temp 37^{o}C.

Procaine concentration (mg.ml^{-1}) o——o, 0.0; x——x, 10.0; □——□, 20.0; Δ——Δ, 25.0; :——:, 30.0; +——+, 35.0; ▼——▼, 40.0; ■——■, 50.0.

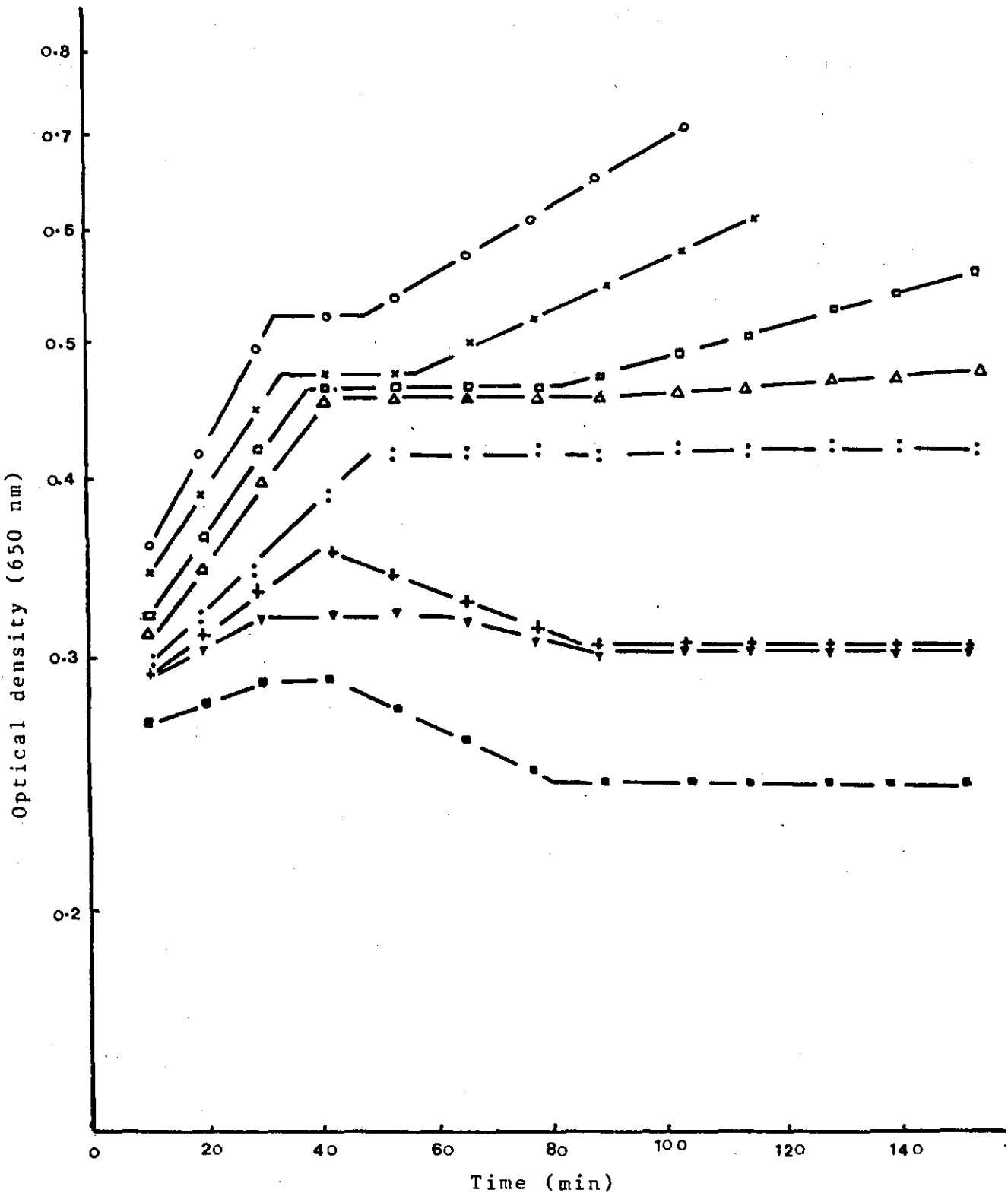

0·8
0·7
0·6
0·5
0·4
0·3
0·2
Optical density (650 nm)
0
20
40
60
80
100
120
140
Time (min)

Fig 3.28 Diauxic growth of E.coli in growth medium containing glucose-galactose (0.15 $mg.ml^{-1}$) with cinchocaine added 10 min before the addition of carbohydrates. Initial cell concentration 3,8 x 10^8 $cells.ml^{-1}$. Temp $37^{o}C$. Cinchocaine concentration ($mg.ml^{-1}$): o——o, 0.0; ▲——▲, 0.20; x——x, 0.30; ▫——▫, 0.40; Δ——Δ, 0.60; :——:, 0.80; ▼——▼, 1.00.

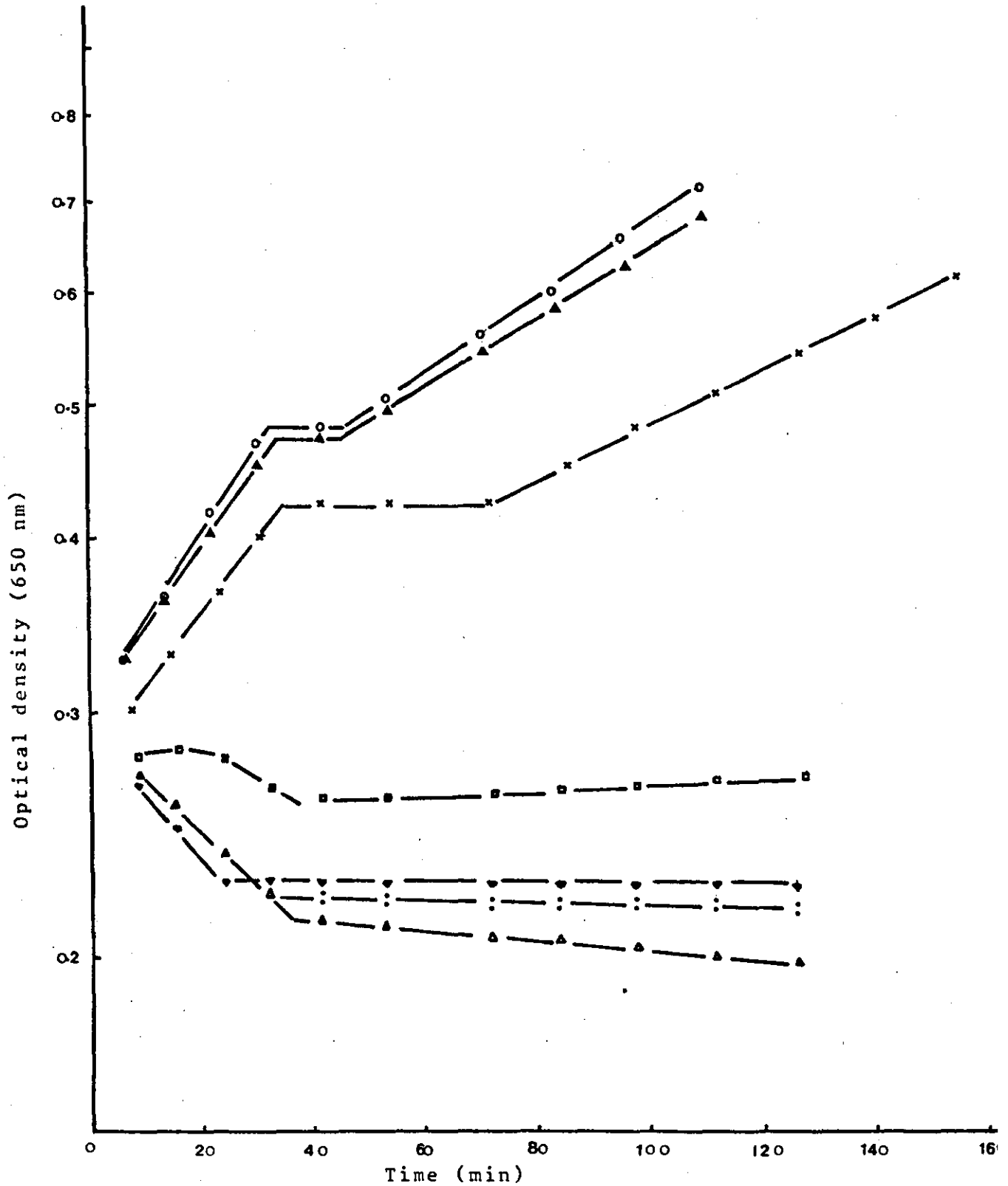

Optical density (650 nm)
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0
20
40
60
80
100
120
140
Time (min)

Fig 3.29 Diauxic growth of E.coli in growth medium containing glucose-galactose (0.15 mg.ml^{-1}) with lignocaine added 10 min before the addition of carbohydrates. Initial cell concentration 3.8 x 10^8 cells.ml^{-1}. Temp 37^{o}C. Lignocaine concentration (mg.ml^{-1}):
o——o, 0.0; x——x, 10.0; ▫——▫, 15.0; Δ——Δ, 20.0;
:——:, 25.0; ▼——▼, 30.0; +——+, 40.0.

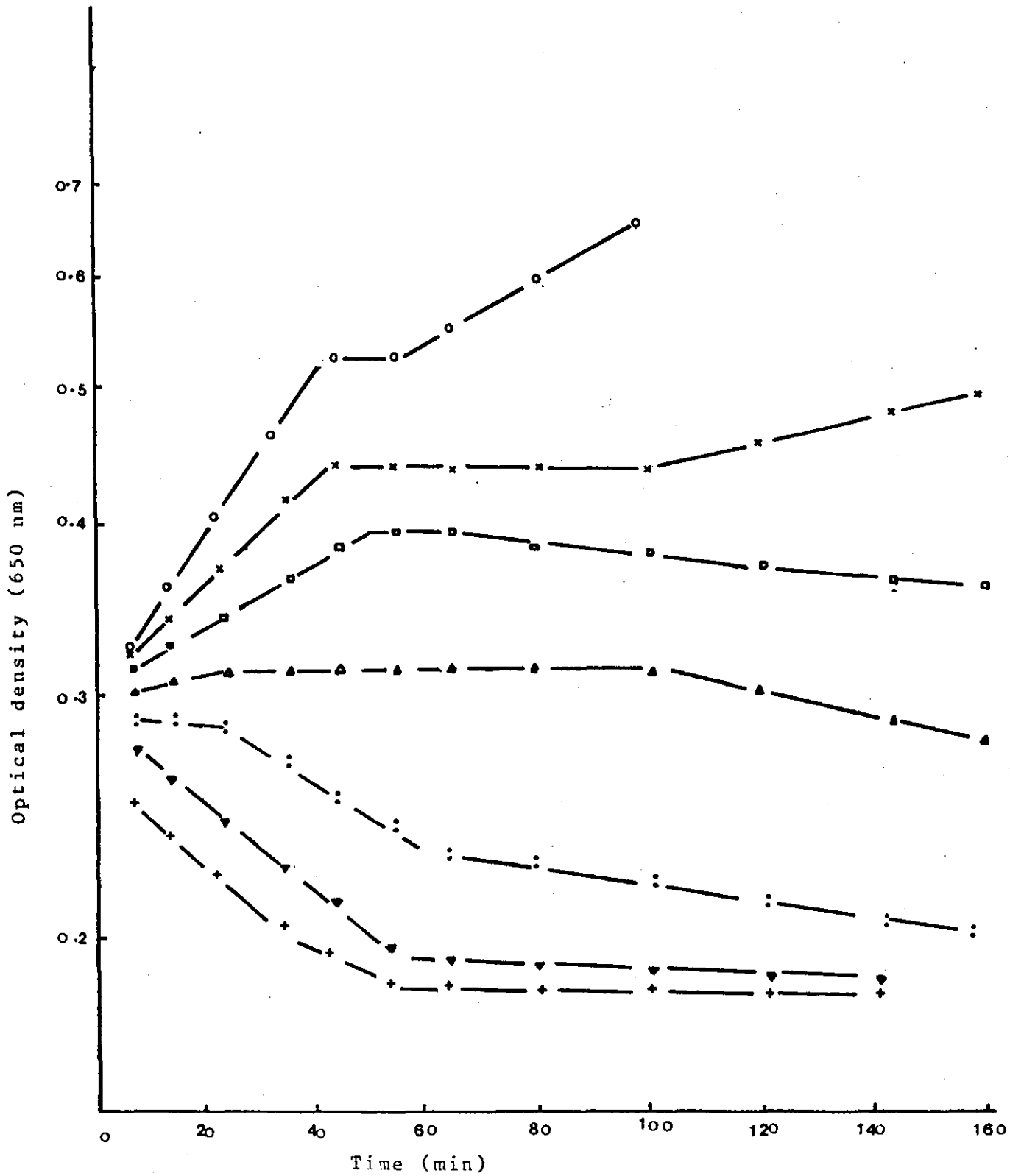

Optical density (650 nm)
0·7
0·6
0·5
0·4
0·3
0·2
0
20
40
60
80
100
120
140
160
Time (min)

Fig 3.30 Diauxic growth of E.coli in growth medium containing glucose-galactose (0.15 mg.ml^{-1}) with chloramphenicol added 10 min before the addition of carbohydrates. Initial cell concentration 3.8 x 10^8 cells.ml^{-1}. Temp 37^{o}C. Chloramphenicol concentration (μg.ml^{-1}): o—o, 0.0; x—x, 2.0; □—□, 4.0; Δ—Δ, 8.0; :—:, 10.0.

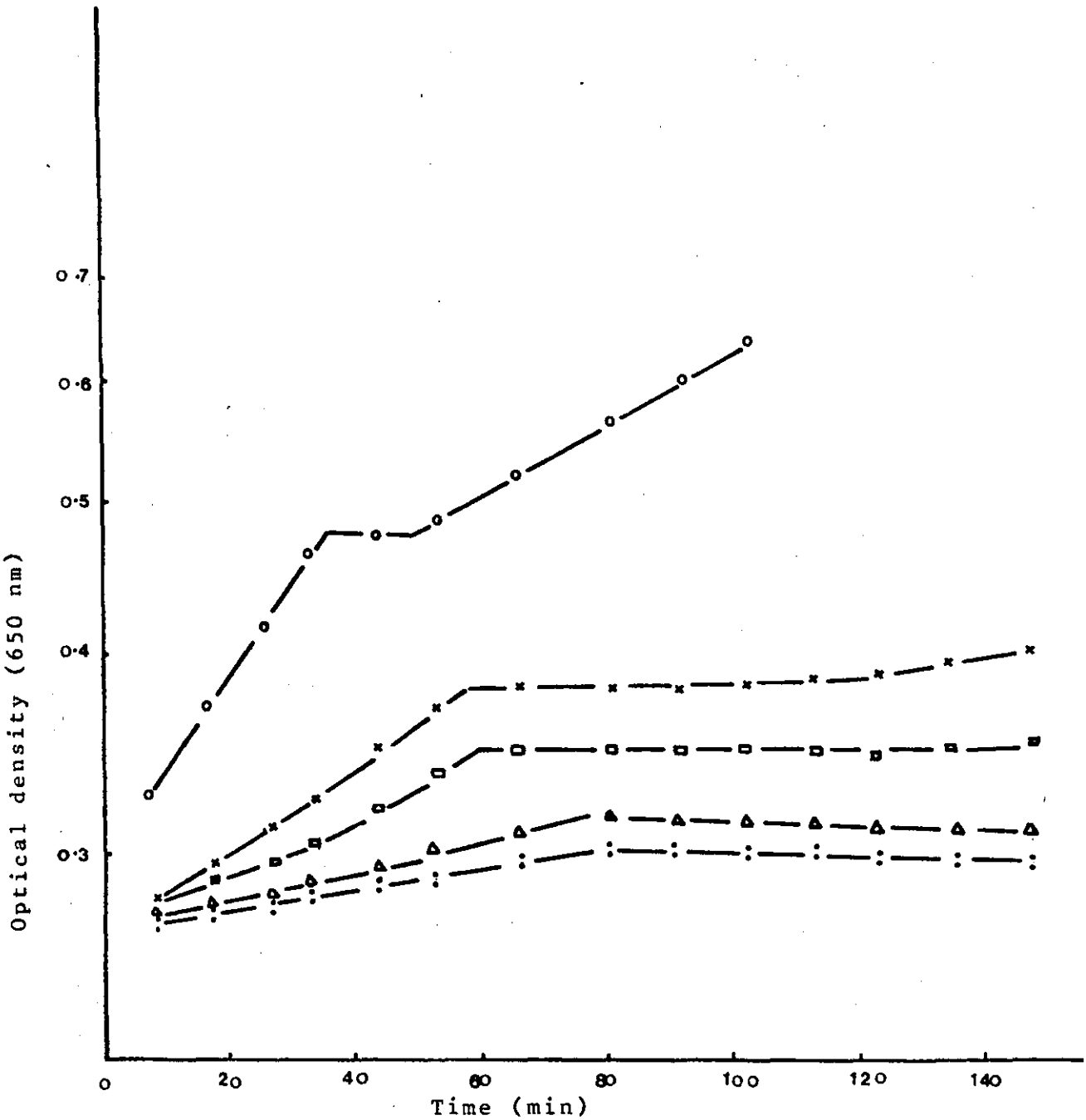

Optical density (650 nm)
0·7
0·6
0·5
0·4
0·3
0
20
40
60
80
100
120
140
Time (min)

Fig 3.31 Diauxic growth of E.coli in growth medium containing glucose-galactose (0.15 mg.ml^{-1}) with ampicillin added 10 min before the addition of carbohydrates. Initial cell concentration 3.8 x 10^8 cells.ml^{-1}. Temp 37^{o}C. Ampicillin concentration (µg.ml^{-1}): o——o, 0.0; x——x, 1.0; □——□, 2.5; Δ——Δ, 3.0; :——:, 4.0; ▼——▼, 5.0; +——+, 7.5.

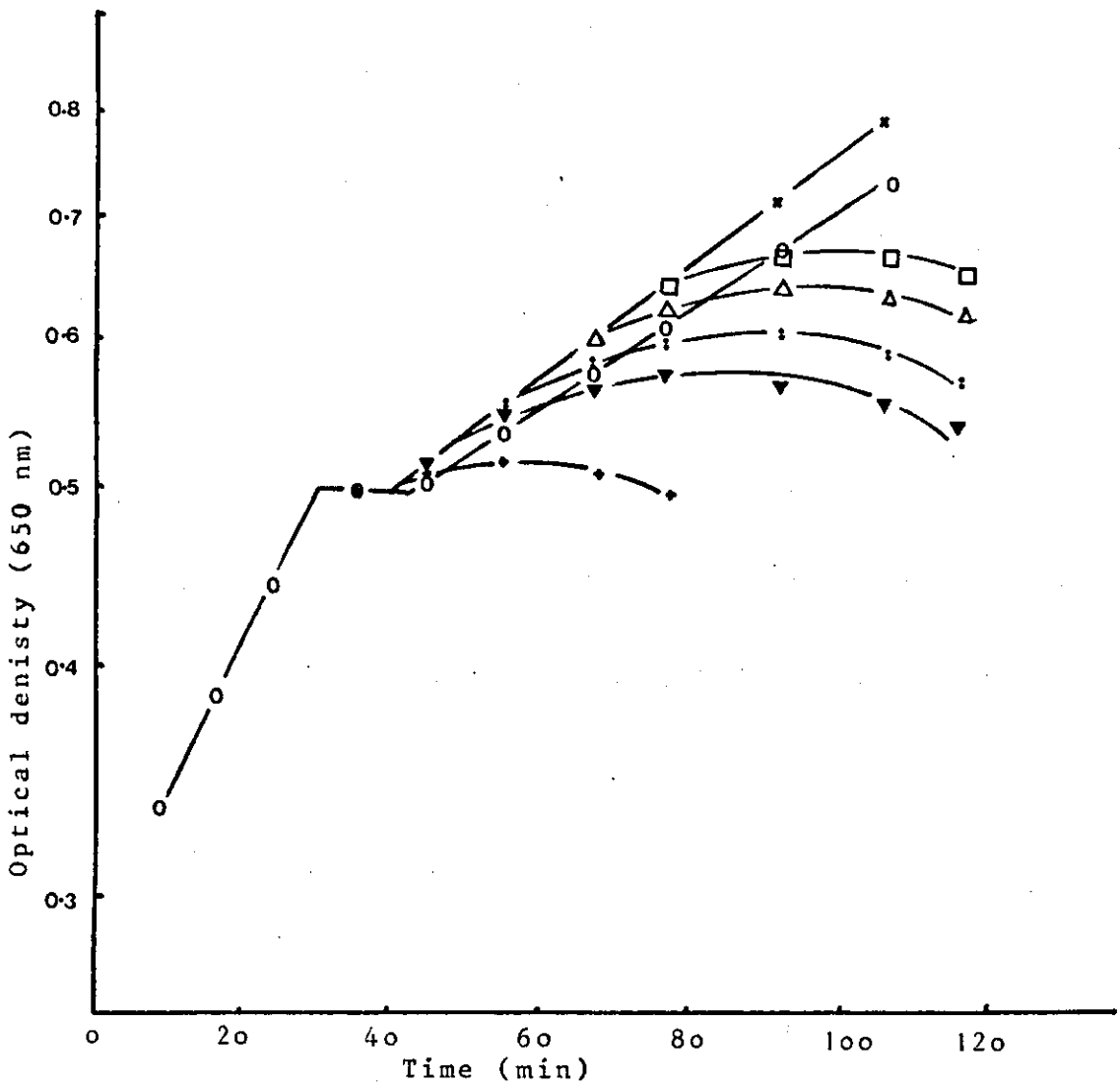

0·8
0·7
0·6
0·5
0·4
0·3
Optical denisty (650 nm)
0
2o
4o
6o
8o
1oo
12o
Time (min)

Fig 3.32 Diauxic growth of E.coli in growth medium containing glucose-galactose (0.15 $mg.ml^{-1}$) with cetylpyridinium bromide (CPB) added 10 min before the addition of carbohydrates. Initial cell concentration 3.8×10^8 $cells.ml^{-1}$. Temp $37^{o}C$.

CPB concentration ($\mu g.ml^{-1}$): o——o, 0.0 and 2.0; x—— x, 3.0; □——□, 4.0; Δ——Δ, 6.0; :——:, 10.0; ▼——▼, 15.0.

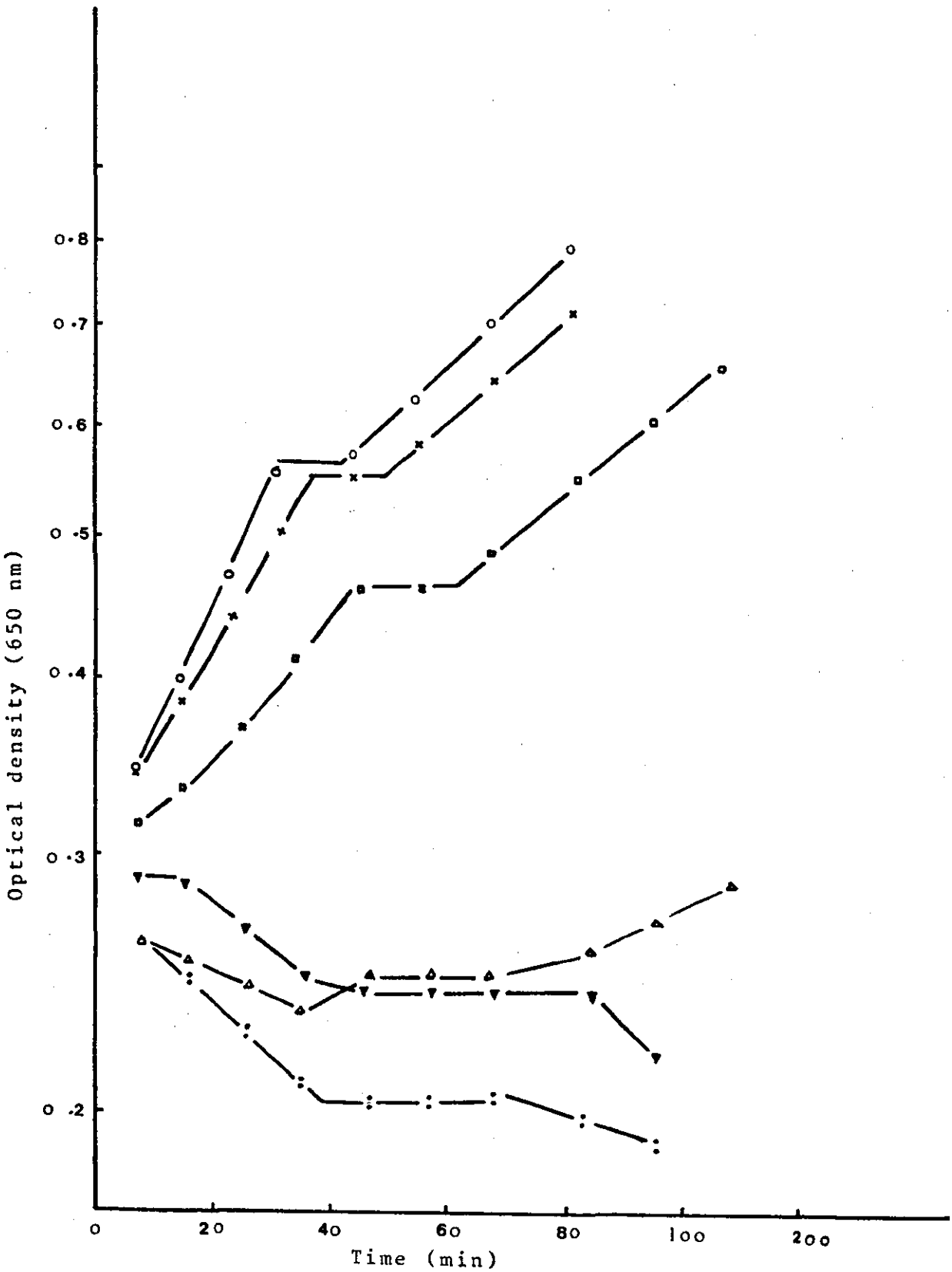

Optical density (650 nm)
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0
20
40
60
80
100
200
Time (min)

Fig 3.33 Diauxic growth of E.coli in growth medium containing glucose-galactose (0.15 mg.ml^{-1}) with amethocaine added 28 min after the addition of carbohydrates.
Initial cell concentration: 3.8 x 10^8 cells.ml^{-1}.
Temp 37^{o}C.
Amethocaine concentration (mg.ml^{-1}) o—o, 0.0; x—x, 0.25; □—□, 0.5; Δ—Δ, 0.75; :—:, 1.0; +—+, 1.5.

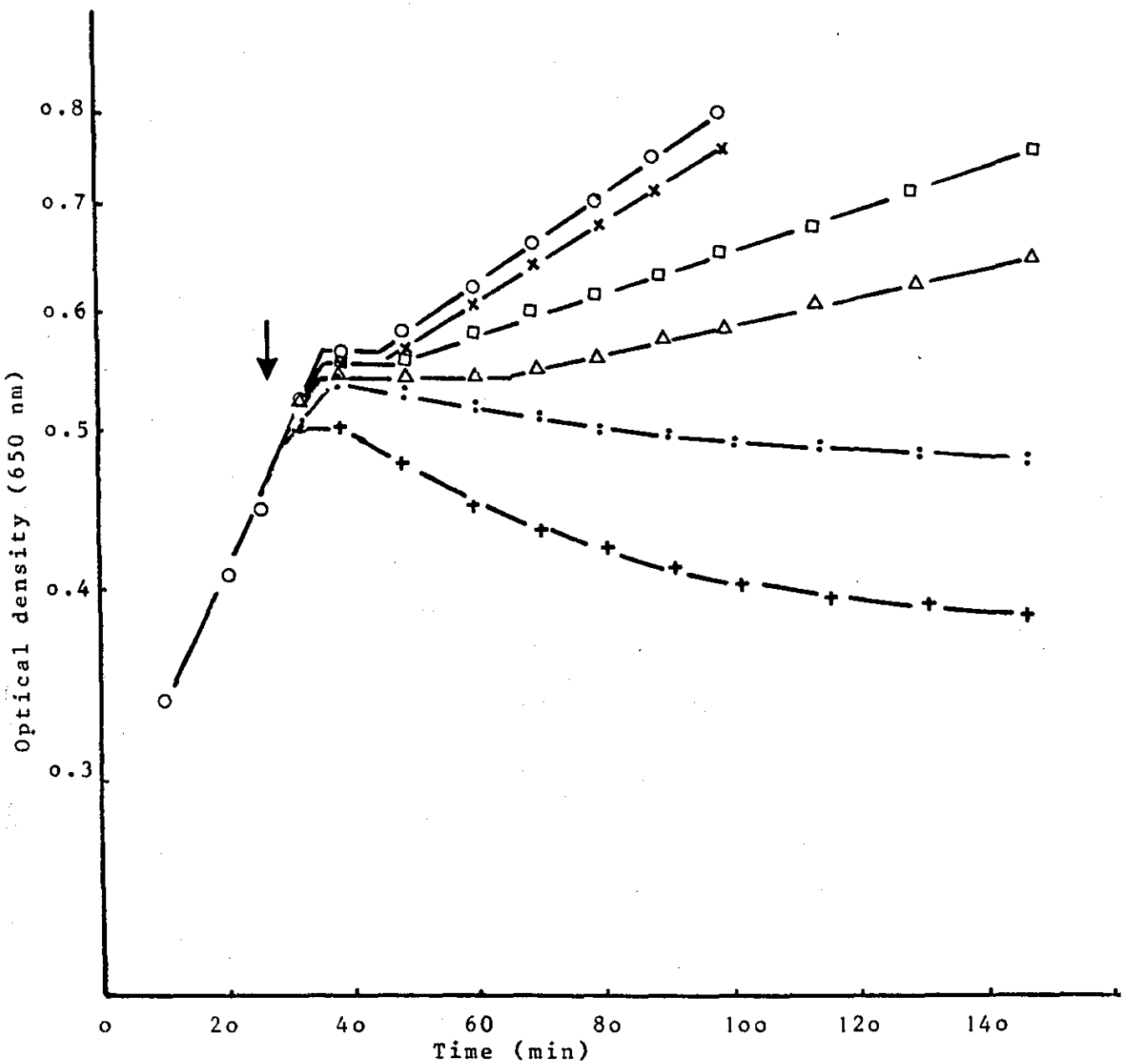

0.8
0.7
0.6
0.5
0.4
0.3
Optical density (650 nm)
0
20
40
60
80
100
120
140
Time (min)

Fig 3.34 Diauxic growth of E.coli in growth medium containing glucose-galactose (0.15 $mg.ml^{-1}$) with procaine added 28 min after addition of carbohydrates. Initial cell concentration: 3.8×10^8 $cells.ml^{-1}$. Temp $37^{o}C$. Procaine concentration ($mg.ml^{-1}$): o—o, 0.0; x—x, 10.0; □—□, 20.0; ▲—▲, 25.0; :—:, 30.0; +—+, 35.0.

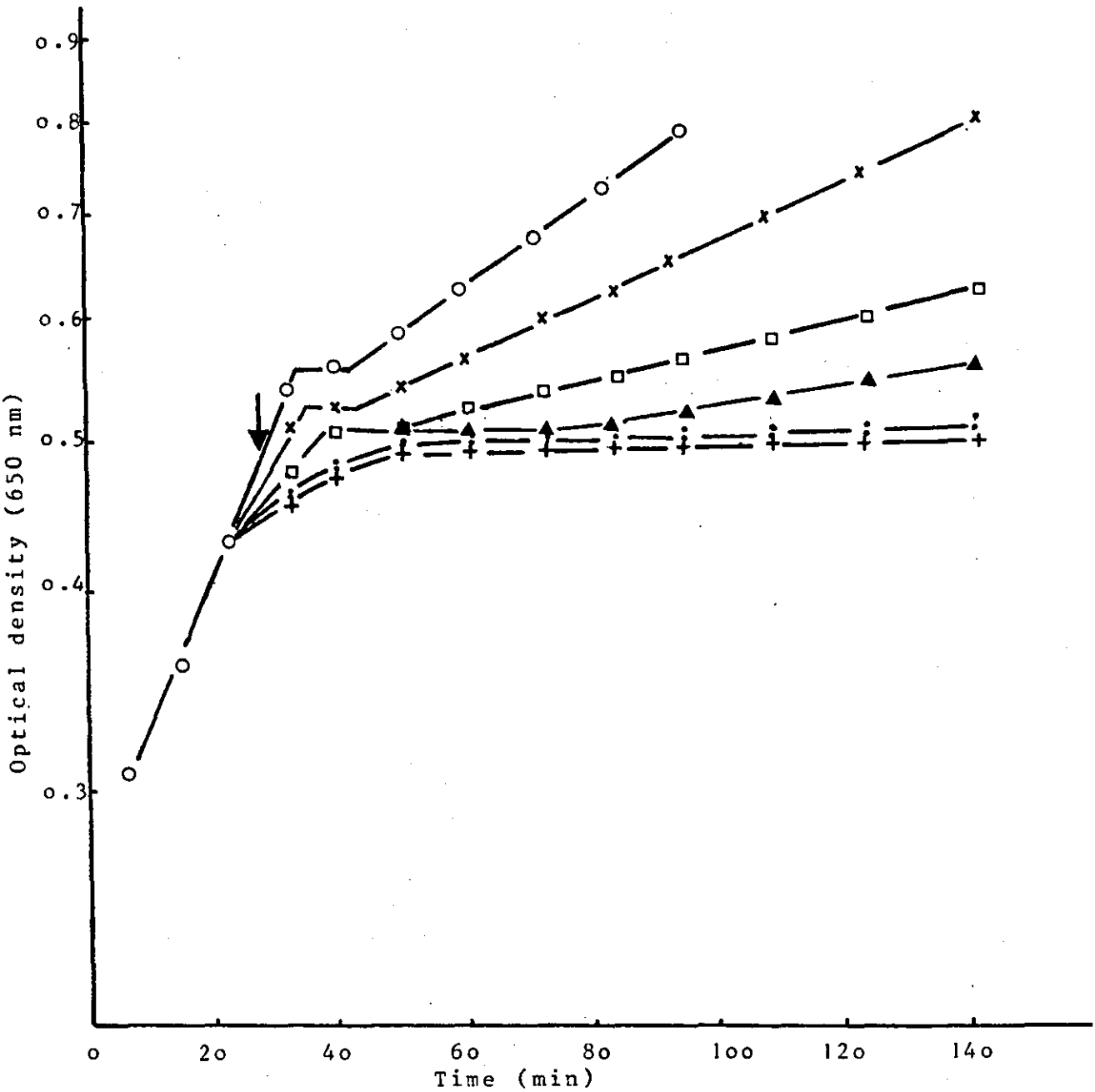

0.9
0.8
0.7
0.6
0.5
0.4
0.3
Optical density (650 nm)
0
20
40
60
80
100
120
140
Time (min)

Fig 3.35 Diauxic growth of E.coli in growth medium containing glucose-galactose (0.15 mg.ml^{-1}) with cinchocaine added 30 min after addition of carbohydrates. Initial cell concentration: 3.8 x 10^8 cells.ml^{-1}. Temp 37^{o}C. Cinchocaine concentration (mg.ml^{-1}): o—o, 0.0 and 0.10; x—x, 0.30; □——□, 0.40; Δ—Δ 0.50; :—:, 0.60.

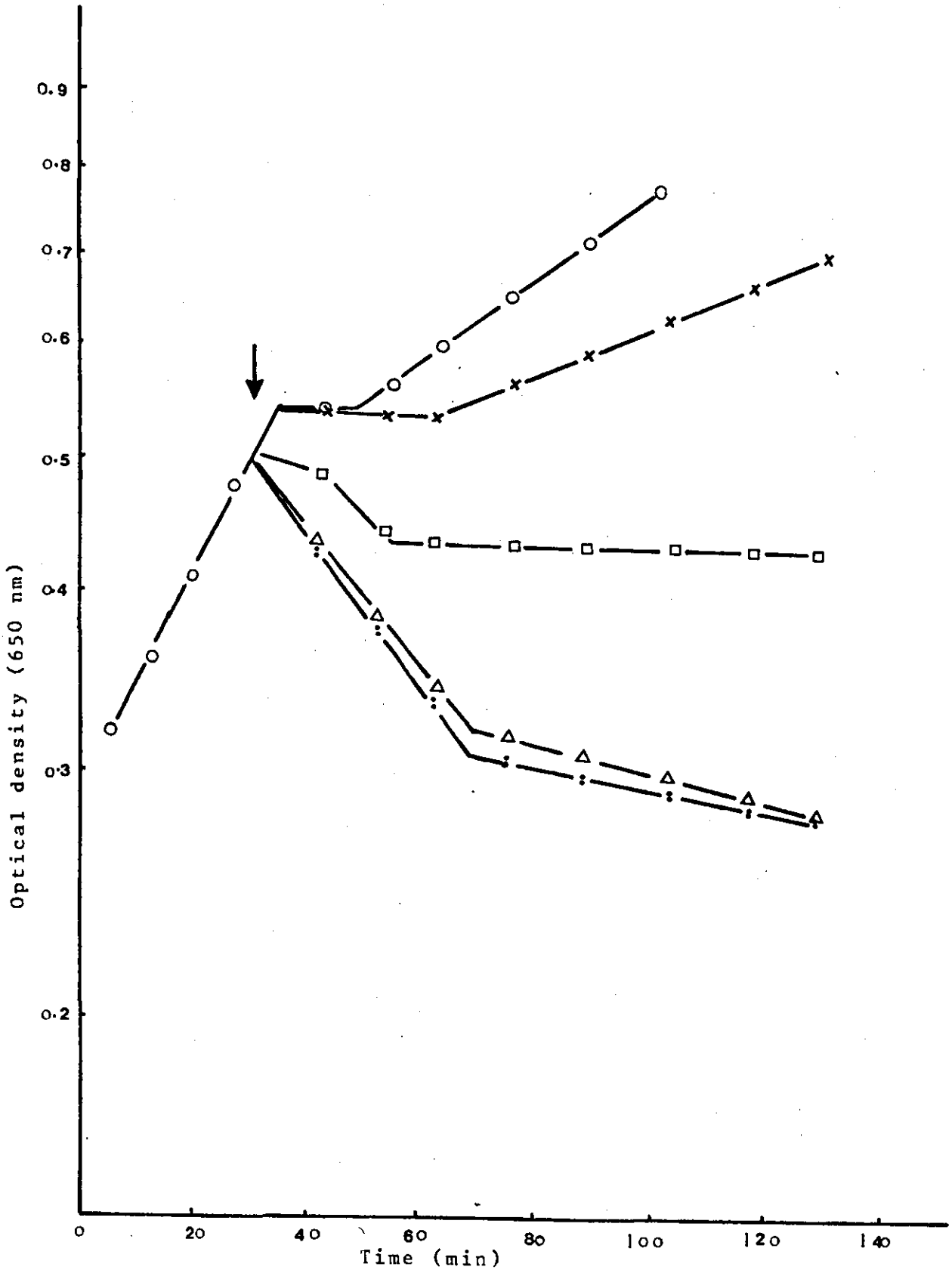
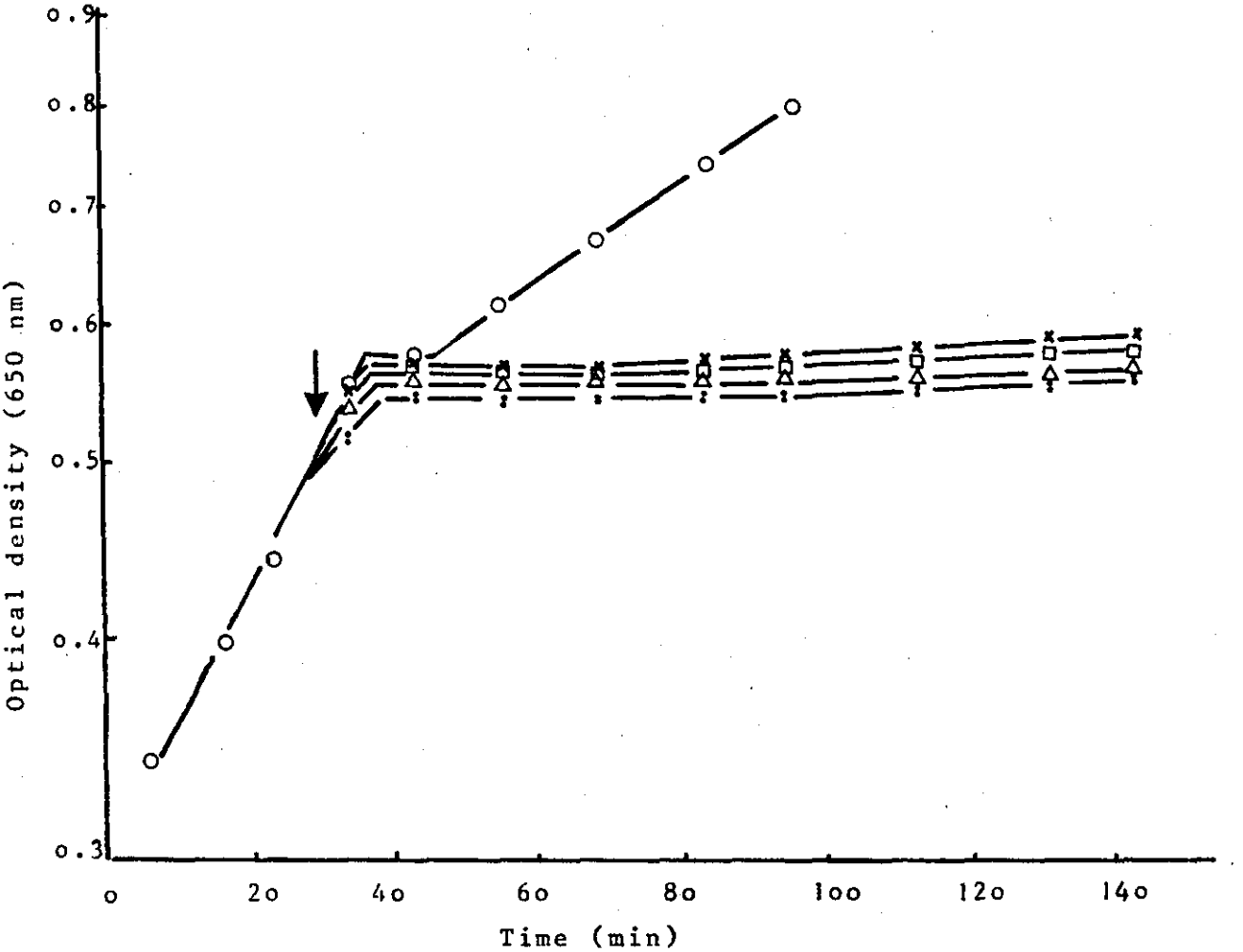

0.9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
Optical density (650 nm)
0
20
40
60
80
100
120
140
Time (min)

Fig 3.36 Diauxic growth of E.coli in growth medium containing glucose-galactose (0.15 $mg.ml^{-1}$) with chloramphenicol added 30 min after the addition of carbohydrates. Initial cell concentration: 3.8×10^8 $cells.ml^{-1}$. Temp 37°C. Chloramphenicol concentration ($\mu g.ml^{-1}$): o—o, 0.0; x—x, 2.5; □—□ 5.0; Δ—Δ 7.5 and 10.0; :—:, 15.0, 20.0 and 25.0.

Fig 3.37 Diauxic growth of E.coli in growth medium containing glucose-galactose (0.15 $mg.ml^{-1}$) with puromycin added 30 min after the addition of carbohydrates. Initial cell concentration: 3.8×10^8 $cells.ml^{-1}$. Temp 37°C Puromycin concentration ($\mu g.ml^{-1}$): o—o, 0.0; x—x, 100; □—□, 200 and 300; Δ—Δ500; :—: 600.


Optical density (650 nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0
20
40
60
80
100
120
140
Time (min)

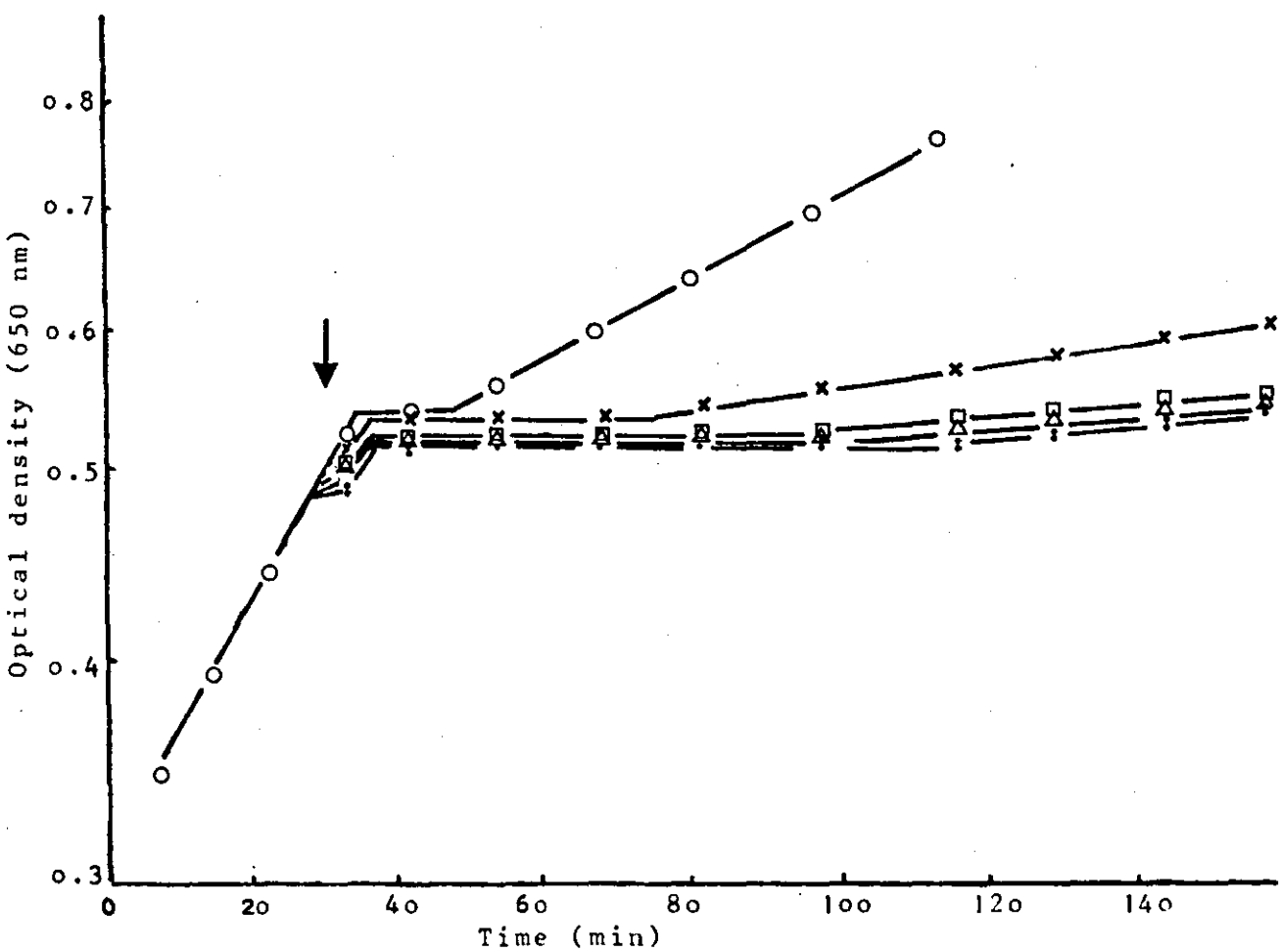
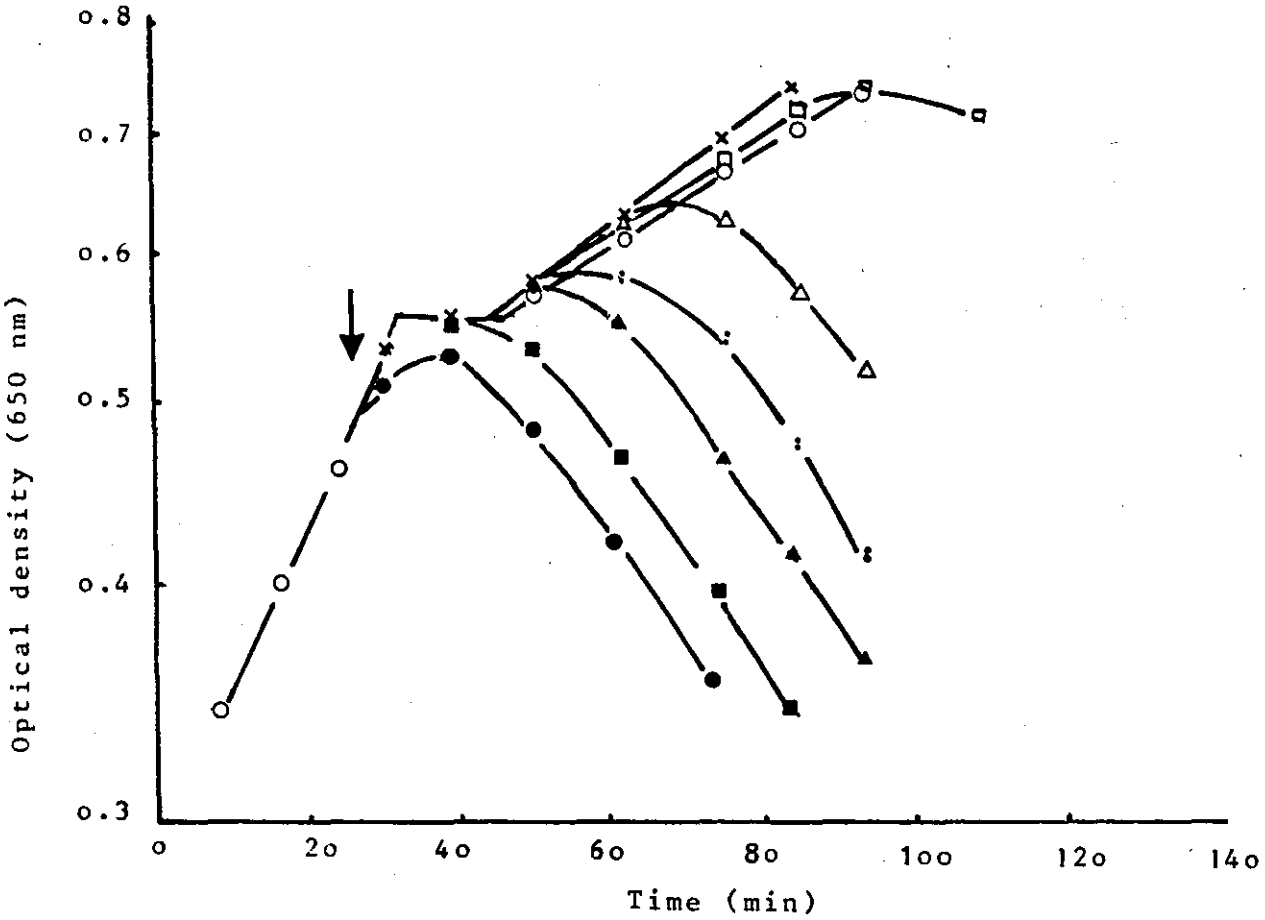

Optical density (650 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0
20
40
60
80
100
120
140
Time (min)

Fig 3.38 Diauxic growth of E.coli in growth medium containing glucose-galactose (o.15 mg.ml^{-1}) with ampicillin added 28 min after the addition of carbohydrates. Initial cell concentration: 3.8 x 10^8 cells.ml^{-1}. Temp 37^{o}C. Ampicillin concentration (µg.ml^{-1}): o——o, 0.0; x——x, 1.0;▫———▫, 2.5; Δ—Δ, 5.0; :——:, 7.5; ▲——▲, 10.0;■——■, 15.0; ●—●, 20.0.

Fig 3.39 Diauxic growth of E.coli in growth medium containing glucose-galactose (0.15 mg.ml^{-1}) with cetylpyridinium bromide added 28 min after the addition of carbohydrates. Initial cell concentration: 3.8 x 10^8 cells.ml^{-1}. Temp 37^{o}C.
Cetylpyridinium bromide concentration (µg.ml^{-1}): o—o, 0.0 and 2.0; x——x, 3.0;▫——▫, 4.0; Δ—Δ, 5.0; :——:, 6.0.


0.8
0.7
0.6
0.5
0.4
0.3
Optical density (650 nm)
0
20
40
60
80
100
120
140
Time (min)

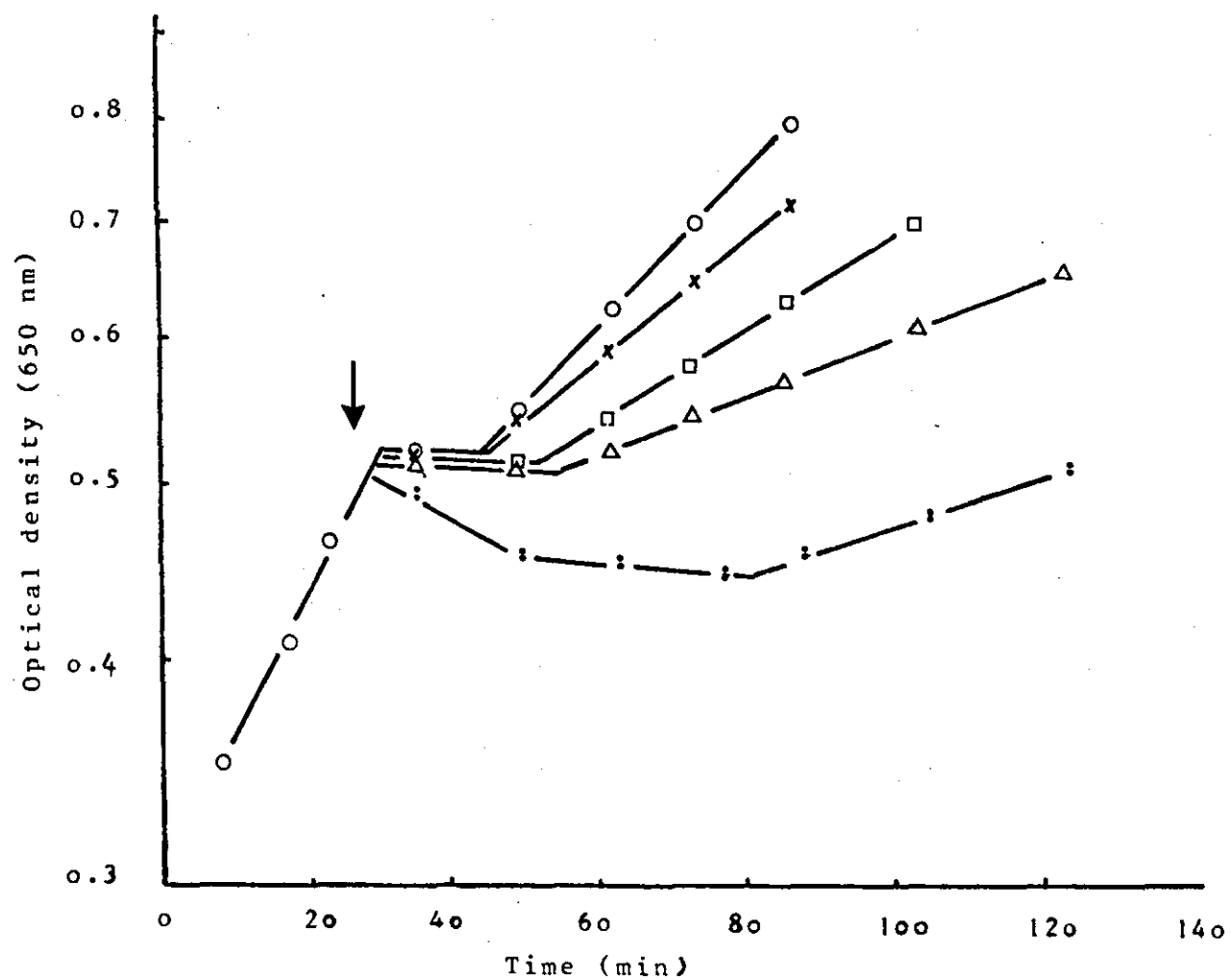
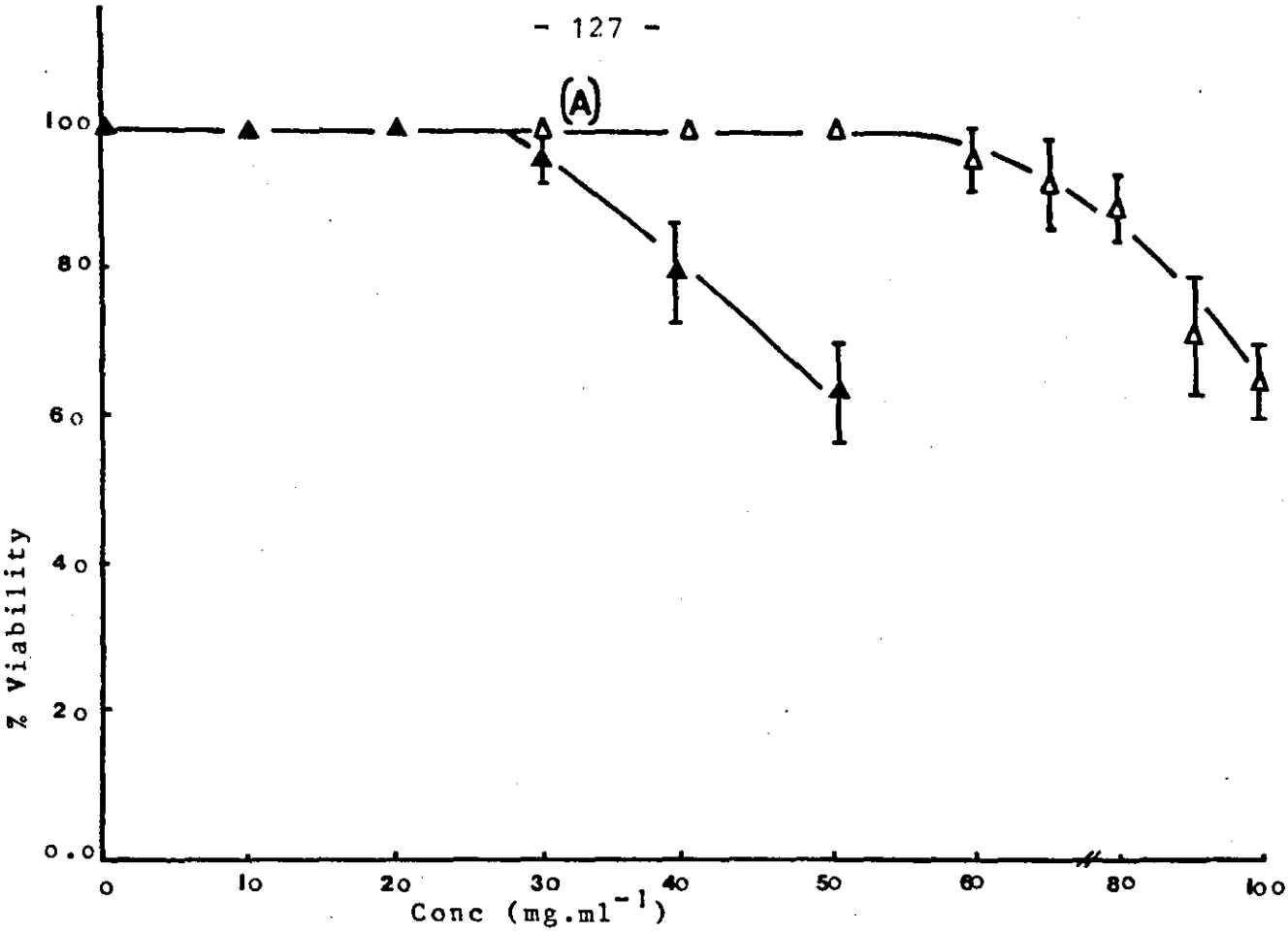

0.8
0.7
0.6
0.5
0.4
0.3
Optical density (650 nm)
0
20
40
60
80
100
120
140
Time (min)

Fig 3.40 The effect of (A) procaine Δ—Δ and lignocaine ▲—▲; (B) amethocaine, o—o and cinchocaine ●—● concentration on the percentage viability of cells of E.coli suspended in CFM. Cell concentration 3.8×10^8 cells.ml^{-1}. Contact time 10 min. Temp 22^oC.


(A)
100
80
60
40
20
0.0
% Viability
0
10
20
30
40
50
60
80
100
Conc (mg.ml^{-1})

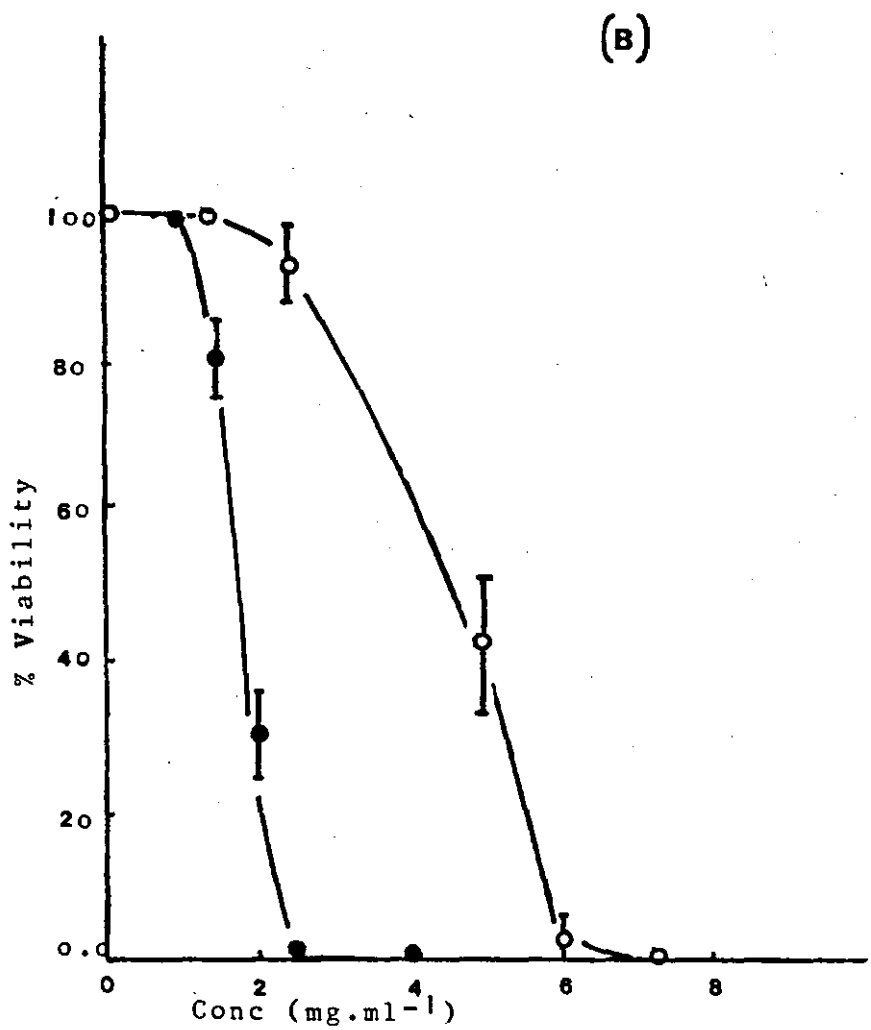
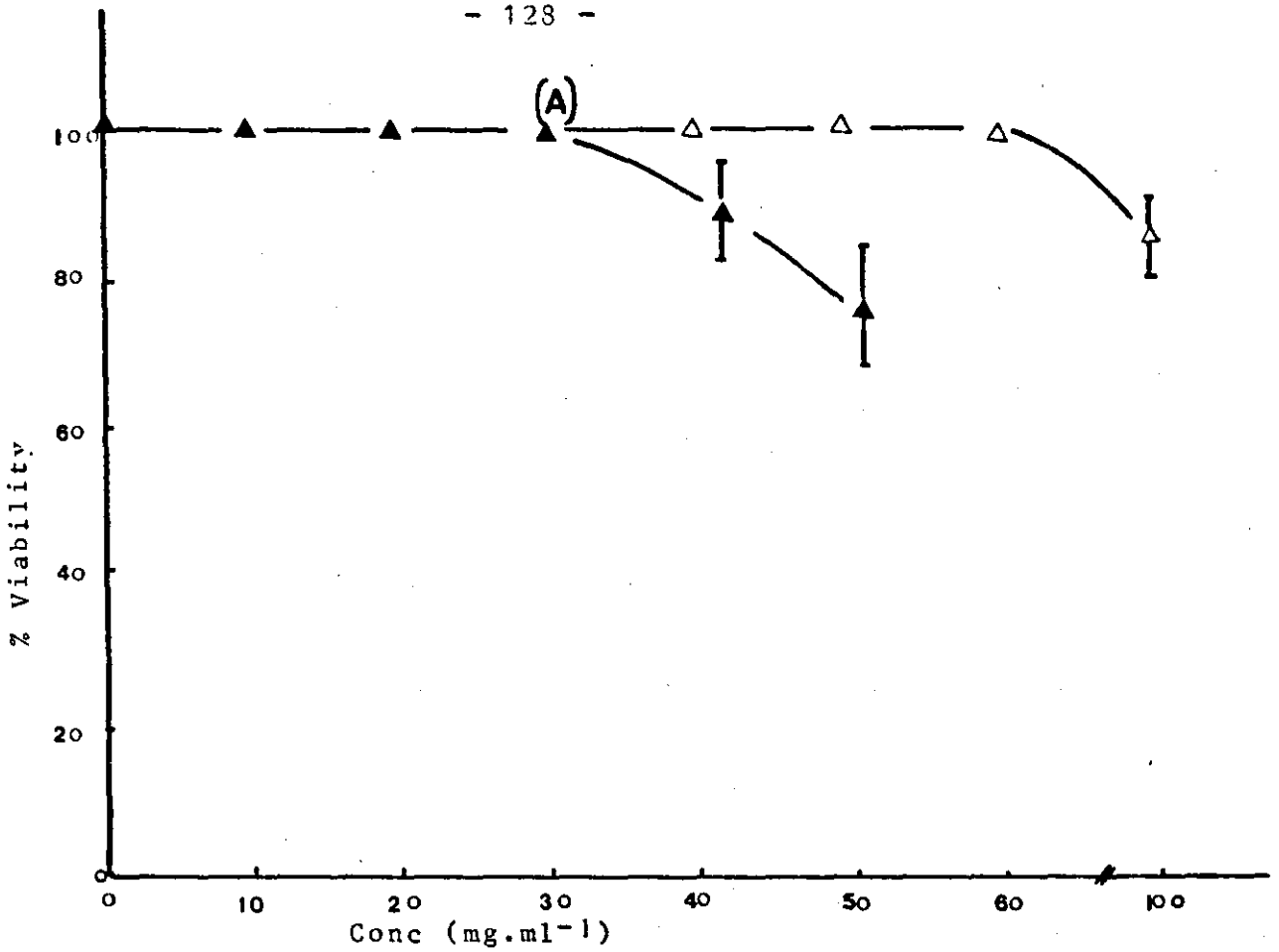

(B)
100
80
60
40
20
0.0
% Viability
0
2
4
6
8
Conc (mg.ml^{-1})

Fig 3.41 The effect of (A) procaine Δ——Δ and lignocaine ▲——▲; (B) amethocaine o——o and cinchocaine ●——● concentration on the percentage viability of cells of K.aerogenes suspended in CFM. Initial cell concentration 0.12 $mg.ml^{-1}$. Contact time 10 min. Temp $22^{o}C$.


(A)
% Viability
100
80
60
40
20
0
0
10
20
30
40
50
60
100
Conc (mg.ml⁻¹)

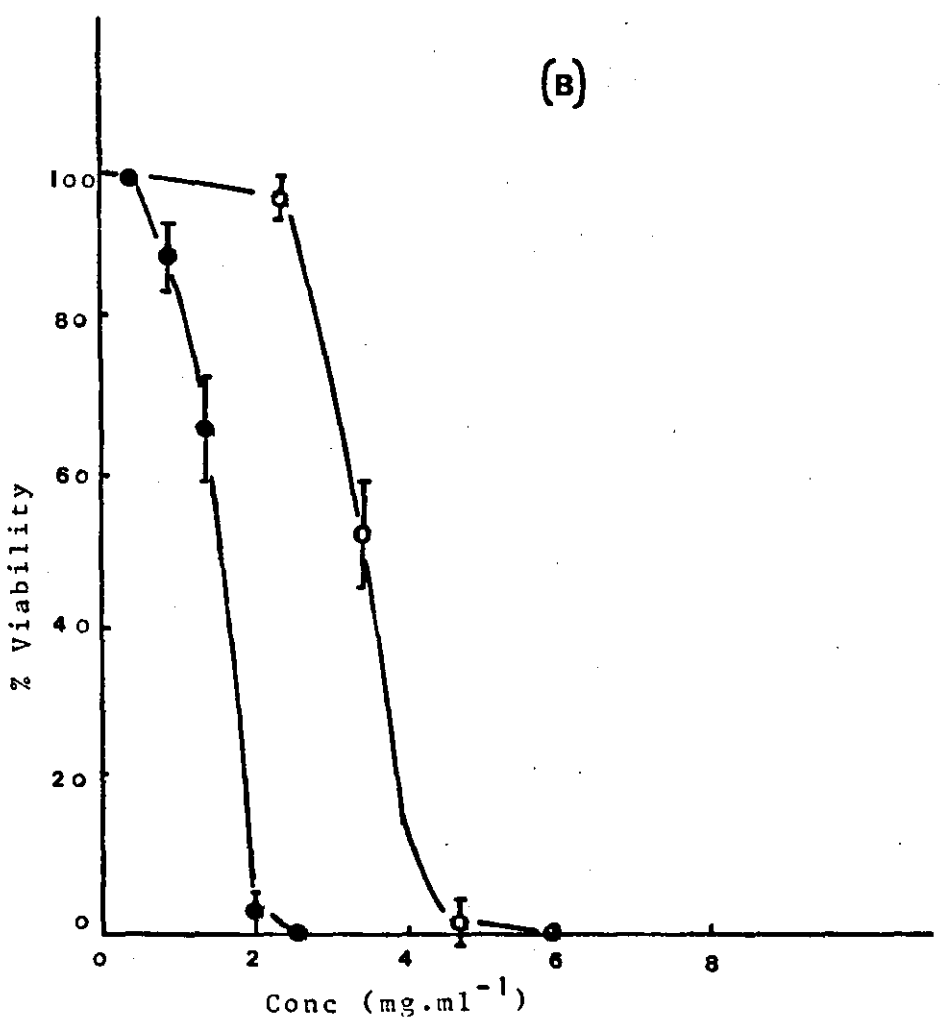
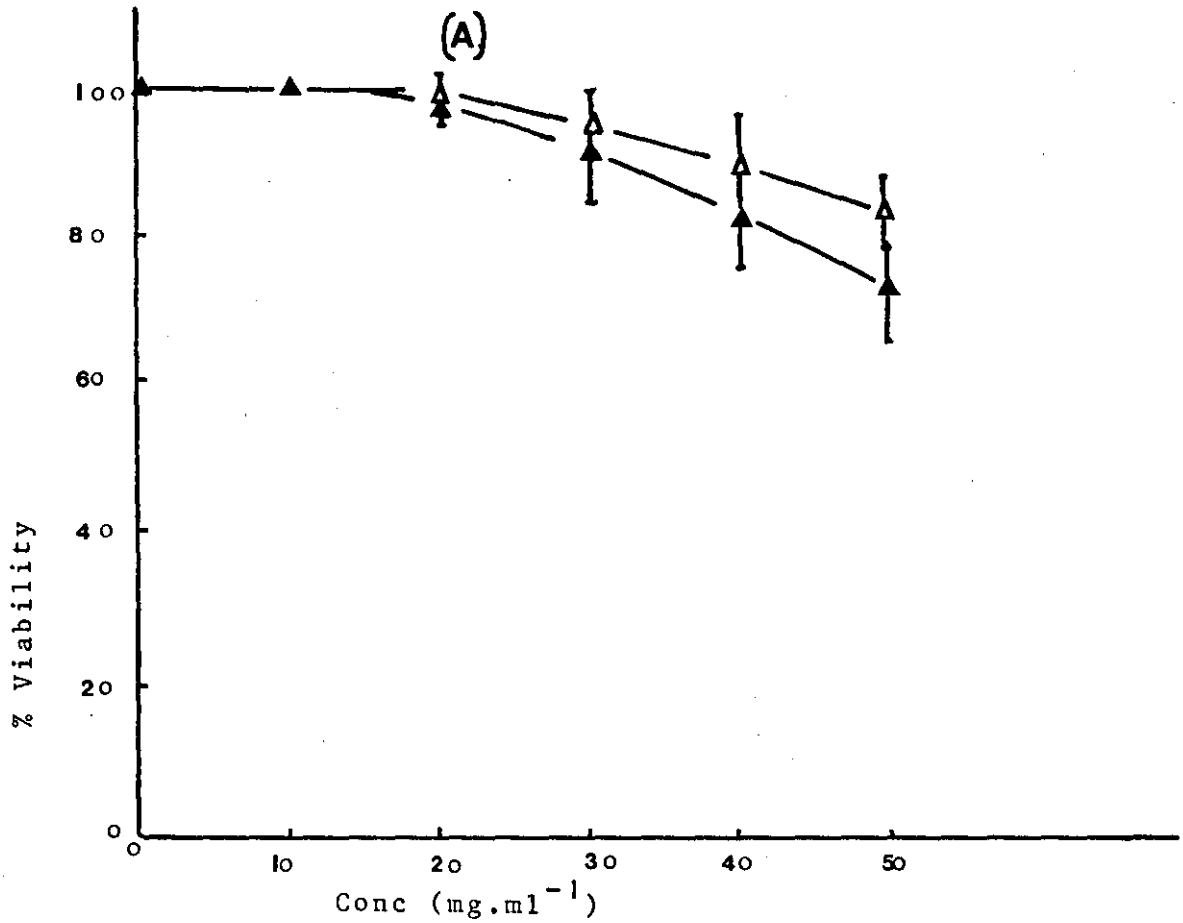

(B)
% Viability
100
80
60
40
20
0
0
2
4
6
8
Conc (mg.ml⁻¹)

Fig 3.42 The effect of (A) procaine Δ——Δ and lignocaine ▲——▲; (B) amethocaine o——o and cinchocaine ●——●, concentration on the percentage viability of cells of P.aeruginosa suspended in CFM. Initial cell concentration 0.125 $mg.ml^{-1}$. Contact time 10 min. Temp $22^{o}C$.


(A)
100
80
60
40
20
0
% Viability
0
10
20
30
40
50
Conc (mg.ml^{-1})

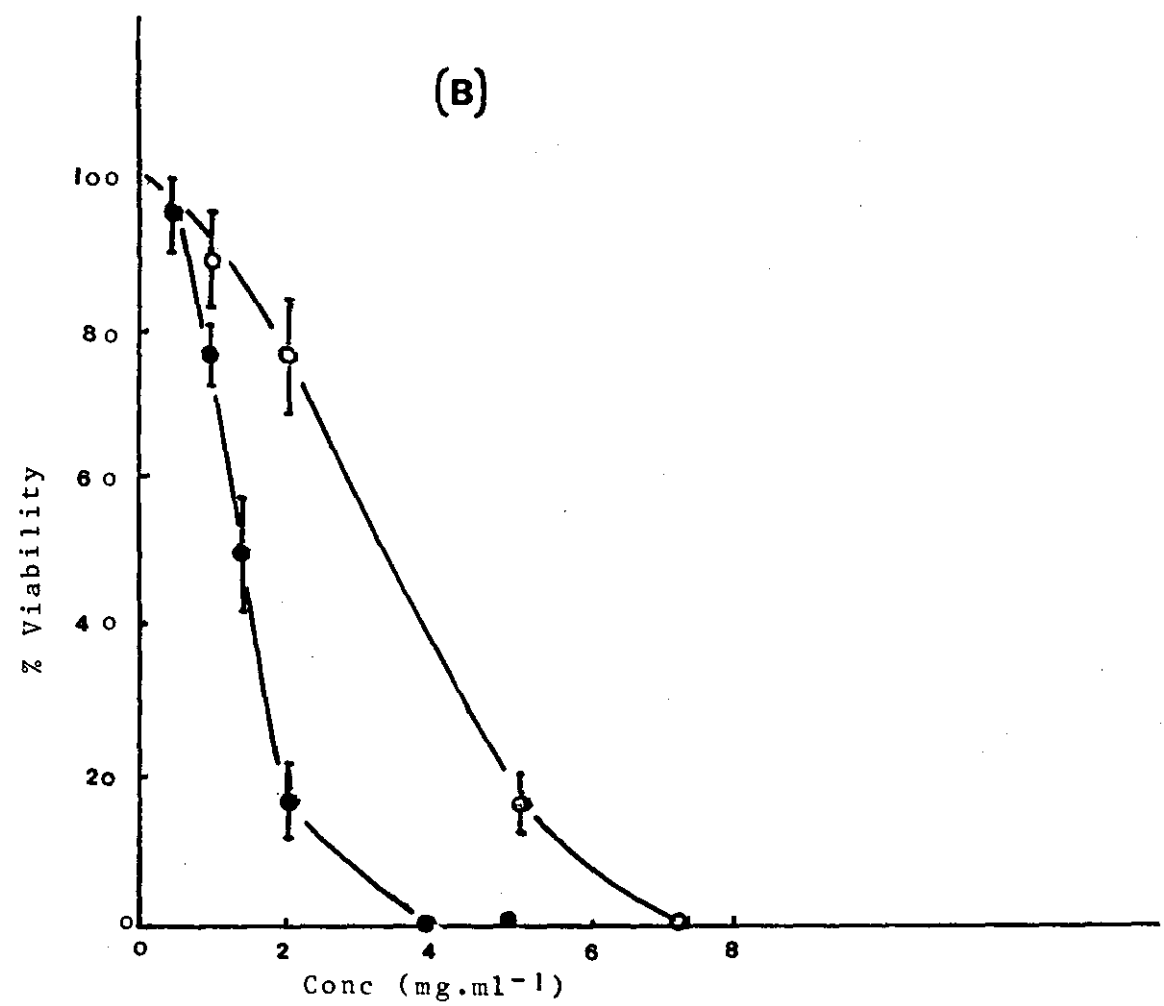
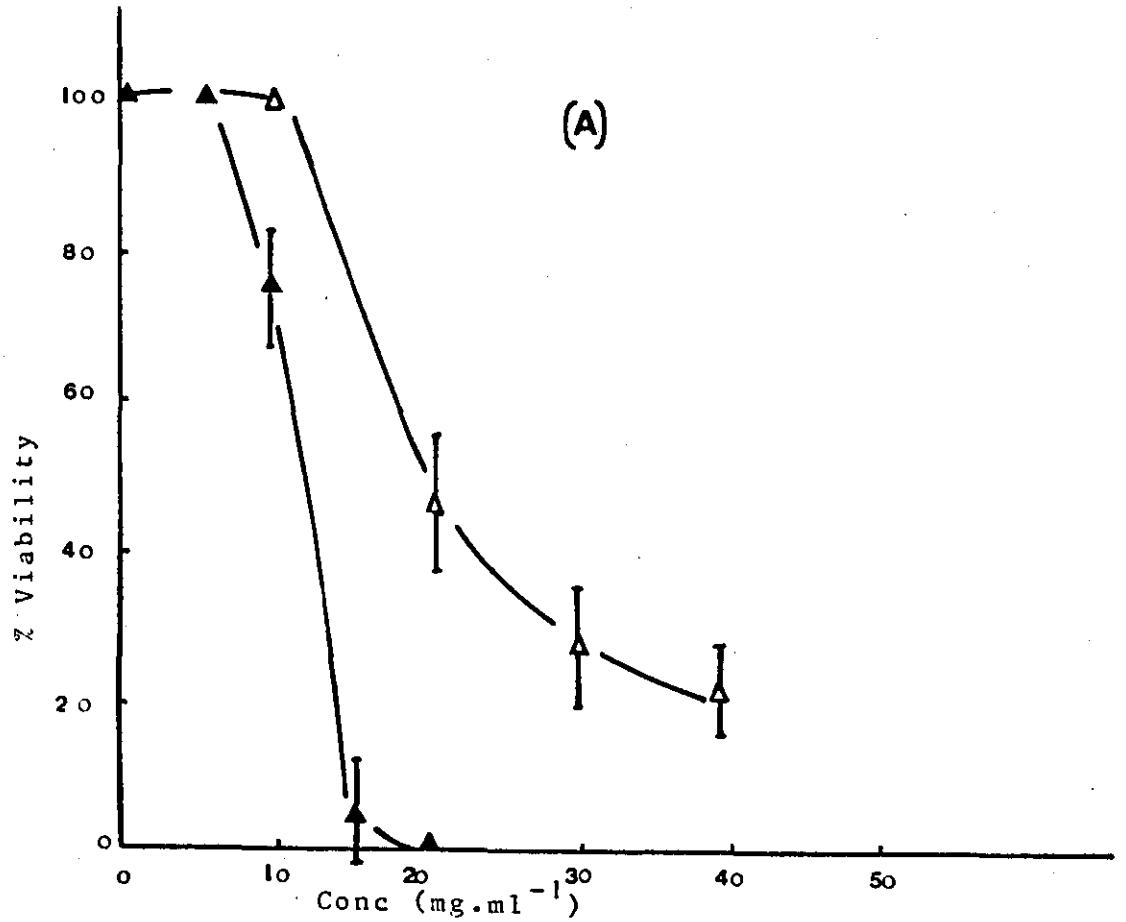

(B)
100
80
60
40
20
0
% Viability
0
2
4
6
8
Conc (mg.ml^{-1})

Fig 3.43 The effect of (A) procaine Δ——Δ and lignocaine ▲——▲; (B) amethocaine, o——o and cinchocaine, ●——●; concentration on the percentage viability of cells of B.megaterium suspended in CFM. Initial cell concentration 0.070 $mg.ml^{-1}$. Contact time 10 min. Temp $22^{o}C$.


(A)
% Viability
100
80
60
40
20
0
0
10
20
30
40
50
Conc (mg.ml^{-1})

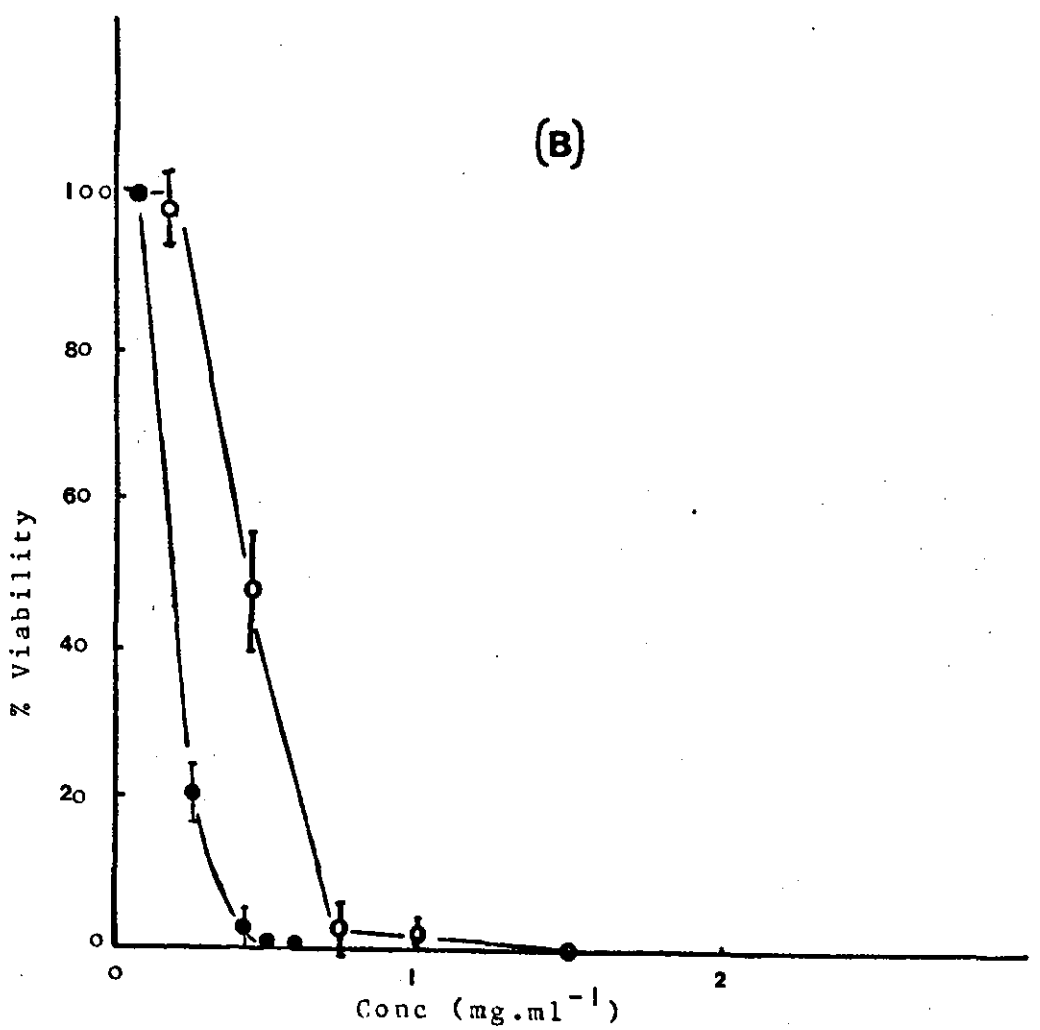
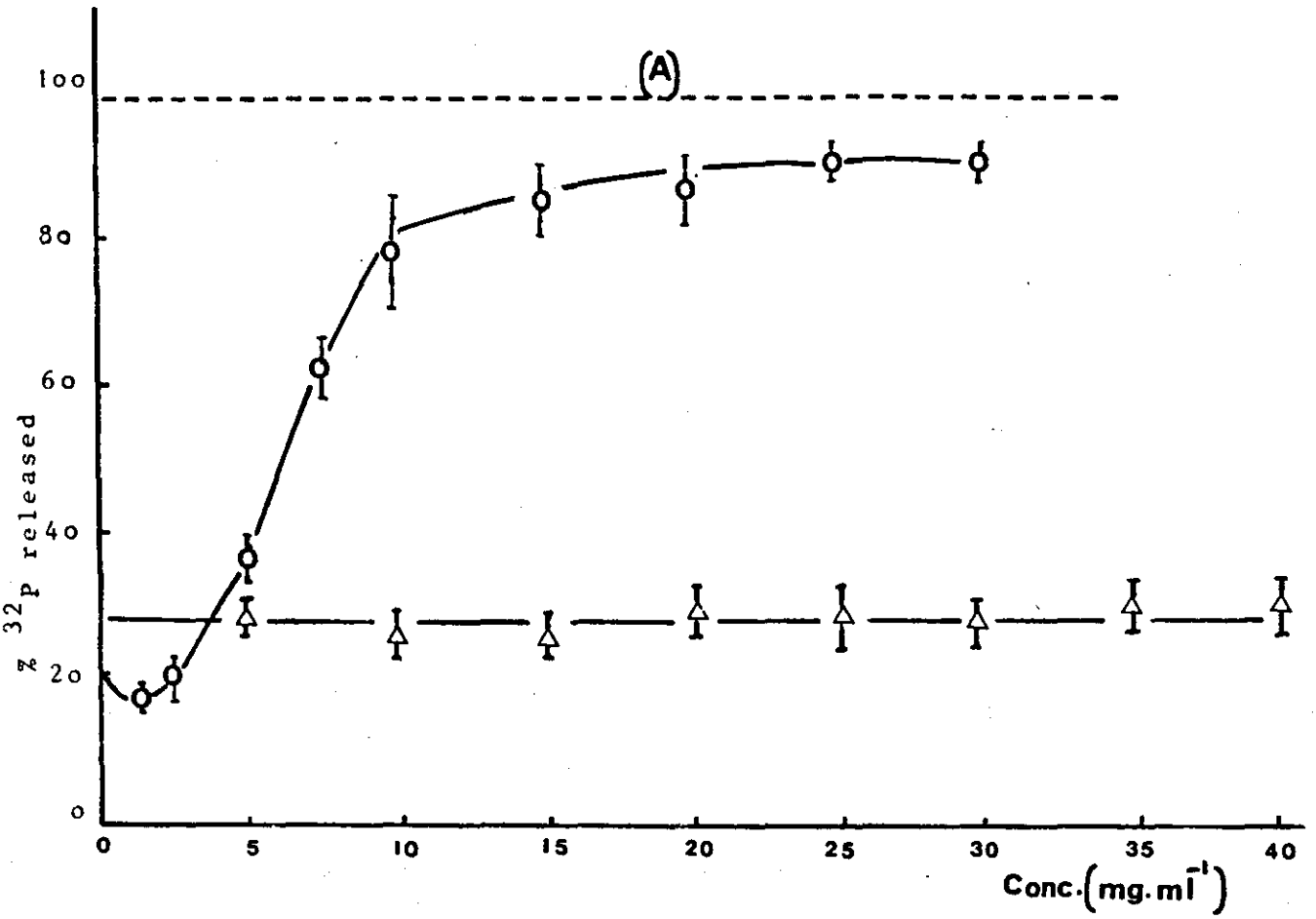

(B)
% Viability
100
80
60
40
20
0
0
1
2
Conc (mg.ml^{-1})

Fig 3.44 The effect of treatment with local anaesthetics for 10 min at 22°C on the ^{32}P content of labelled cells of E.coli suspended in CFM. Cell concentration 3.8 x10^8cells. ml^{-1}.

(A) o——o Amethocaine; Δ——Δ Procaine

(B) ●——● Cinchocaine; ▲——▲ Lignocaine

---- radioactivity released from the cells exposed to 5% trichloroacetic acid.


(A)
100
80
60
40
20
0
% 32P released
0
5
10
15
20
25
30
35
40
Conc.(mg.ml-1)

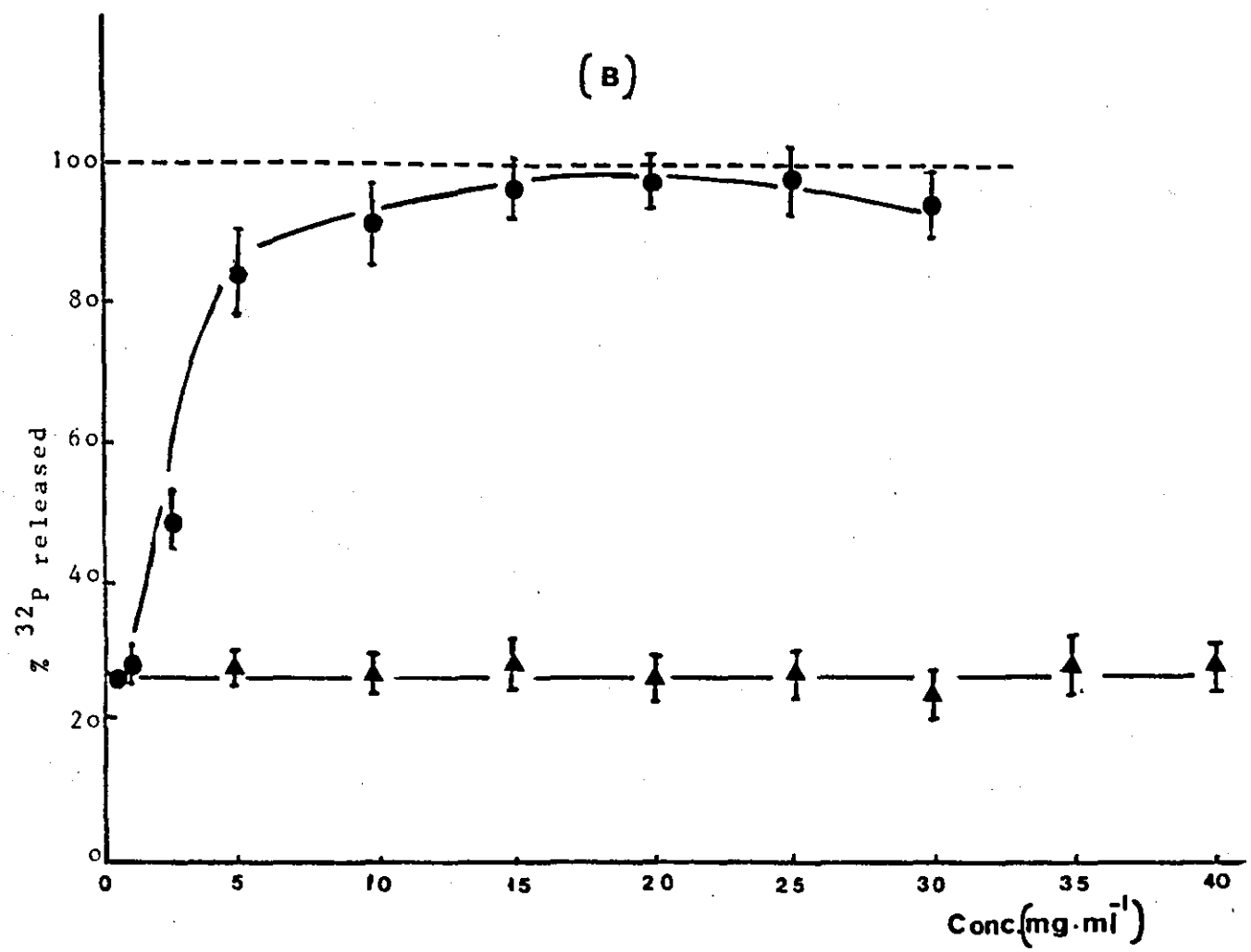
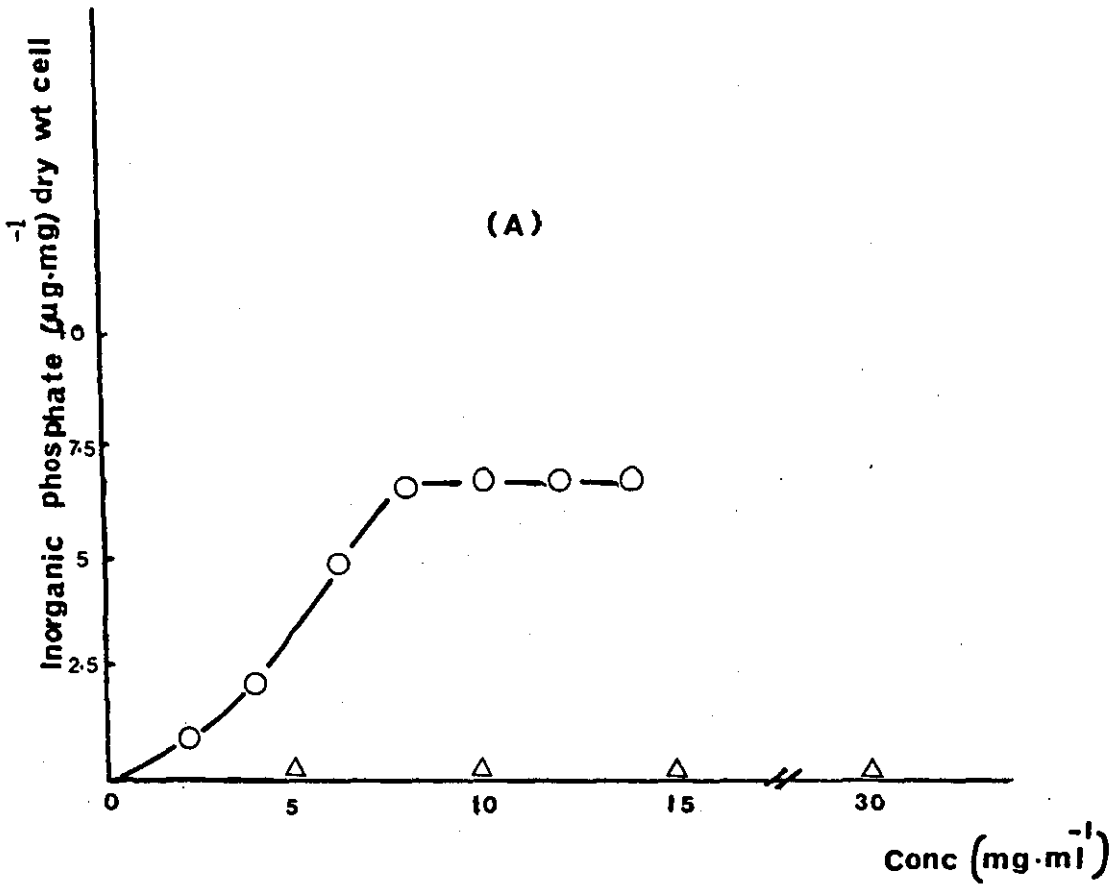

(B)
100
80
60
40
20
0
% 32P released
0
5
10
15
20
25
30
35
40
Conc.(mg.ml-1)

Fig 3.45 The release of inorganic phosphate from E.coli cells suspended in LPM after 10 min exposure to different concentrations of local anaesthetics. Cell concentration 3.8×10^9 cells.ml^{-1}. Temp 22°C.

(A) o——o Amethocaine; Δ——Δ Procaine

(B) ●——● Cinchocaine; ▲——▲ Lignocaine


(A)
Inorganic phosphate (µg·mg)$^{-1}$ dry wt cell
0
7·5
5
2·5
0
5
10
15
30
Conc (mg·ml^{-1})

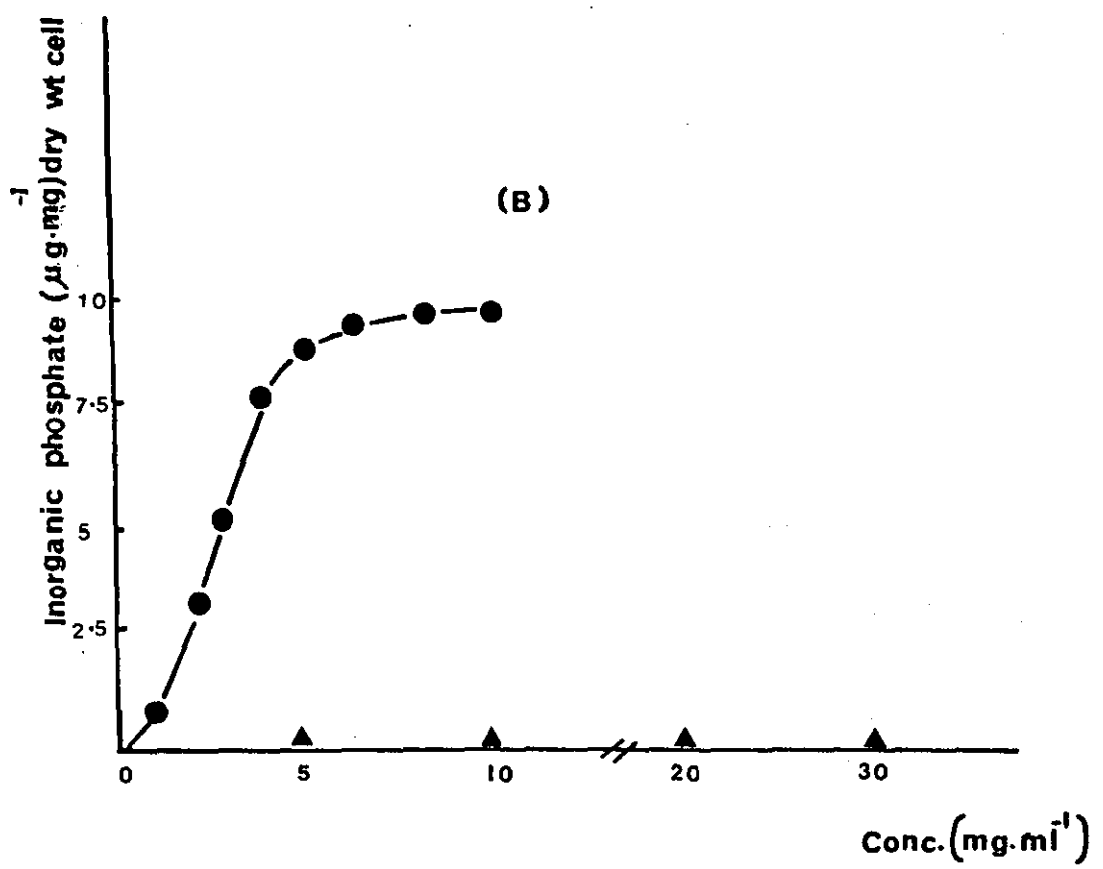
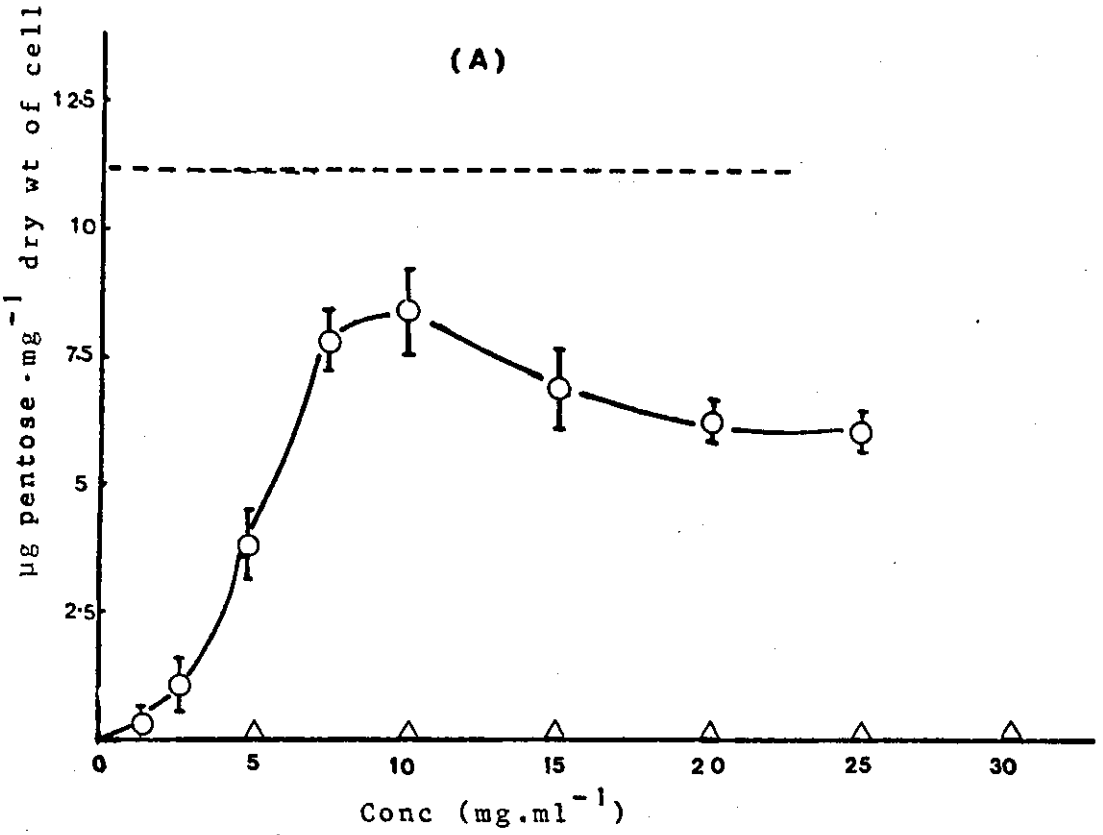

(B)
Inorganic phosphate (µg·mg)$^{-1}$ dry wt cell
10
7·5
5
2·5
0
5
10
20
30
Conc.(mg.ml^{-1})

Fig 3.46 The release of pentose from E.coli cells suspended in CFM after 15 min exposure to different concentrations of local anaesthetics. Cell concentration 3.8×10^9 cells.ml^{-1}. Temp $22^{\circ}C$.

(A) o——o Amethocaine; Δ——Δ Procaine

(B) ●——● Cinchocaine; ▲——▲ Lignocaine

---- Pentose released from the cells after boiling the cells for 10 min.


(A)
µg pentose · mg⁻¹ dry wt of cell
12·5
10
7·5
5
2·5
0
5
10
15
20
25
30
Conc (mg.ml⁻¹)

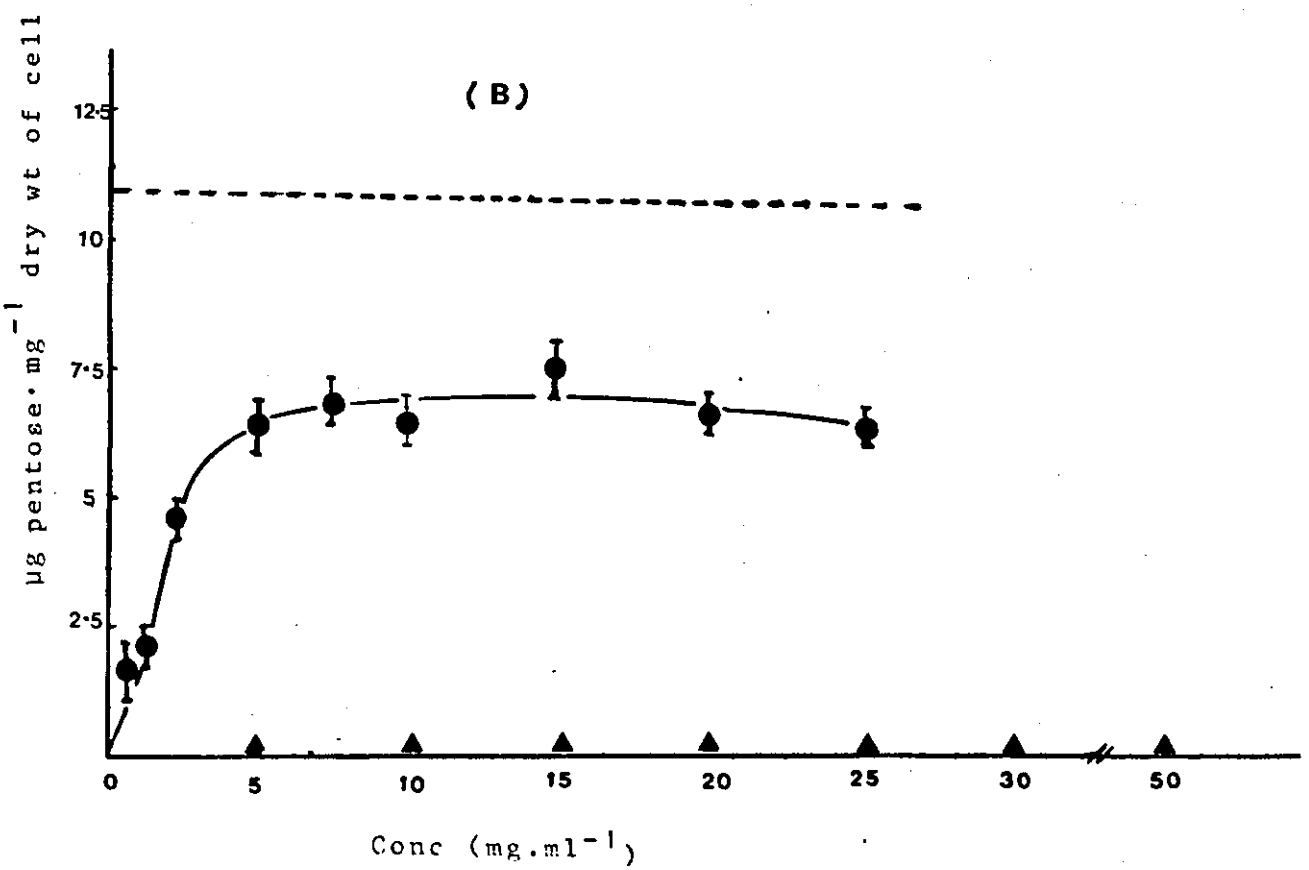
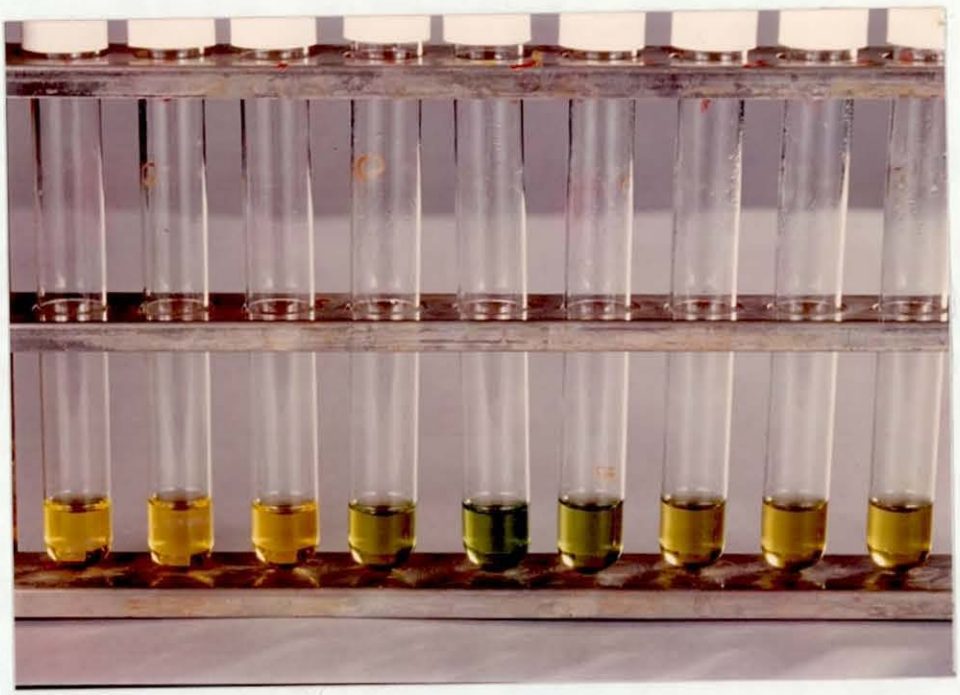

(B)
µg pentose · mg⁻¹ dry wt of cell
12·5
10
7·5
5
2·5
0
5
10
15
20
25
30
50
Conc (mg.ml⁻¹)

Plate 3.1 Effect of amethocaine on pentose release from E.coli cells after 15 min contact time at 22°C, as a change in colour from yellow to green and greenish yellow, corresponding to increase and then decrease in released pentose. Cell concentration: 3.8×10^9 cells.ml^{-1}. Concentration of amethocaine (mg.ml^{-1}), from left to right: 0.0, 1.25, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 25.0.

Plate 3.2 Effect of procaine on pentose release from E.coli cells after 15 min contact time at 22°C, no colour changes can be observed, i.e. no pentose released. Cell concentration: 3.8×10^9 cells.ml^{-1}. Concentration of procaine (mg.ml^{-1}), from left to right: 0.0, 10.0, 20.0, 25.0, 30.0,35.0, 40.0, 50.0



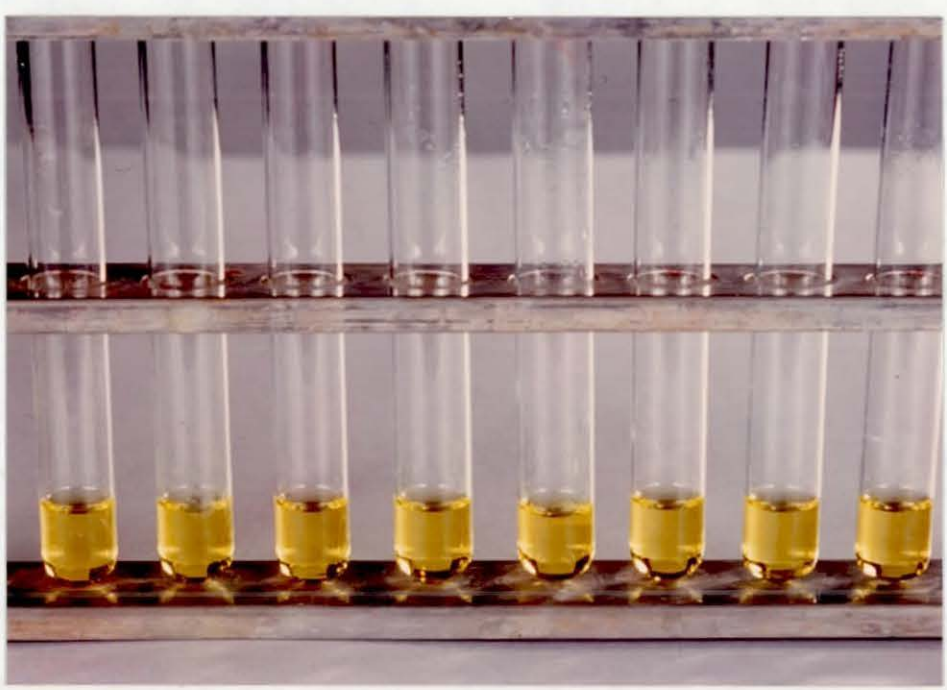
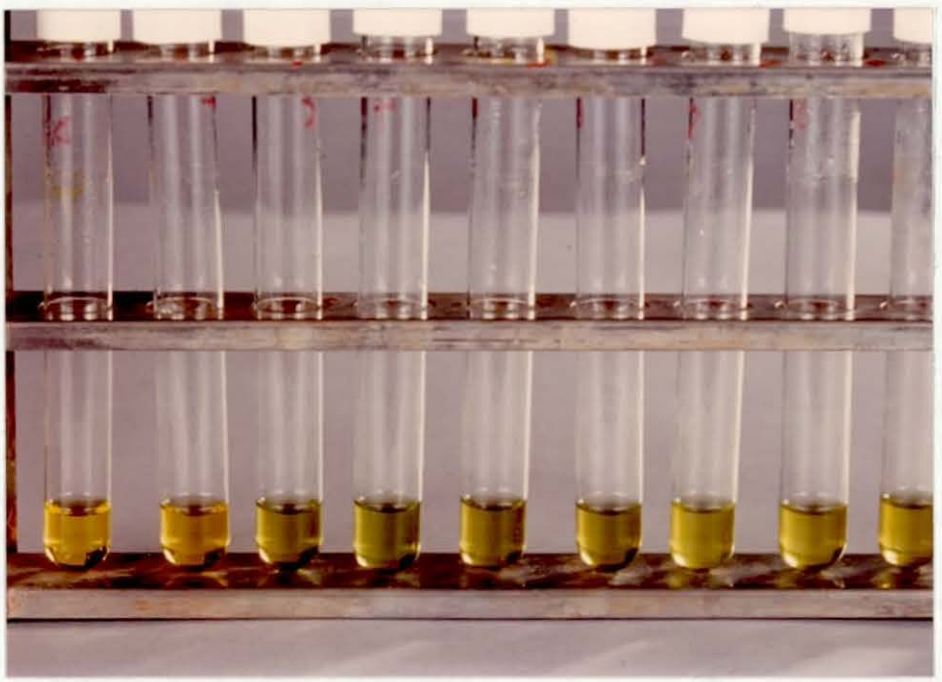


Plate 3.3 Effect of cinchocaine on pentose release from E.coli cells after 15 min contact time at 22°C, as a change in colour from yellow to green and greenish yellow, corresponding to increase and then decrease in the released pentose. Cell concentration: 3.8×10^9 cells.ml^{-1}. Concentration of cinchocaine (mg.ml^{-1}) from left to right:
0.0, 1.25, 2.5, 5.0, 7.5, 10.0, 15.0,20,0, 25.0.

Plate 3.4 Effect of lignocaine on pentose release from E.coli cells after 15 min contact time at 22°C. No colour changes were observed, i.e. no pentose released. Cell concentration: 3.8×10^9 cells.ml^{-1}. Concentration of lignocaine (mg.ml^{-1}) from left to right:
0.0, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0.



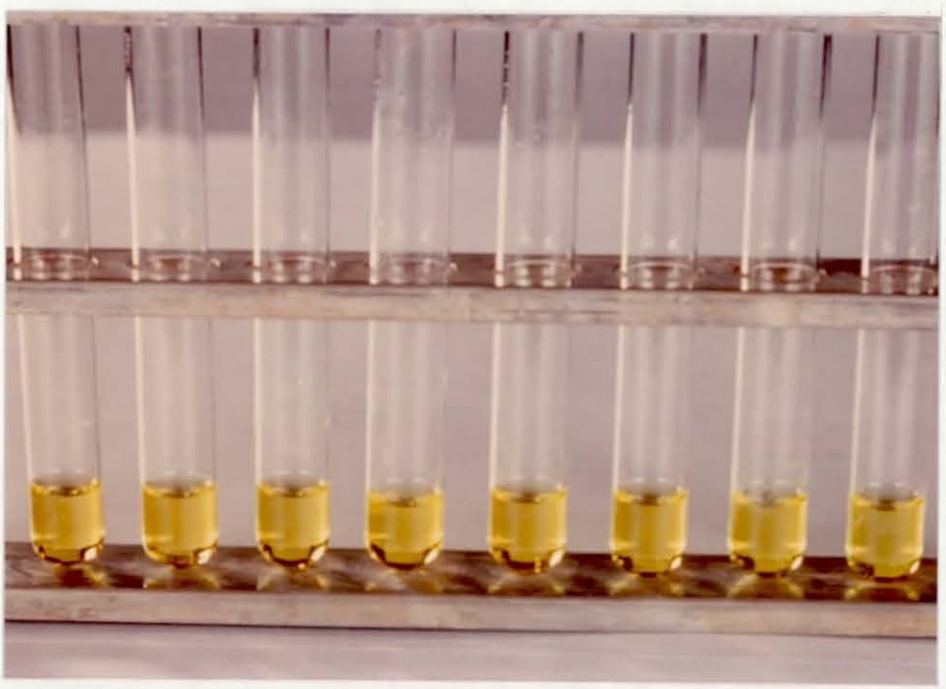


Fig 3.47 The effect of LA concentration on the release of pentose from E.coli cells suspended in CFM after different contact times at 22°C. Cell concentration, 3.8×10^9 cells. ml^{-1}.

(A)

o—o 10 $mg.ml^{-1}$ amethocaine

□—□ 30 $mg.ml^{-1}$ amethocaine

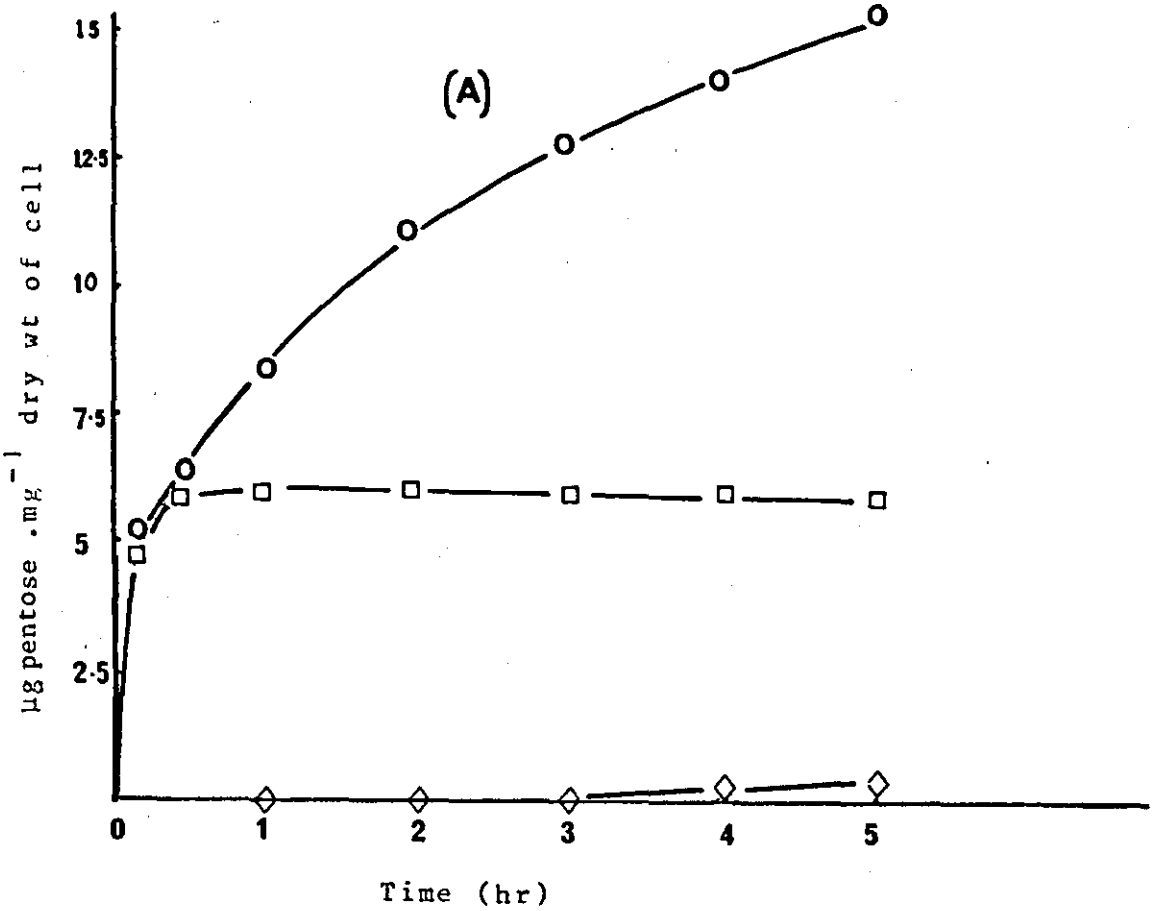
◇—◇ 50 $mg.ml^{-1}$ procaine

(B)

●—● 5 $mg.ml^{-1}$ cinchocaine

■—■ 20 $mg.ml^{-1}$ cinchocaine

◆—◆ 40 $mg.ml^{-1}$ lignocaine


(A)
15
12·5
10
7·5
5
2·5
µg pentose . mg⁻¹ dry wt of cell
0
1
2
3
4
5
Time (hr)

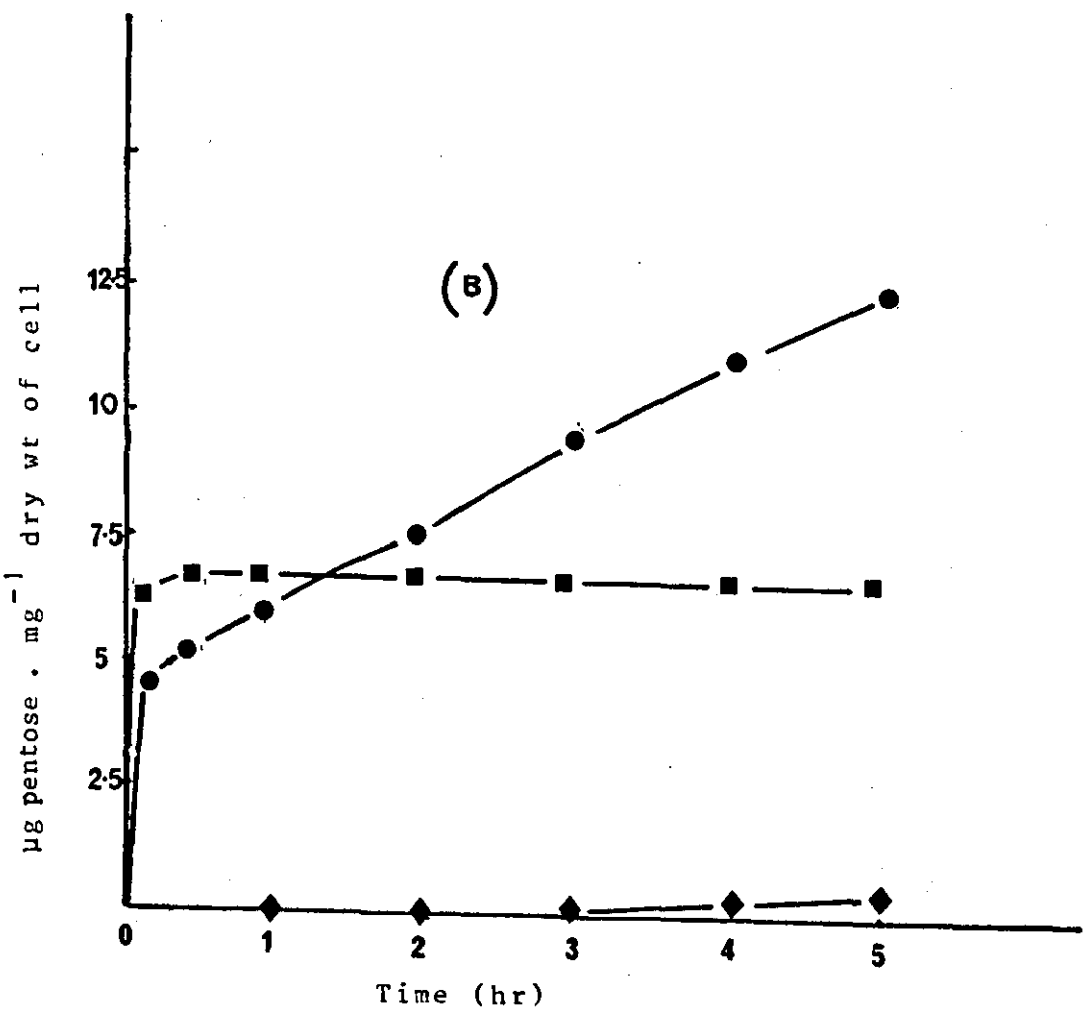

(B)
12·5
10
7·5
5
2·5
µg pentose . mg⁻¹ dry wt of cell
0
1
2
3
4
5
Time (hr)

Fig 3.48 The effect of LA concentration on the release of pentose from E.coli cells suspended in CFM after different contact times at 1°C. Cell concentration 3.8×10^9 cells.ml^{-1}.

(A)

o—o 5 mg.ml^{-1} amethocaine

□—□ 10 mg.ml^{-1} amethocaine

◇—◇ 25 mg.ml^{-1} amethocaine

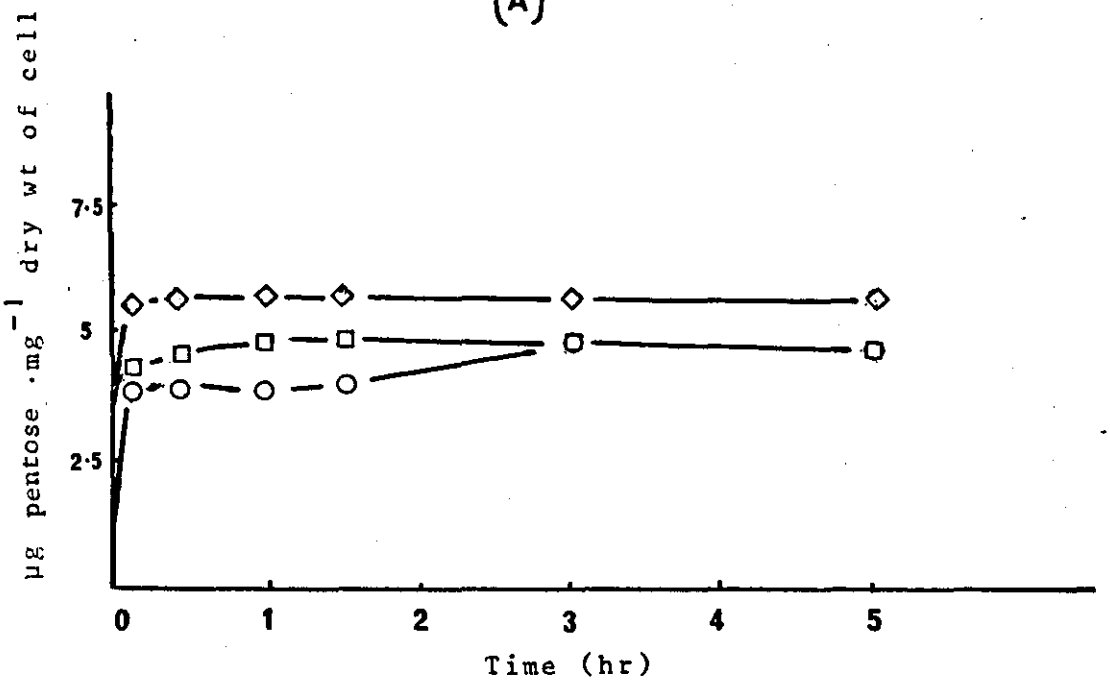
(B)

●—● 5 mg.ml^{-1} cinchocaine

■—■ 10 mg.ml^{-1} cinchocaine

◆—◆ 20 mg.ml^{-1} cinchocaine

(A)


µg pentose ·mg⁻¹ dry wt of cell
7·5
5
2·5
0
1
2
3
4
5
Time (hr)

(B)

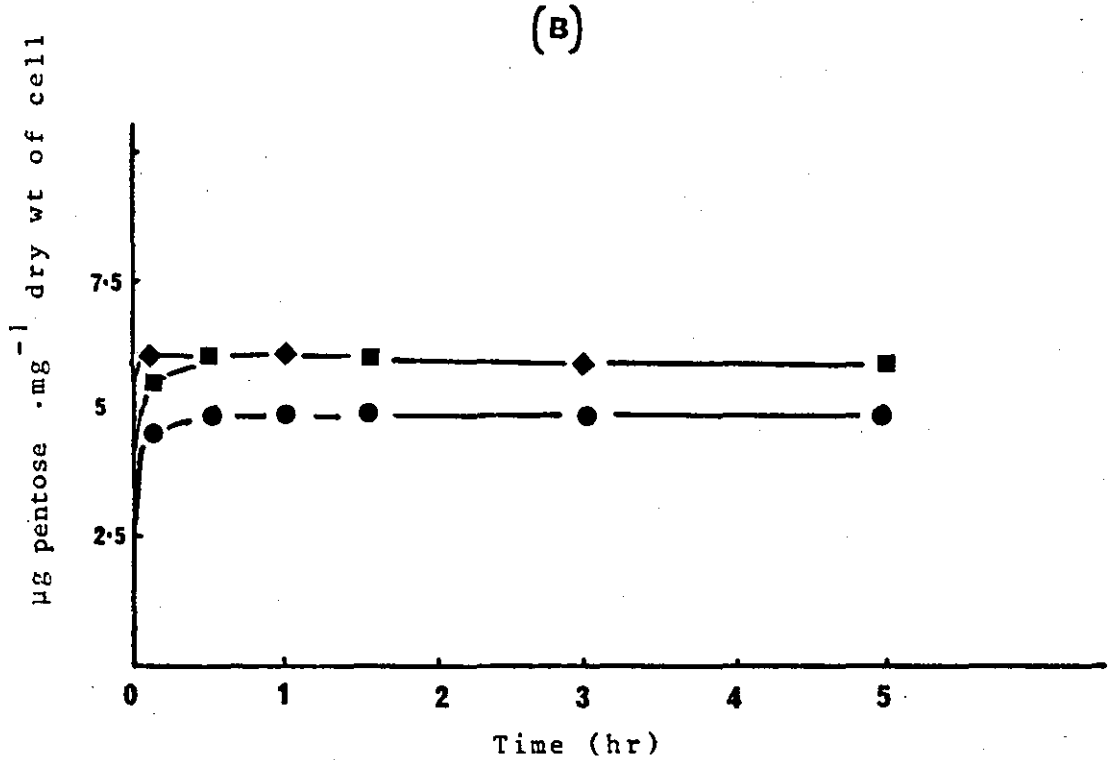
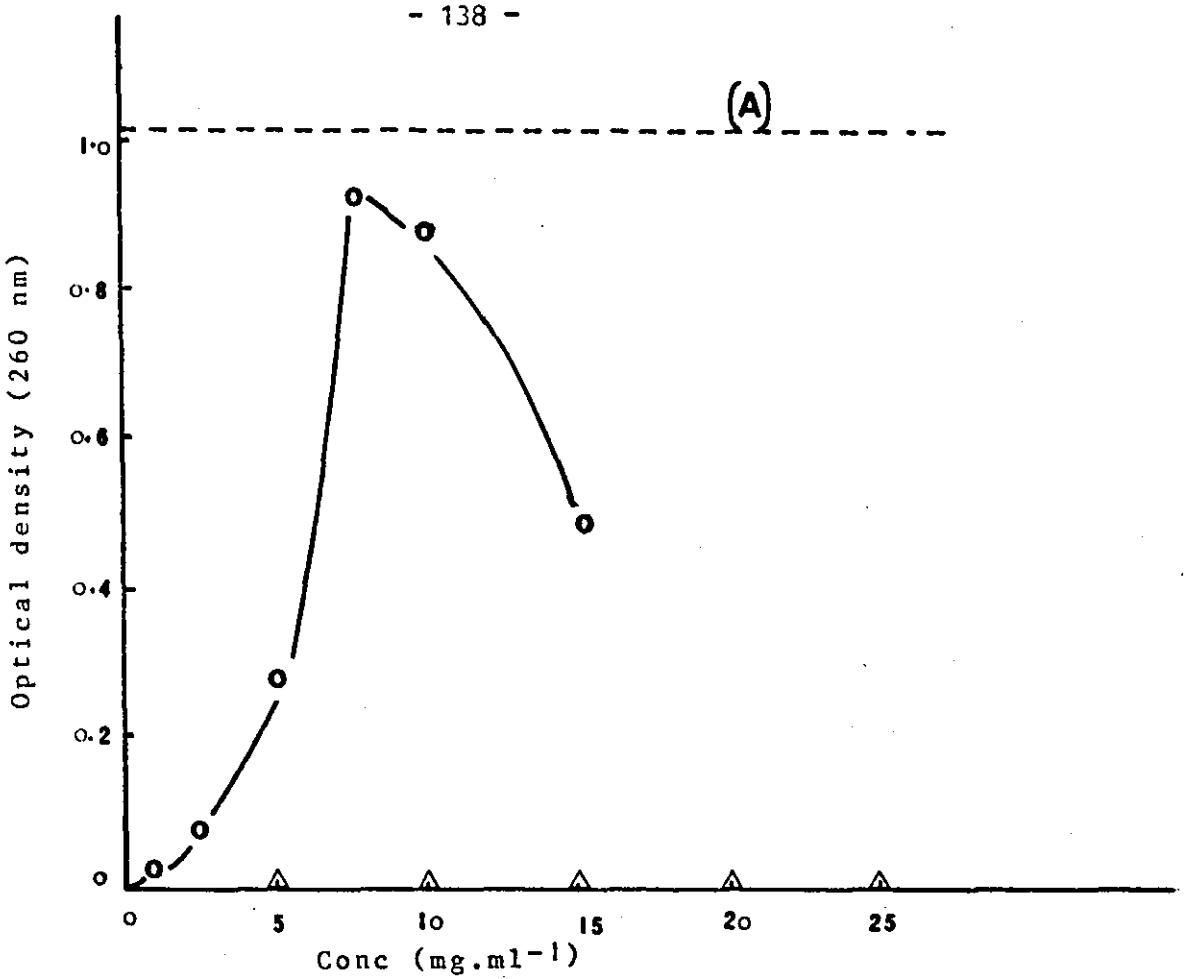

µg pentose ·mg⁻¹ dry wt of cell
7·5
5
2·5
0
1
2
3
4
5
Time (hr)

Fig 3.49 The leakage of 260 nm absorbing material from E.coli cells, suspended in CFM after 30 min exposure to different concentration of LA. Cell concentration 3.8 x 10^9 cells.ml^{-1}. Temp 22^oC.

(A) o——o, Amethocaine; Δ——Δ Procaine

(B) ●——● Cinchocaine; ▲——▲ Lignocaine

---- Amount of 260nm released after boiling of the cells for 10 min.


(A)
Optical density (260 nm)
1·0
0·8
0·6
0·4
0·2
0
0
5
10
15
20
25
Conc (mg.ml⁻¹)

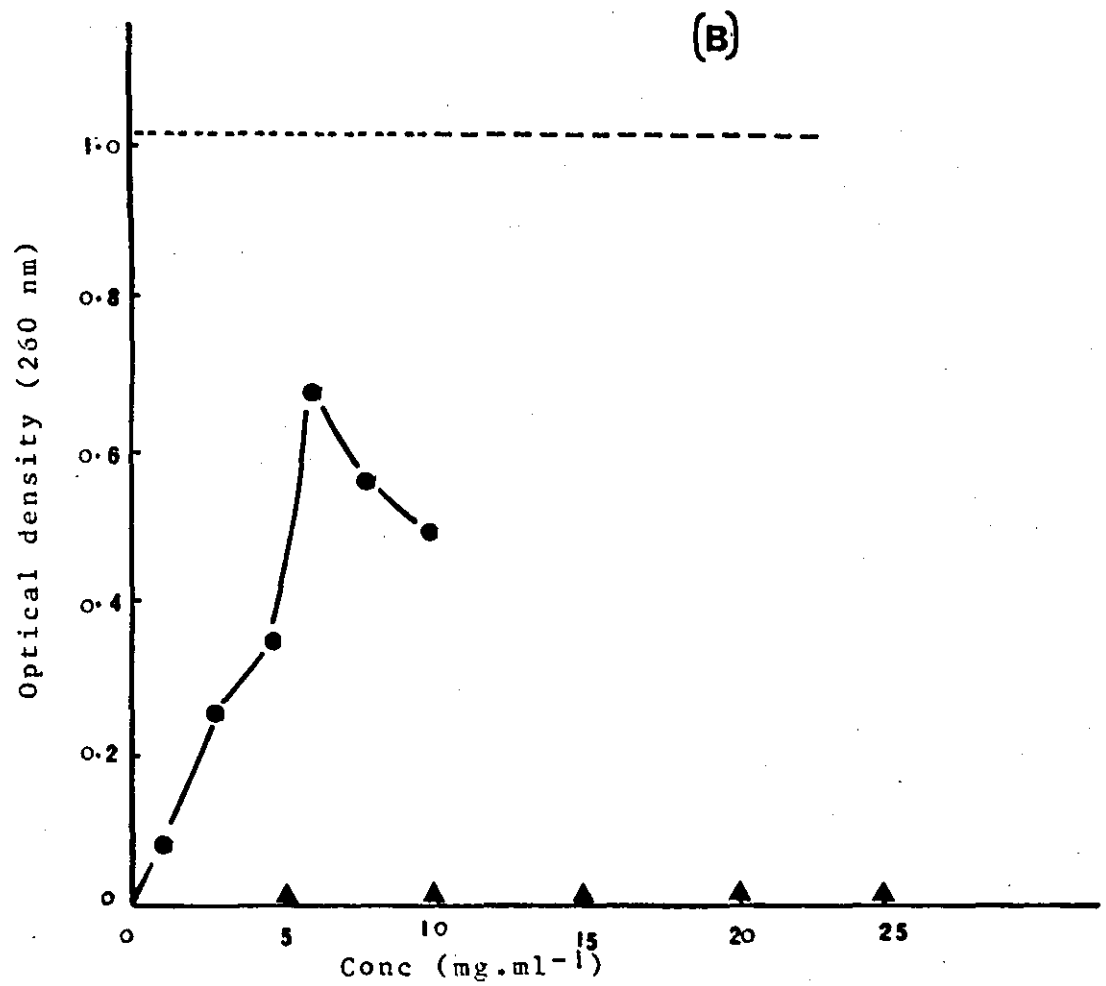
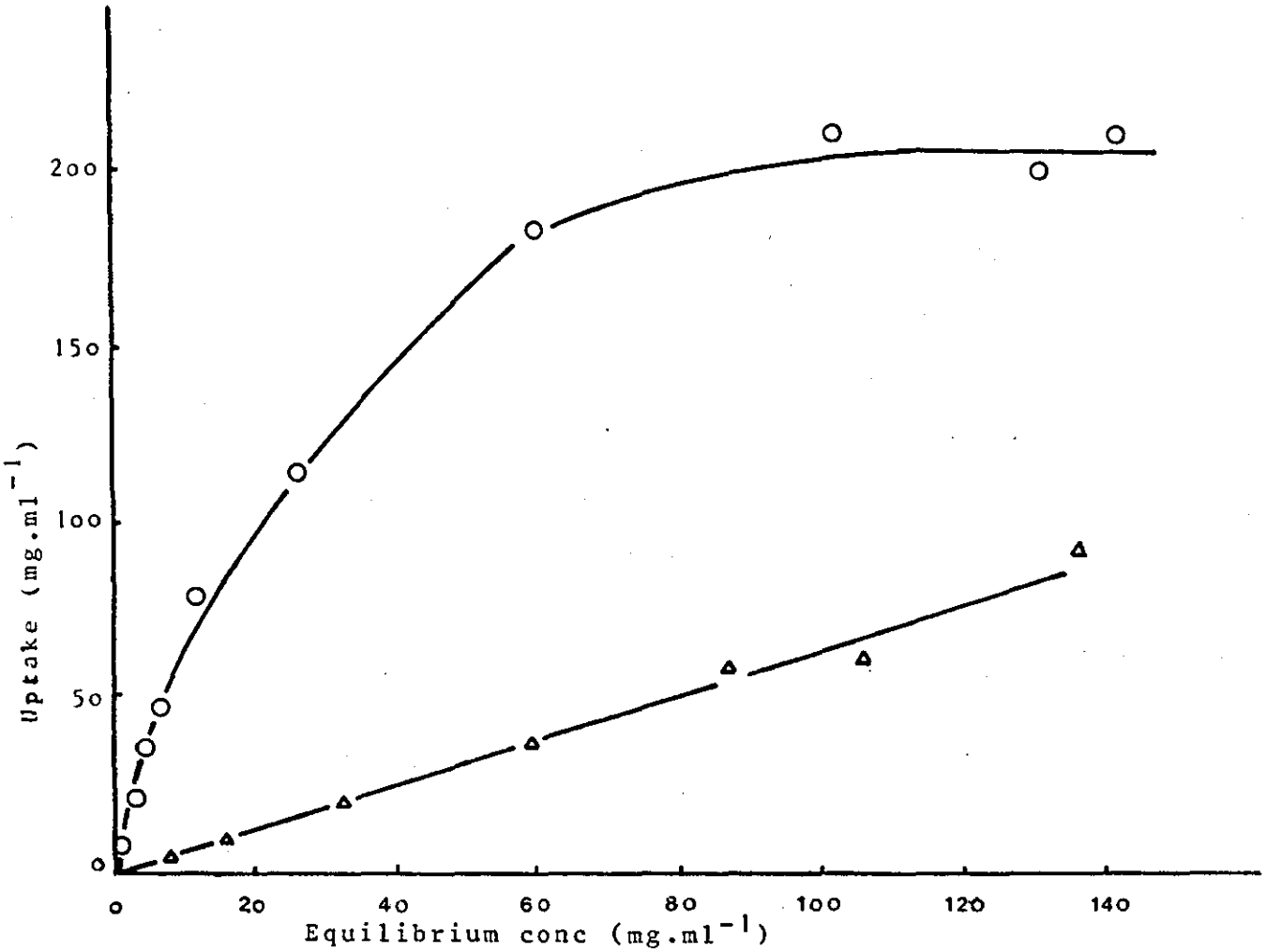

(B)
Optical density (260 nm)
1·0
0·8
0·6
0·4
0·2
0
0
5
10
15
20
25
Conc (mg.ml⁻¹)

Fig 3.50 Uptake of amethocaine (o——o) and procaine (Δ——Δ) by E.coli suspended in CFM plotted as a function of equilibrium concentration. Contact time 30 min; Temp 37°C, cell concentration 3.8 x 10^{10} cells.ml^{-1}.

Fig 3.51 Uptake of amethocaine by E.coli cells suspended in CFM plotted as a function of equilibrium concentration. Contact time 30 min, temp 37°C, cell concentration 3.8 x 10^{10} cells.ml^{-1}.


Uptake (mg.ml^{-1})
200
150
100
50
0
0
20
40
60
80
100
120
140
Equilibrium conc (mg.ml^{-1})

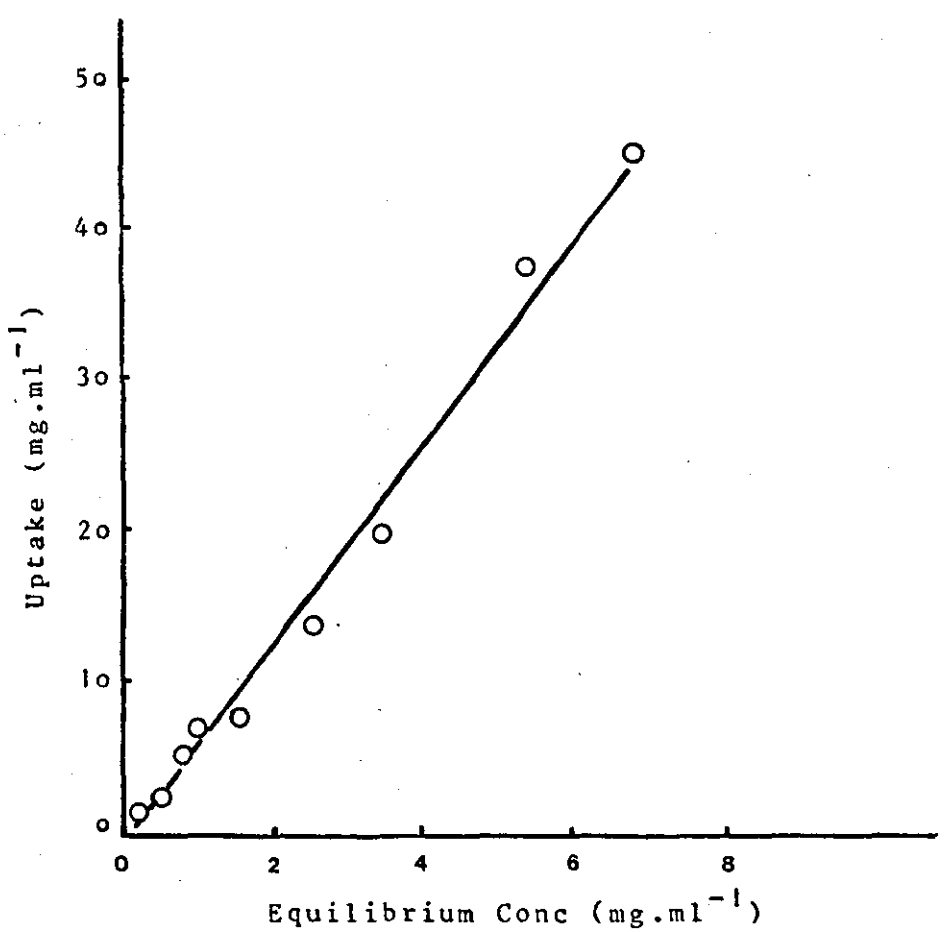

Uptake (mg.ml^{-1})
50
40
30
20
10
0
0
2
4
6
8
Equilibrium Conc (mg.ml^{-1})

Fig 3.52 Uptake of Cinchocaine (●——●) and lignocaine (▲——▲) by E.coli suspended in CFM plotted as a function of equilibrium concentration. Contact time 30 min, temp 37°C. Cell concentration 3.8×10^{10} cells.ml^{-1}.

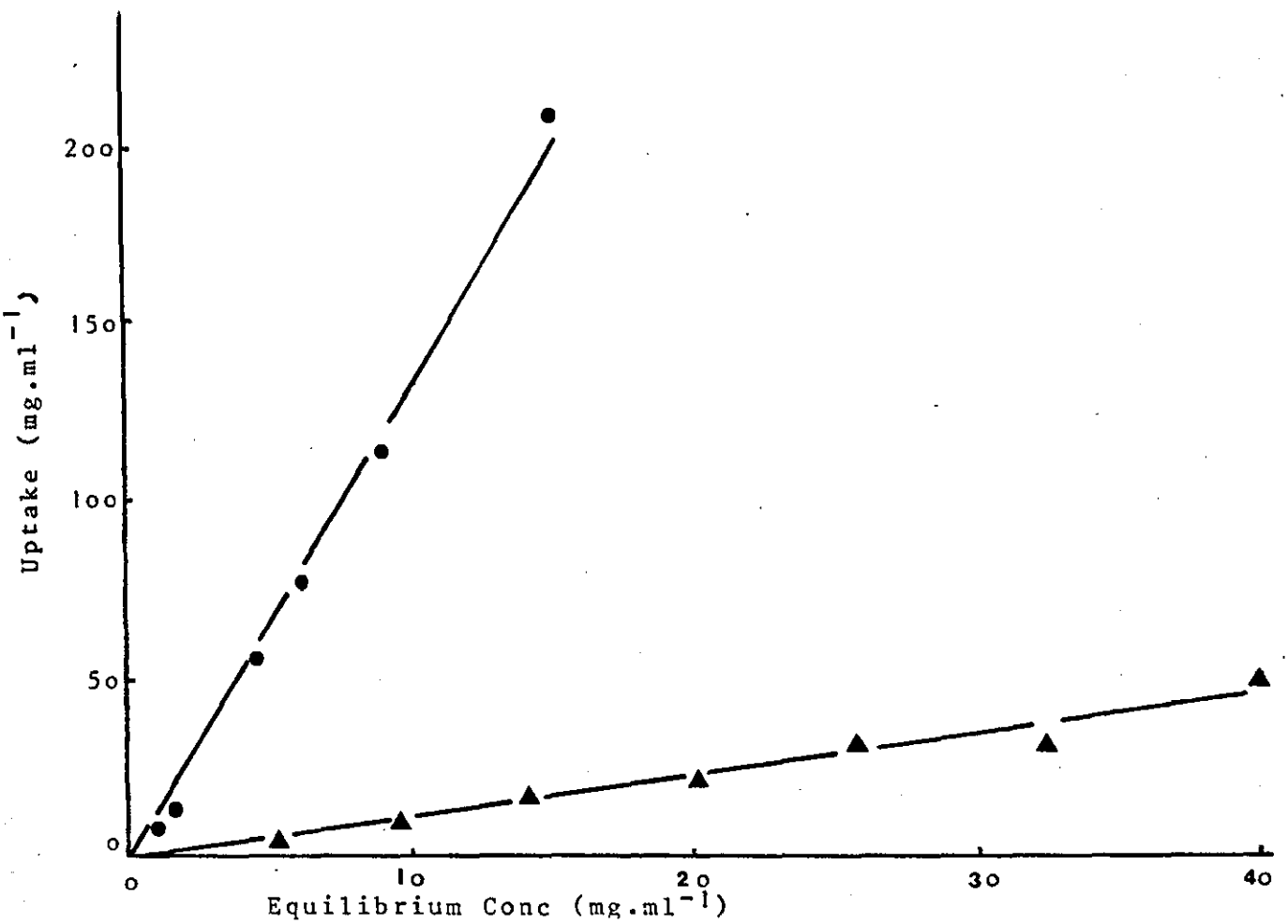
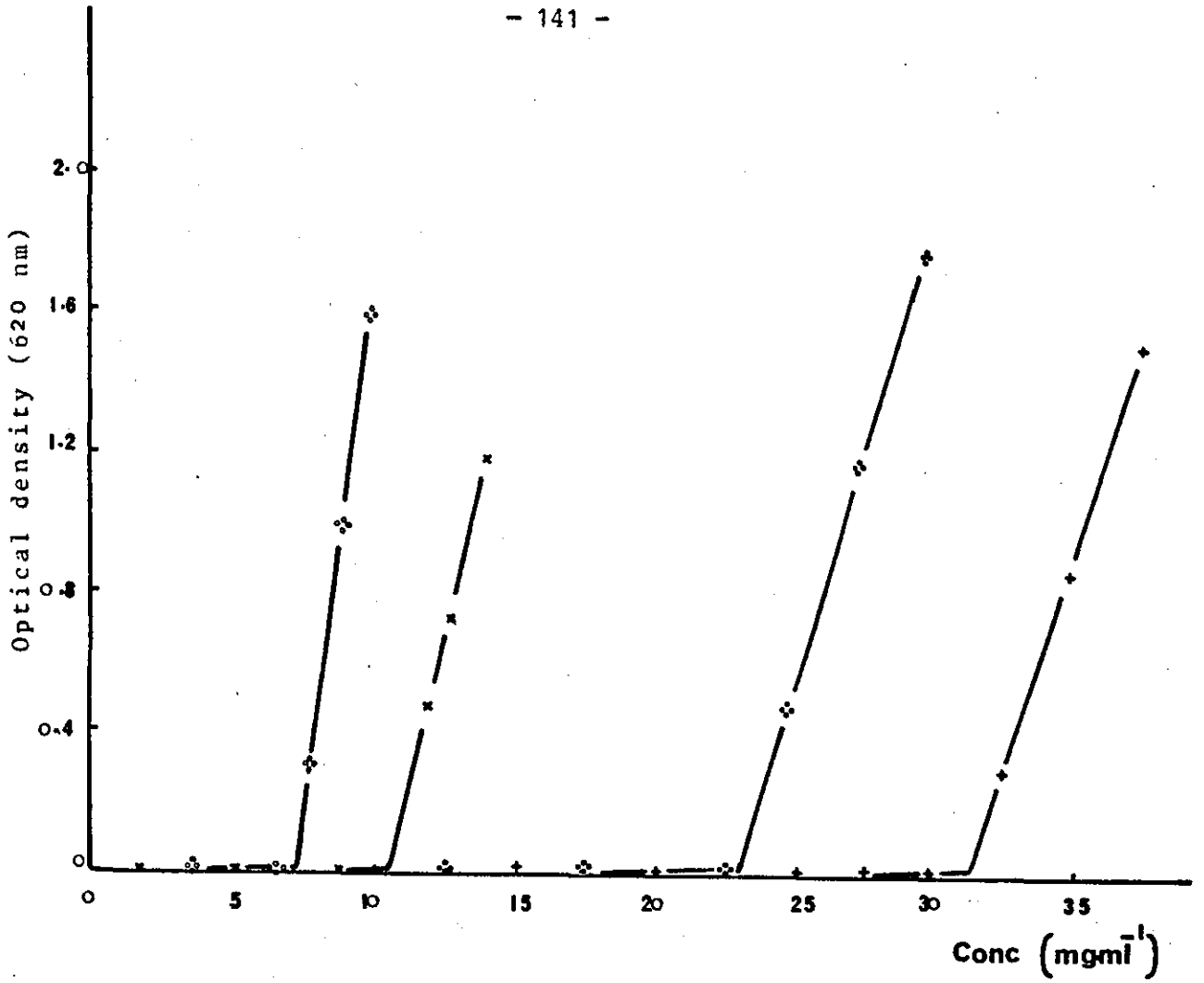

200
150
100
50
0
Uptake (mg.ml^{-1})
0
10
20
30
40
Equilibrium Conc (mg.ml^{-1})

Fig 3.53 Solubilization of Sudan Black B by amethocaine in water +——+ and CFM x——x, cinchocaine in water ∴——∴ and in CFM ⁘——⁘ at 22°C

Fig 3.54 Plot of surface tension (γ) at air-liquid interface for amethocaine in water +——+ and CFM x——x against concentration ($mg.ml^{-1}$) at 22°C.


Optical density (620 nm)
2·0
1·6
1·2
0·8
0·4
0
0
5
10
15
20
25
30
35
Conc (mg·ml⁻¹)

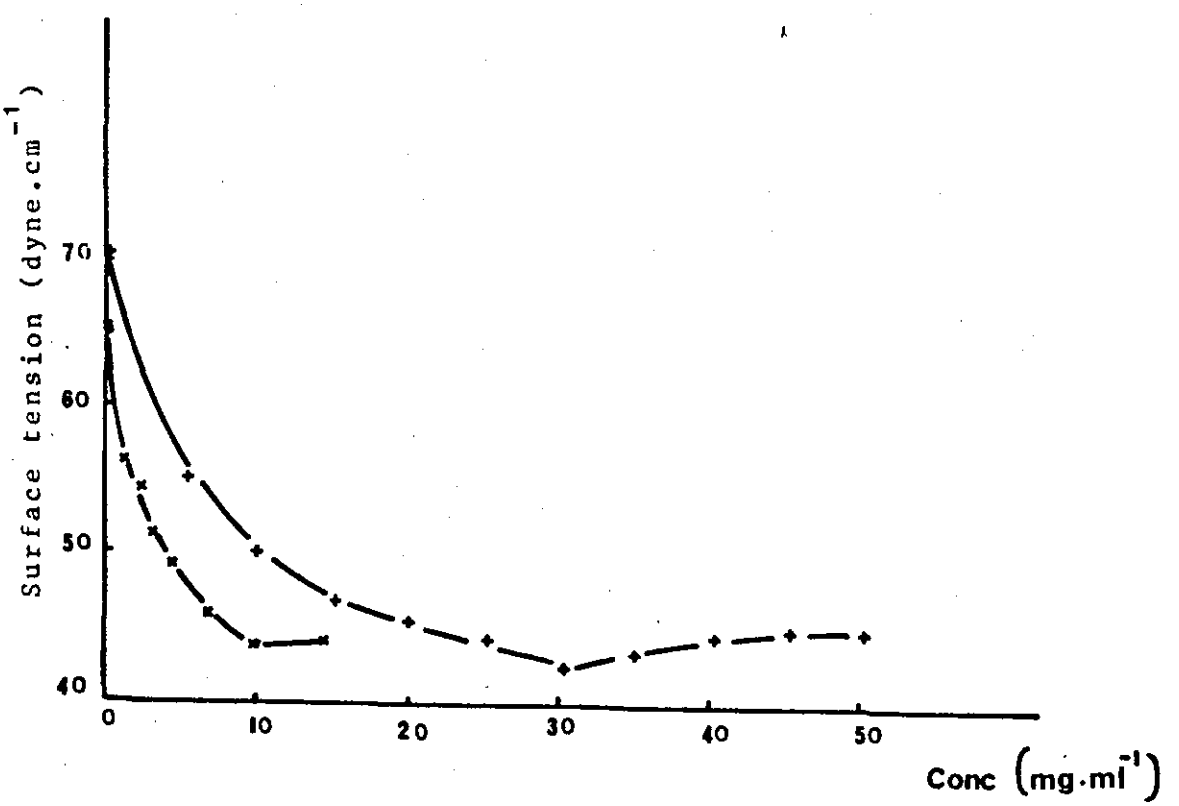
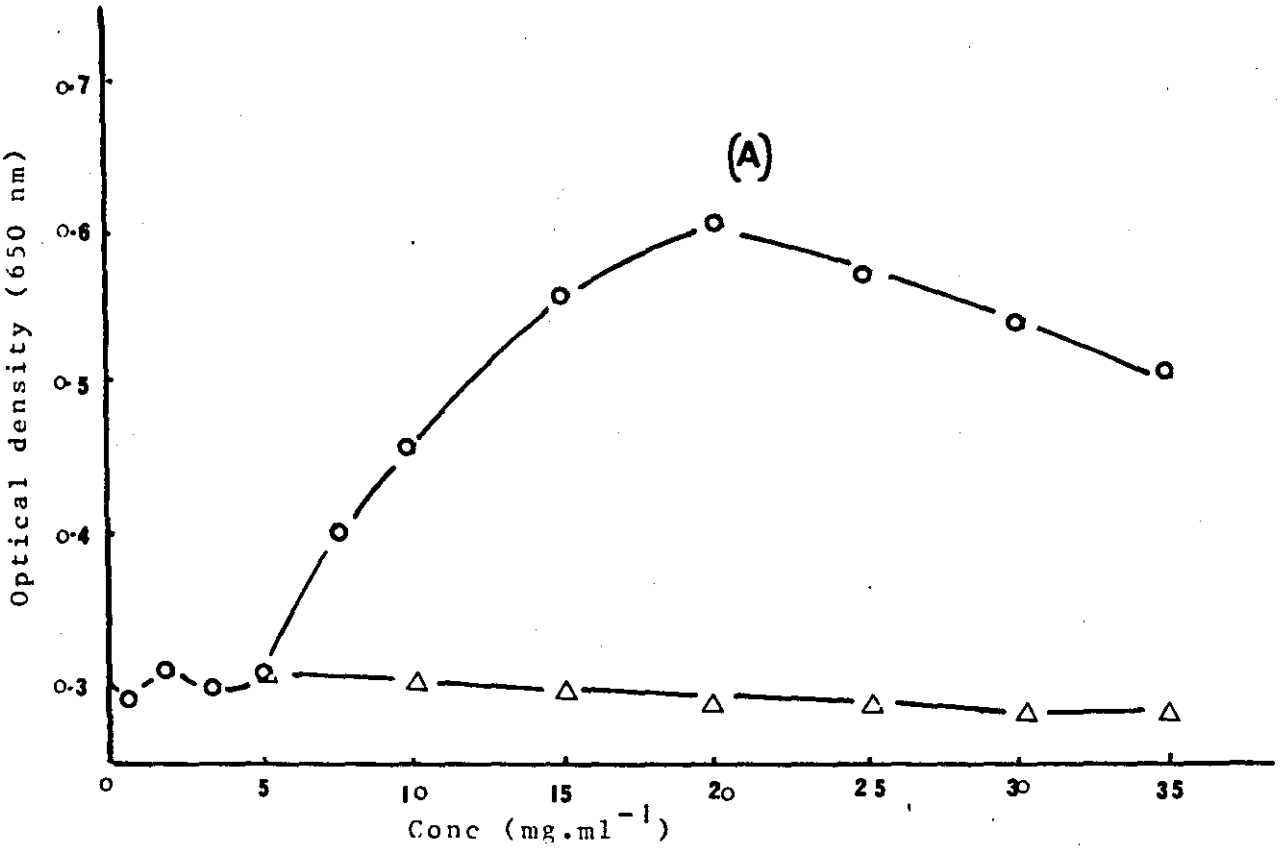

Surface tension (dyne.cm⁻¹)
70
60
50
40
0
10
20
30
40
50
Conc (mg·ml⁻¹)

Fig 3.55 Turbidity changes (optical density at 650 nm) in cells of E.coli suspended in CFM containing different LA as a function of LA concentration. Cell number 3.8×10^8 cells.ml^{-1}. Temp $22^{\circ}C$, contact time 10 min.

(A) o—o Amethocaine; Δ—Δ Procaine

(B) ●—● Cinchocaine; ▲—▲ Lignocaine


(A)
Optical density (650 nm)
0·7
0·6
0·5
0·4
0·3
0
5
10
15
20
25
30
35
Conc (mg.ml^{-1})

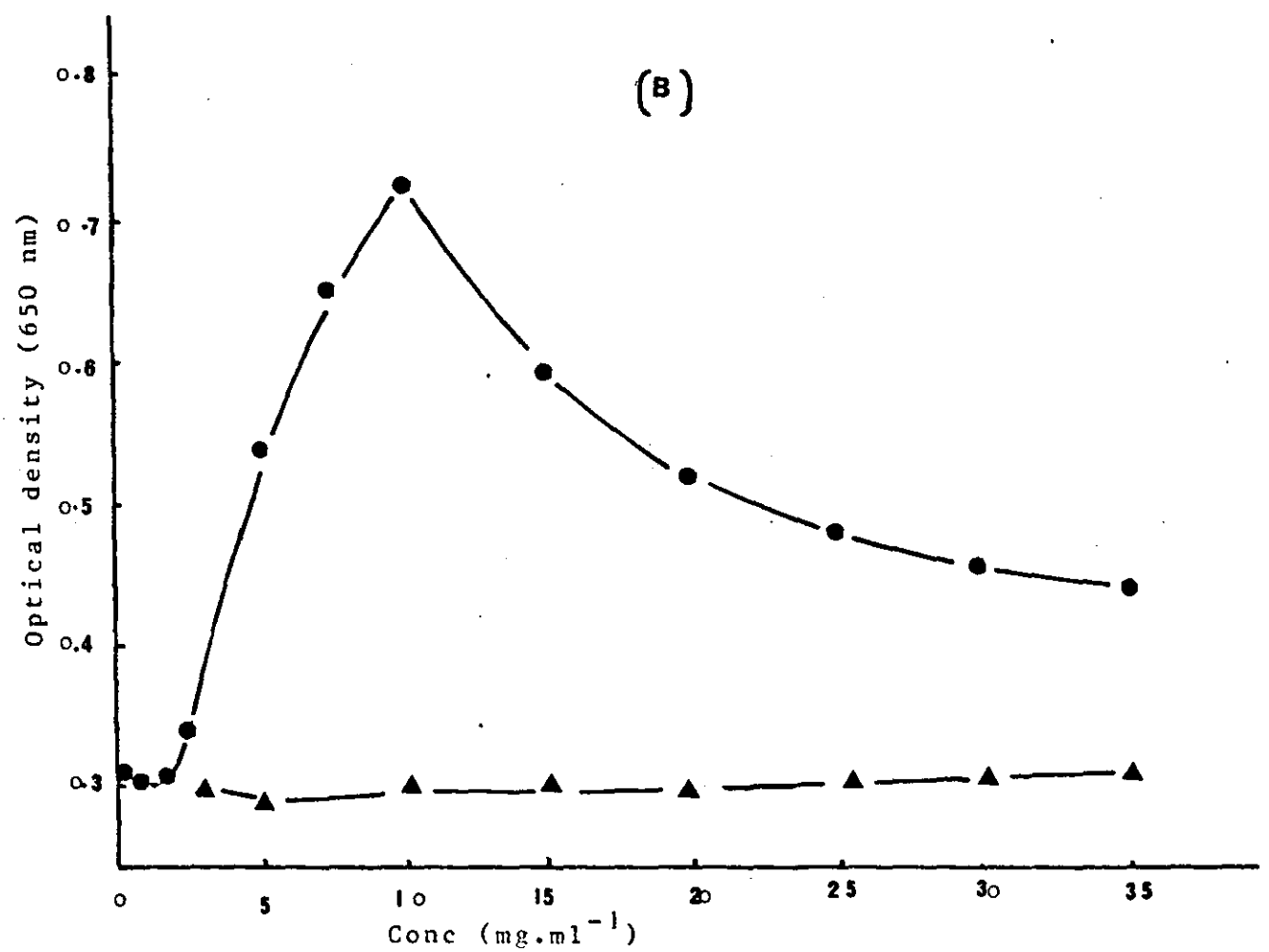
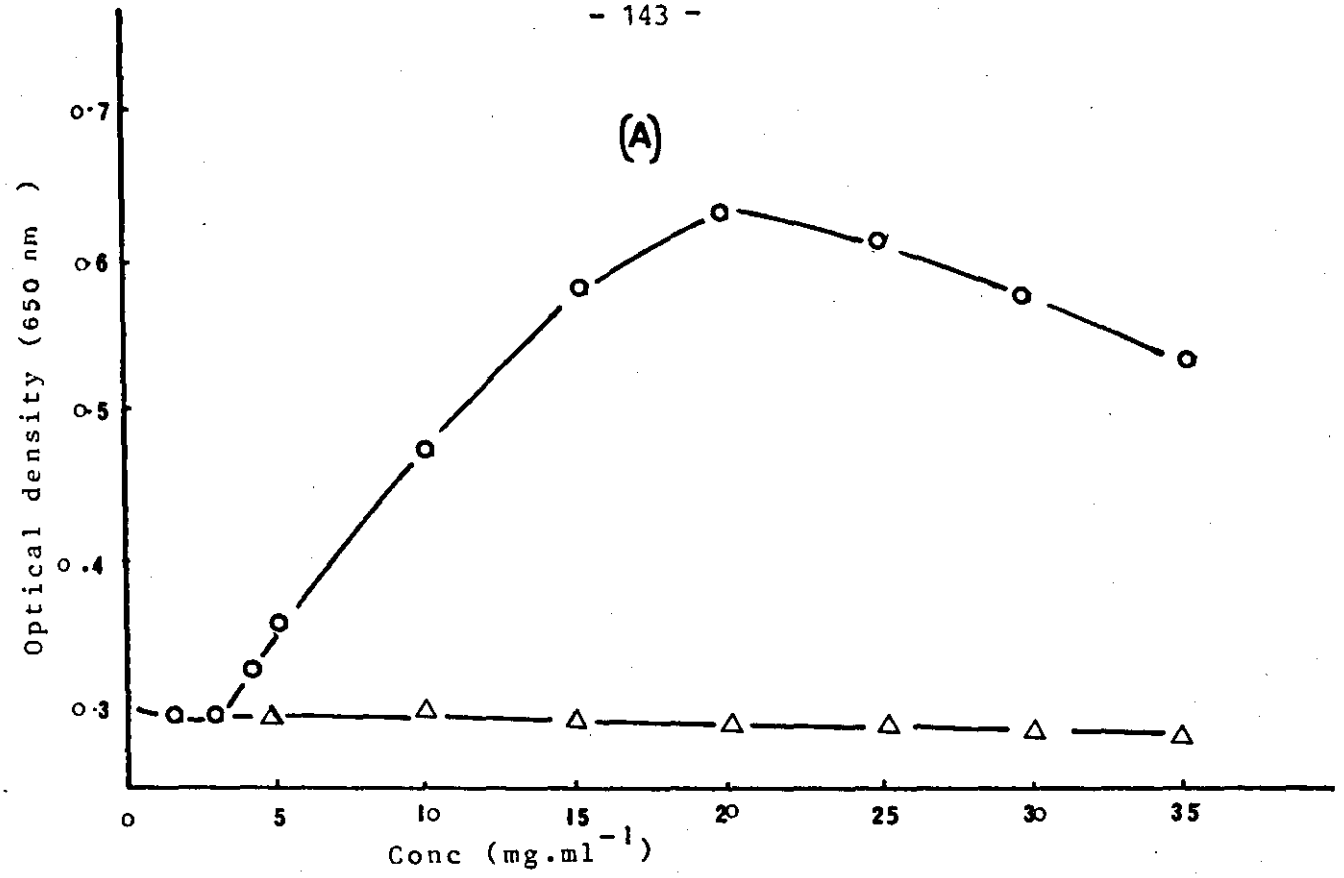

(B)
Optical density (650 nm)
0·8
0·7
0·6
0·5
0·4
0·3
0
5
10
15
20
25
30
35
Conc (mg.ml^{-1})

Fig 3.56 Turbidity changes (optical density at 650 nm) in cells of K. aerogenes suspended in CFM containing different LA as a function of LA concentration. Cell concentration 0.12 $mg.ml^{-1}$. Temp $22^{o}C$, contact time 10 min.

(A) o——o Amethocaine; Δ——Δ Procaine

(B) ●——● Cinchocaine; ▲——▲ Lignocaine


(A)
Optical density (650 nm)
0·7
0·6
0·5
0·4
0·3
0
5
10
15
20
25
30
35
Conc (mg.ml⁻¹)

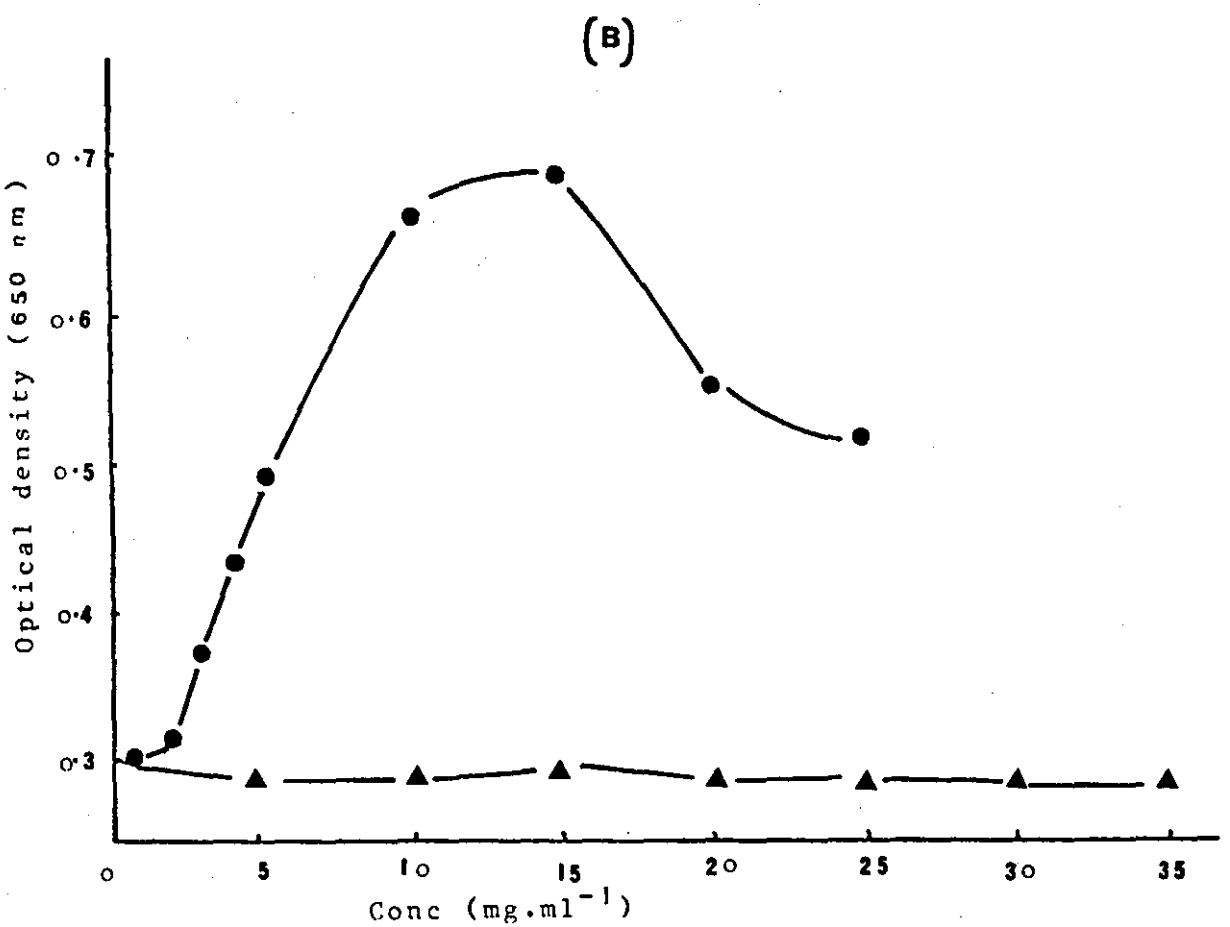
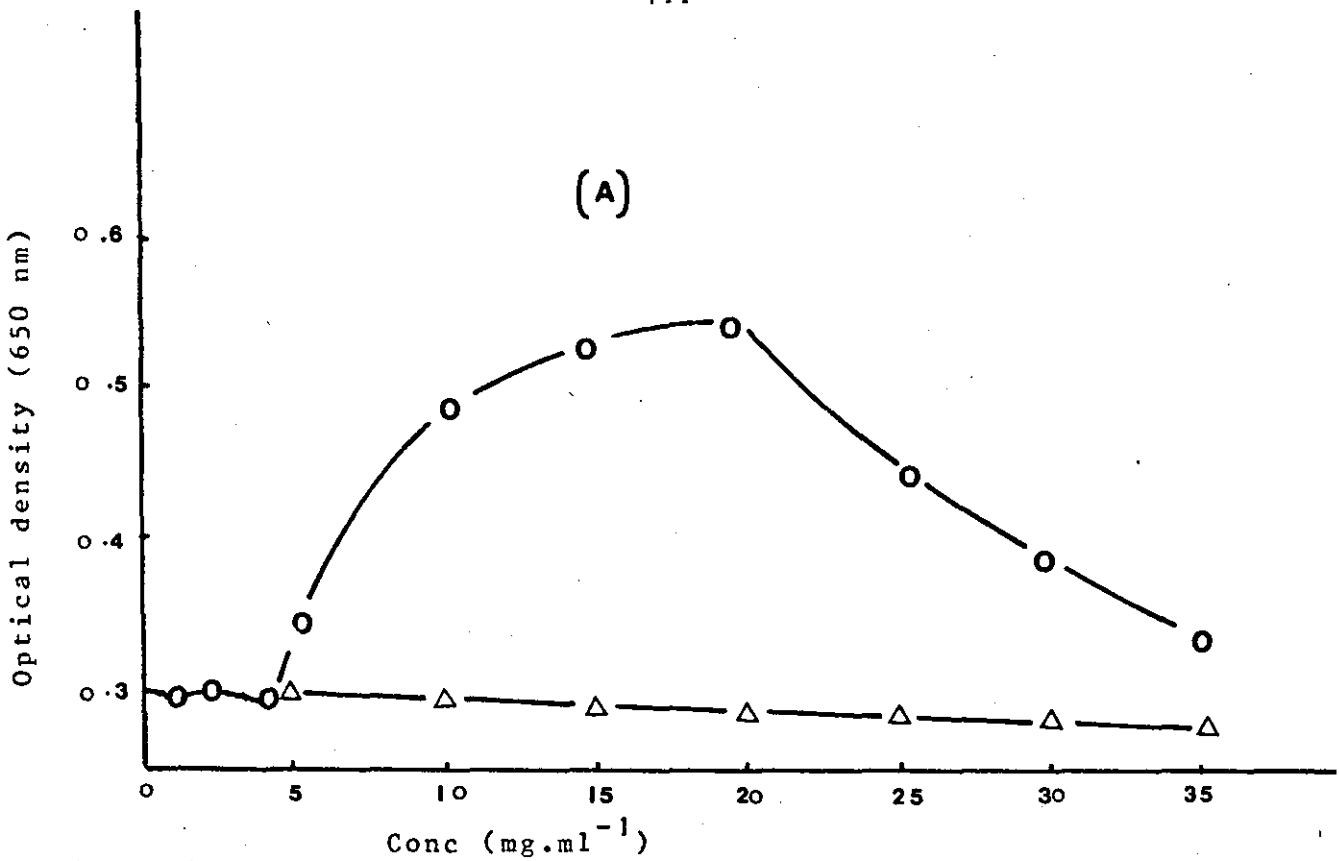

(B)
Optical density (650 nm)
0·7
0·6
0·5
0·4
0·3
0
5
10
15
20
25
30
35
Conc (mg.ml⁻¹)

Fig 3.57 Turbidity changes (optical density at 650 nm) in cells of P.aeruginosa suspended in CFM containing different LA as a function of LA concentration. Cell concentration 0.125 mg.ml^{-1}. Temp 22^{o}C. Contact time 10 min.

(A) o—o Amethocaine; Δ—Δ Procaine

(B) ●—● Cinchocaine; ▲—▲ Lignocaine


(A)
Optical density (650 nm)
0.6
0.5
0.4
0.3
0
5
10
15
20
25
30
35
Conc (mg.ml^{-1})

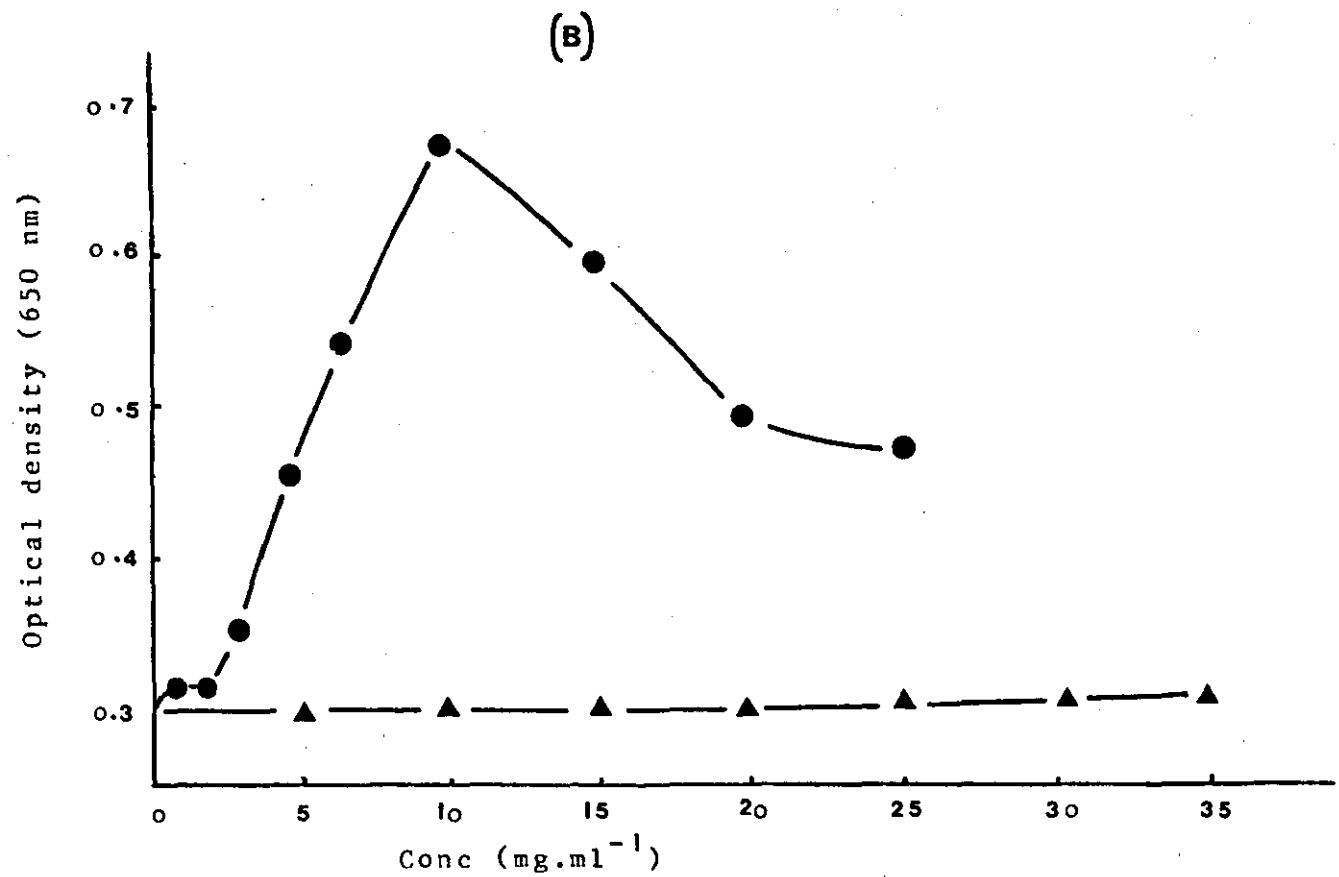
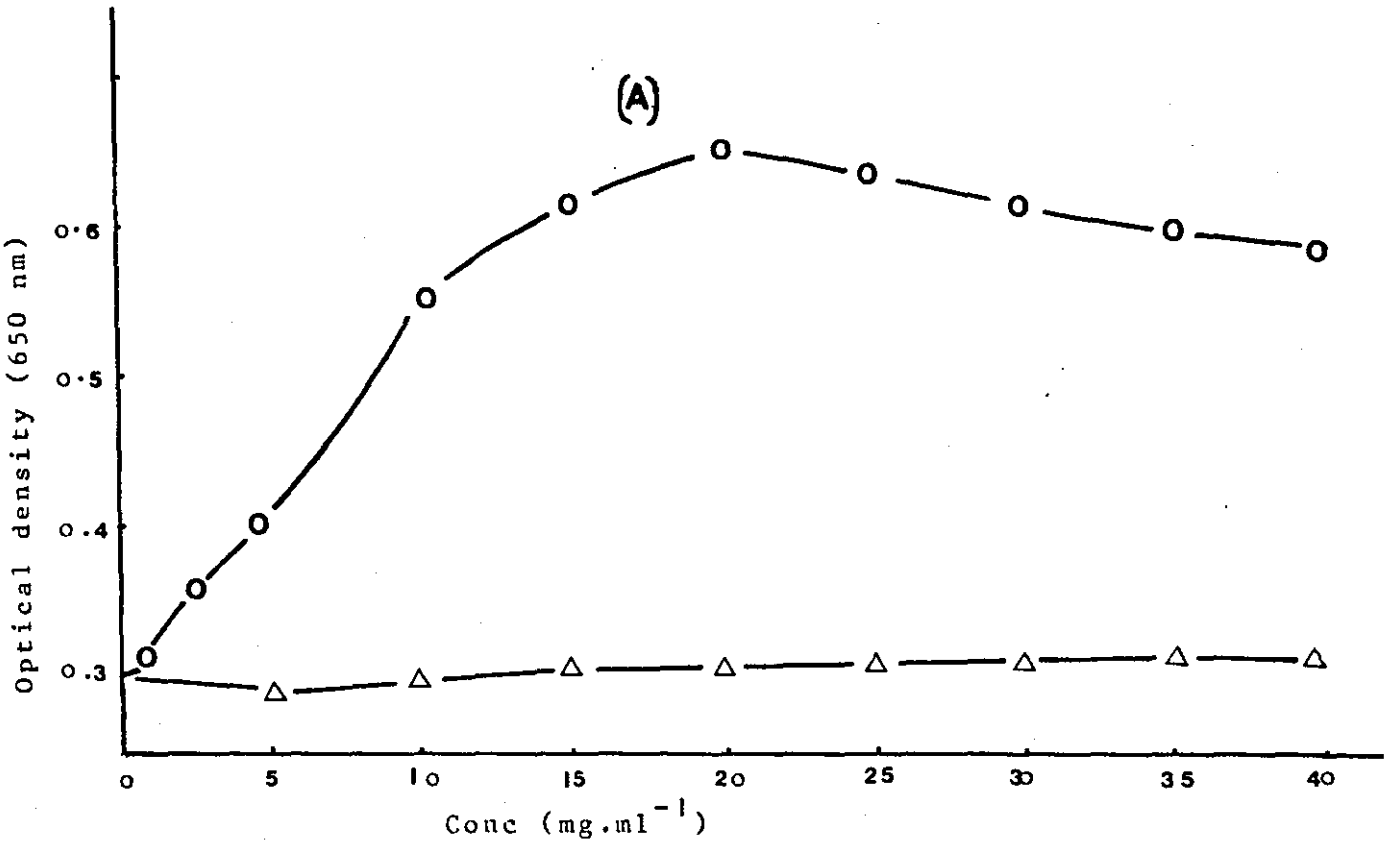

(B)
Optical density (650 nm)
0.7
0.6
0.5
0.4
0.3
0
5
10
15
20
25
30
35
Conc (mg.ml^{-1})

Fig 3.58 Turbidity changes (optical density at 650 nm) in cells of B.megaterium suspended in CFM containing different LA as a function of LA concentration. Cell concentration 0.070 $mg.ml^{-1}$. Temp $22^{o}C$. Contact time 10 min.

(A) o——o Amethocaine; Δ——Δ Procaine

(B) ●——● Cinchocaine; ▲——▲ Lignocaine


(A)
Optical density (650 nm)
0·6
0·5
0·4
0·3
0
5
10
15
20
25
30
35
40
Conc (mg.ml⁻¹)

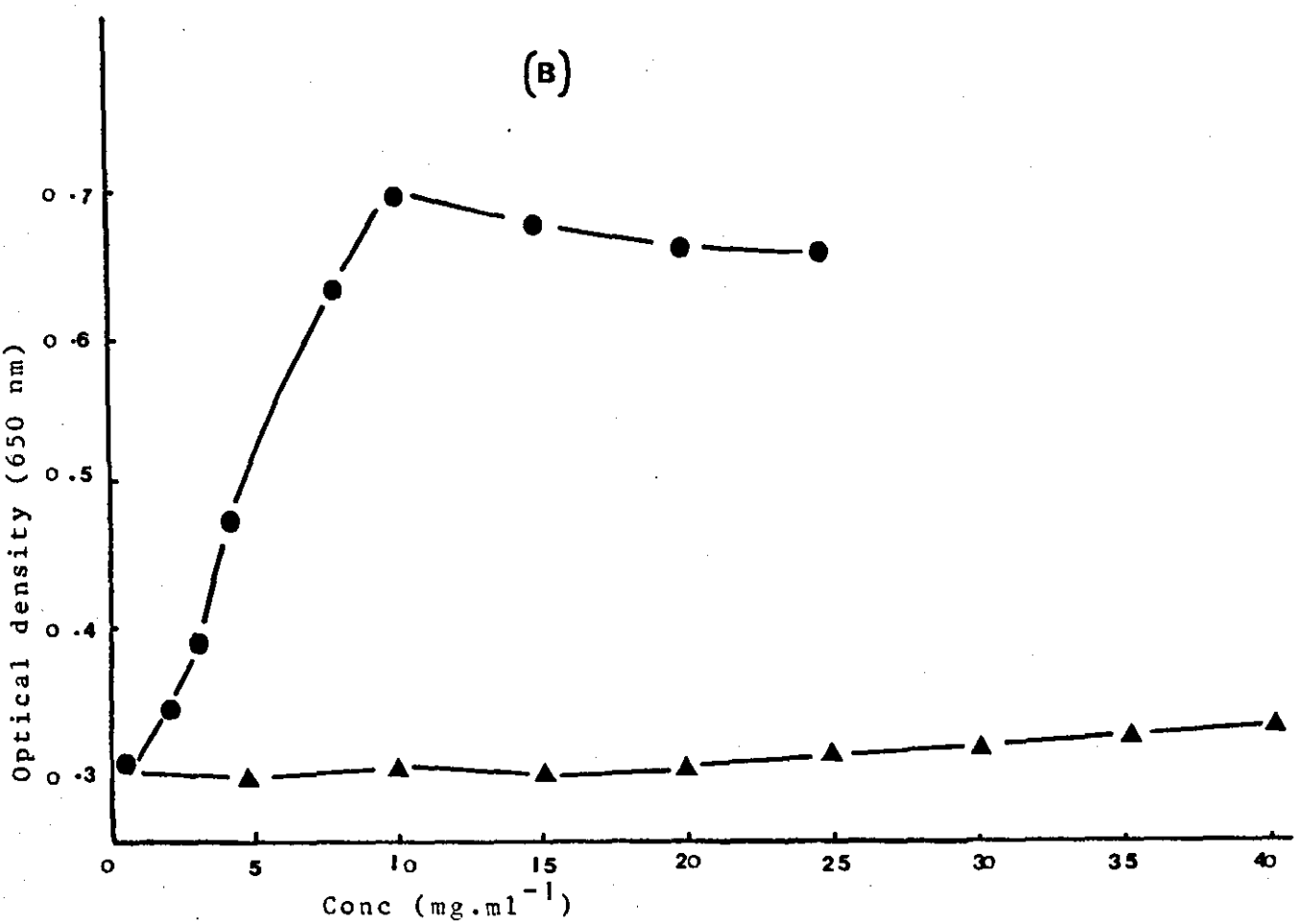

(B)
Optical density (650 nm)
0·7
0·6
0·5
0·4
0·3
0
5
10
15
20
25
30
35
40
Conc (mg.ml⁻¹)

Fig 3.59 Influence of time on the induced turbidity changes in cells of E.coli suspended in CFM containing LAs.

Cell number 3.8×10^8 cells.ml^{-1}. Temp 22^{o}C.

(A)

□—□ 10 mg.ml^{-1} amethocaine

o——o 30 mg.ml^{-1} amethocaine

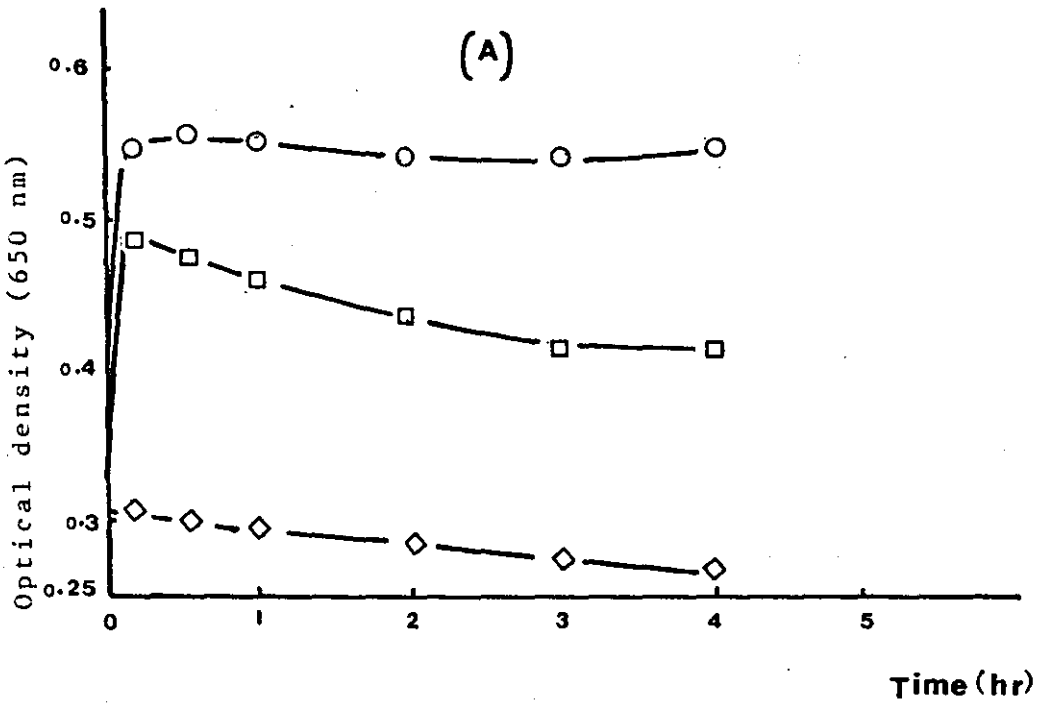
◇——◇ 50 mg.ml^{-1} procaine

(B)

■—■ 5 mg.ml^{-1} cinchocaine

●—● 20 mg.ml^{-1} cinchocaine

◆—◆ 40 mg.ml^{-1} lignocaine


(A)
Optical density (650 nm)
0.6
0.5
0.4
0.3
0.25
0
1
2
3
4
5
Time (hr)

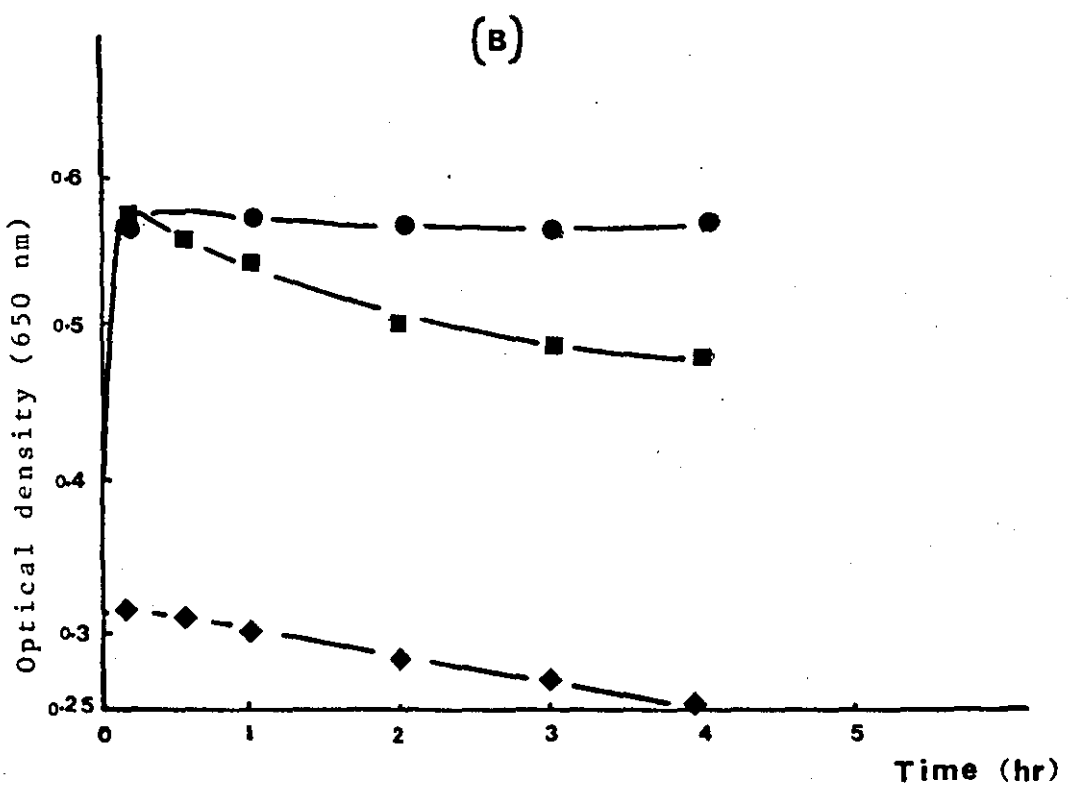
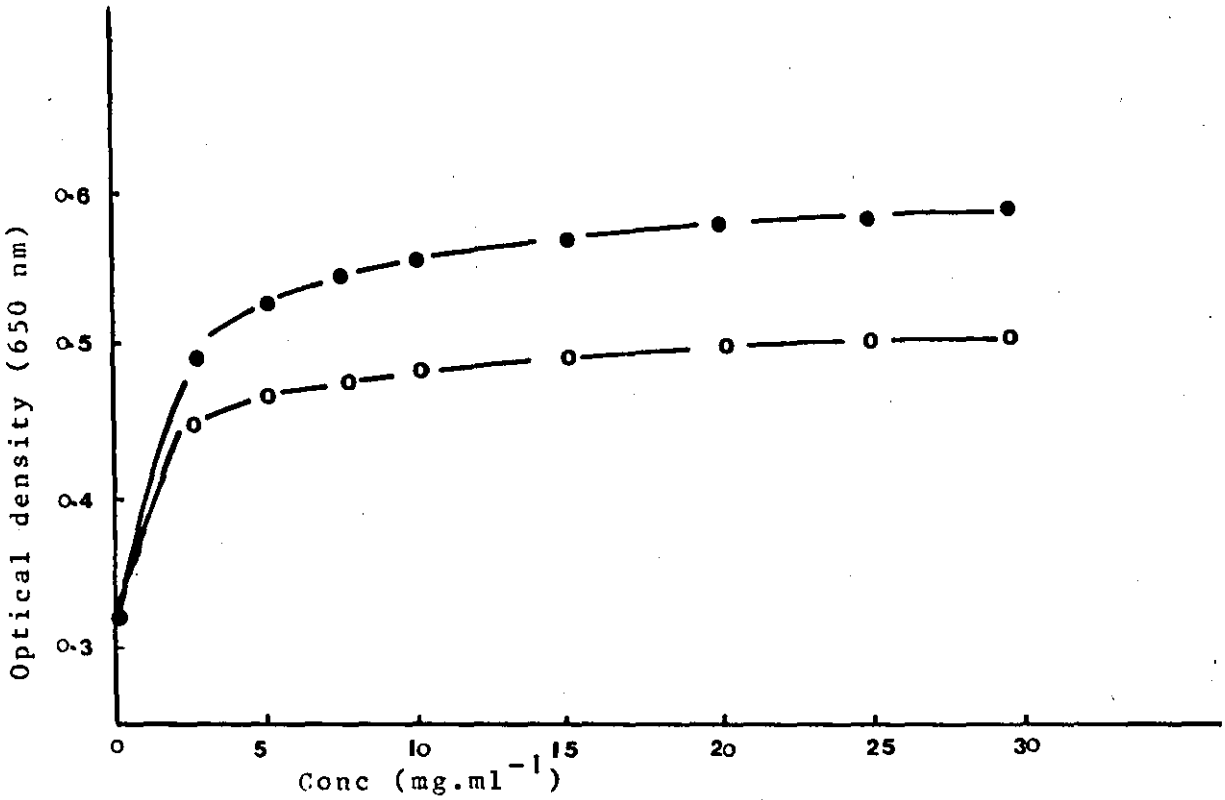

(B)
Optical density (650 nm)
0.6
0.5
0.4
0.3
0.25
0
1
2
3
4
5
Time (hr)

Fig 3.60 Turbidity changes (OD 650 nm) in LA treated cells of E.coli suspended in distilled water. Cell concentration 4.0×10^8 cells.ml^{-1}, temp $22^{o}C$, contact time 10 min

o——o amethocaine, and ●——● cinchocaine,

Fig 3.61 Turbidity changes (OD 650 nm) in LA treated cells of E.coli suspended in phosphate buffer (pH 6.4, 0.2M). Cell concentration 4.0×10^8 cells.ml^{-1}, temp $22^{o}C$, contact time 10 min.

o——o amethocaine, and ●——● cinchocaine.


Optical density (650 nm)
0·6
0·5
0·4
0·3
0
5
10
15
20
25
30
Conc (mg.ml^{-1})

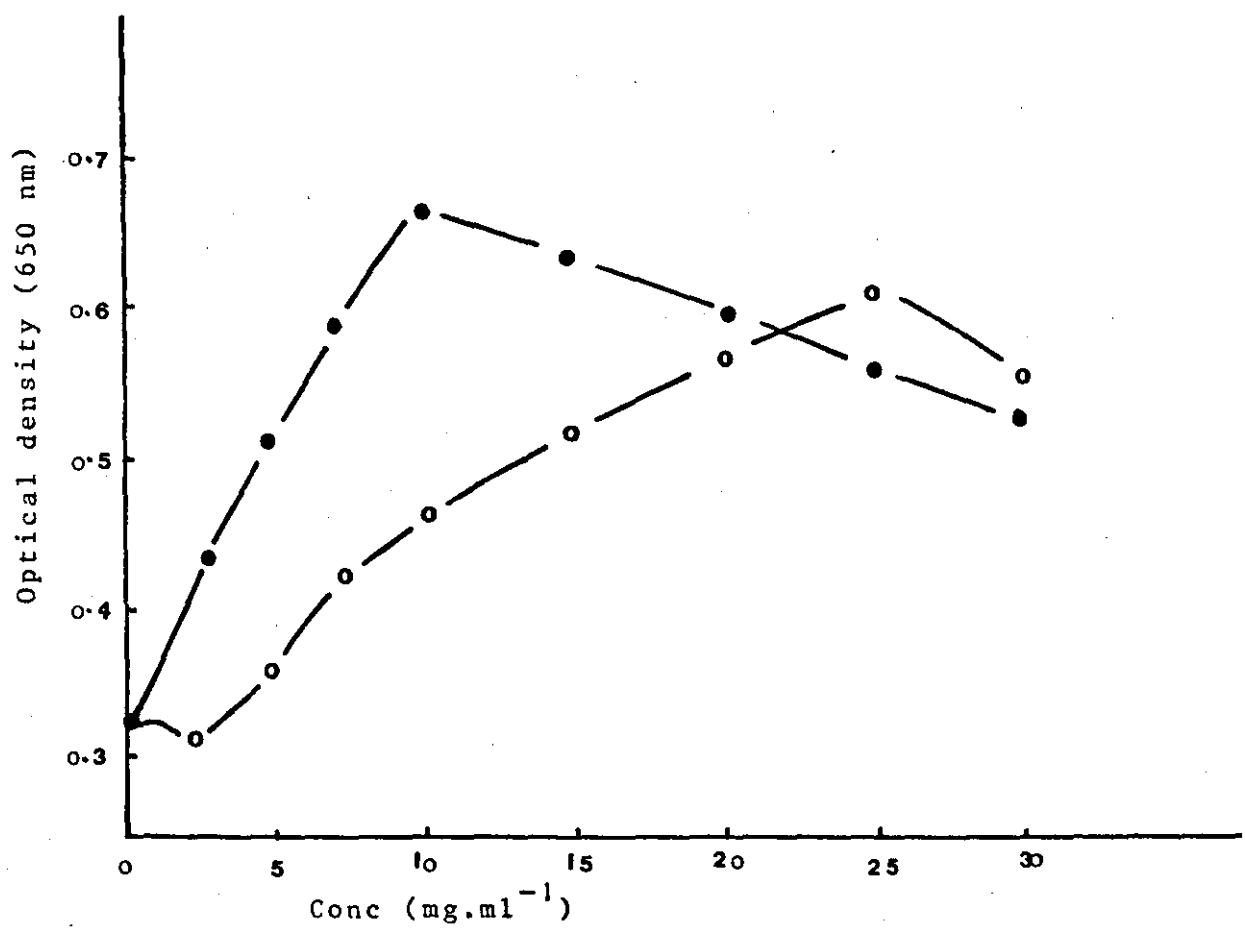

Optical density (650 nm)
0·7
0·6
0·5
0·4
0·3
0
5
10
15
20
25
30
Conc (mg.ml^{-1})

Fig 3.62 Turbidity changes (OD 650 nm) in amethocaine treated cells of E.coli suspended in CFM containing different concentrations of Mg^{++}. Cell concentration 3.8×10^8 cells.ml^{-1}. Temp $22^{\circ}C$, contact time 10 min. Mg^{++} concentration: ▽—▽ $\frac{M}{10,000}$; x—x $\frac{M}{2000}$; □—□ $\frac{M}{1000}$; o—o $\frac{M}{200}$; ▲—▲ $\frac{M}{100}$; ●—● $\frac{M}{20}$; +—+ $\frac{M}{10}$

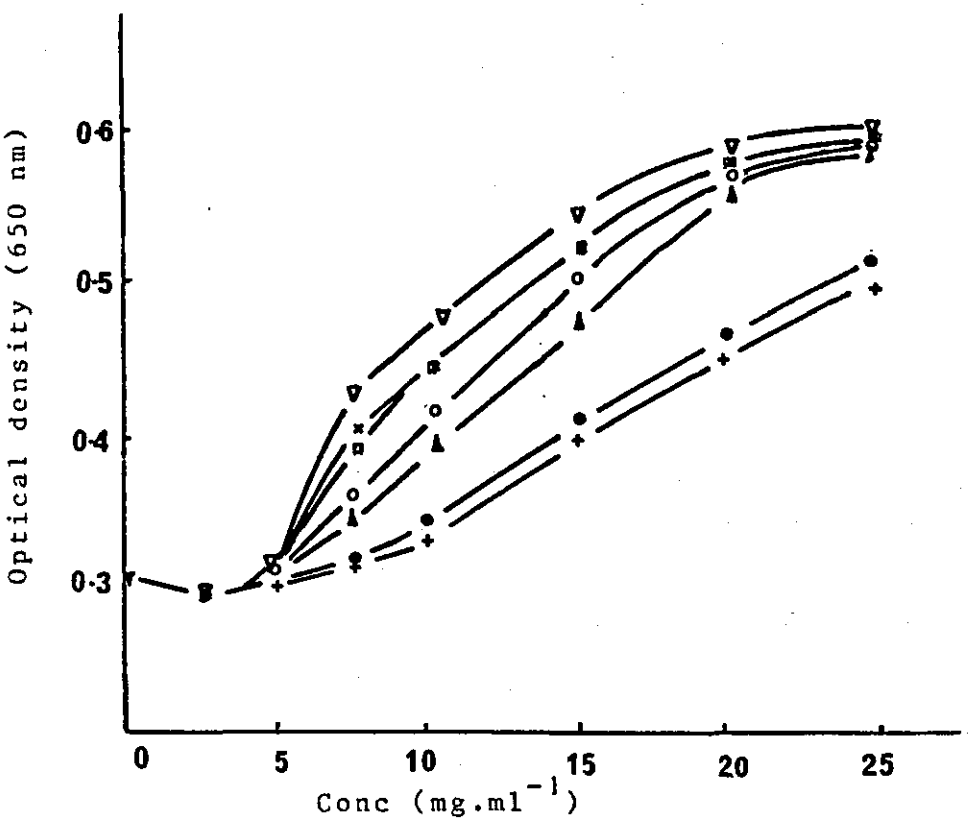
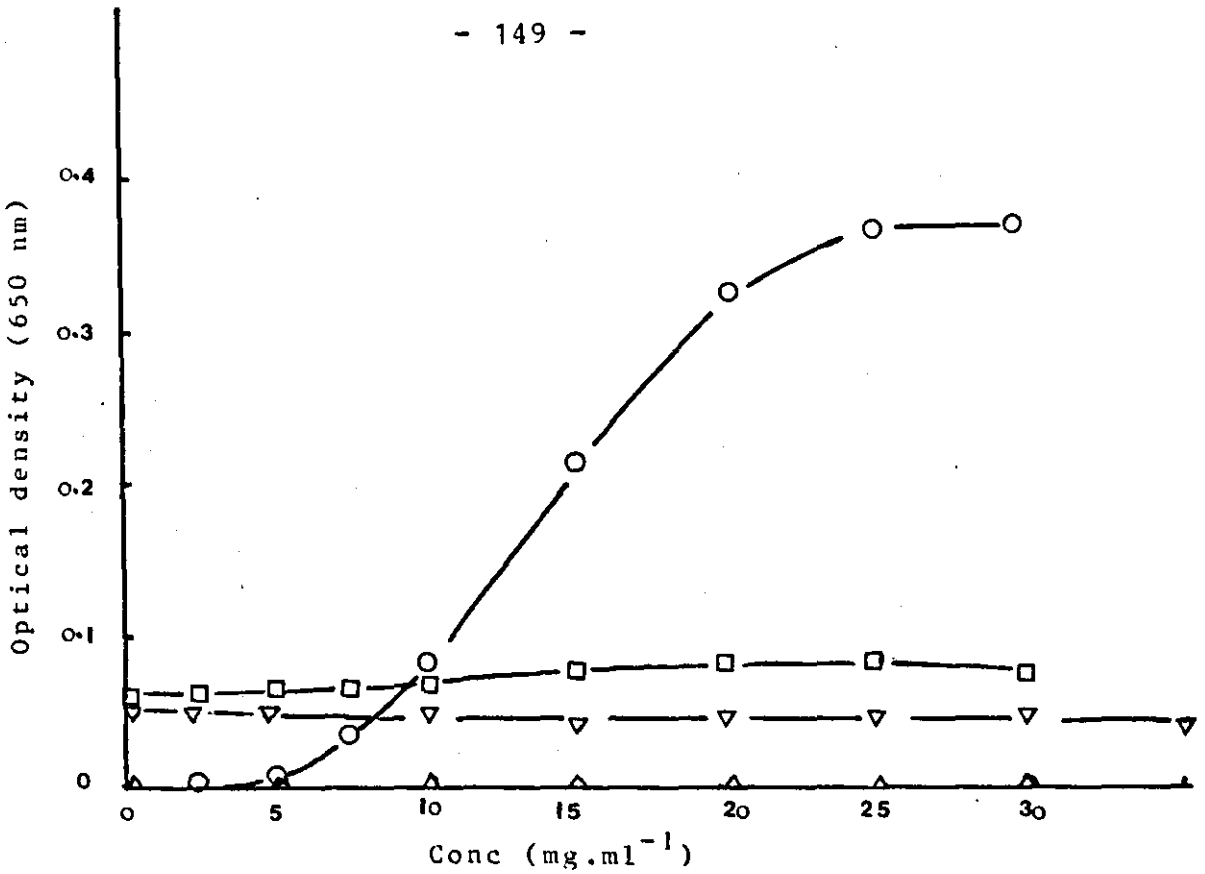

Optical density (650 nm)
0·6
0·5
0·4
0·3
0
5
10
15
20
25
Conc (mg.ml^{-1})

Fig 3.63 The effect of amethocaine and procaine on optical density (650 nm) of E.coli cell envelope preparation (□—□,amethocaine; ▽—▽,procaine) and intracellular material (o—o, amethocaine; Δ—Δ,procaine) in CFM. Cell concentration ca. 3.8×10^8 cells.ml^{-1}. Contact time 10 min. Temp 22°C.

Fig 3.64 The effect of cinchocaine and lignocaine on optical density (650 nm) of E.coli cell envelope preparation (■—■,cinchocaine; ▼—▼,lignocaine) and intracellular material (●—●,cinchocaine; ▲—▲,lignocaine) in CFM. Cell concentration ca. 3.8×10^8 cells.ml^{-1}. Contact time 10 min. Temp 22°C.


Optical density (650 nm)
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
Conc (mg.ml^{-1})

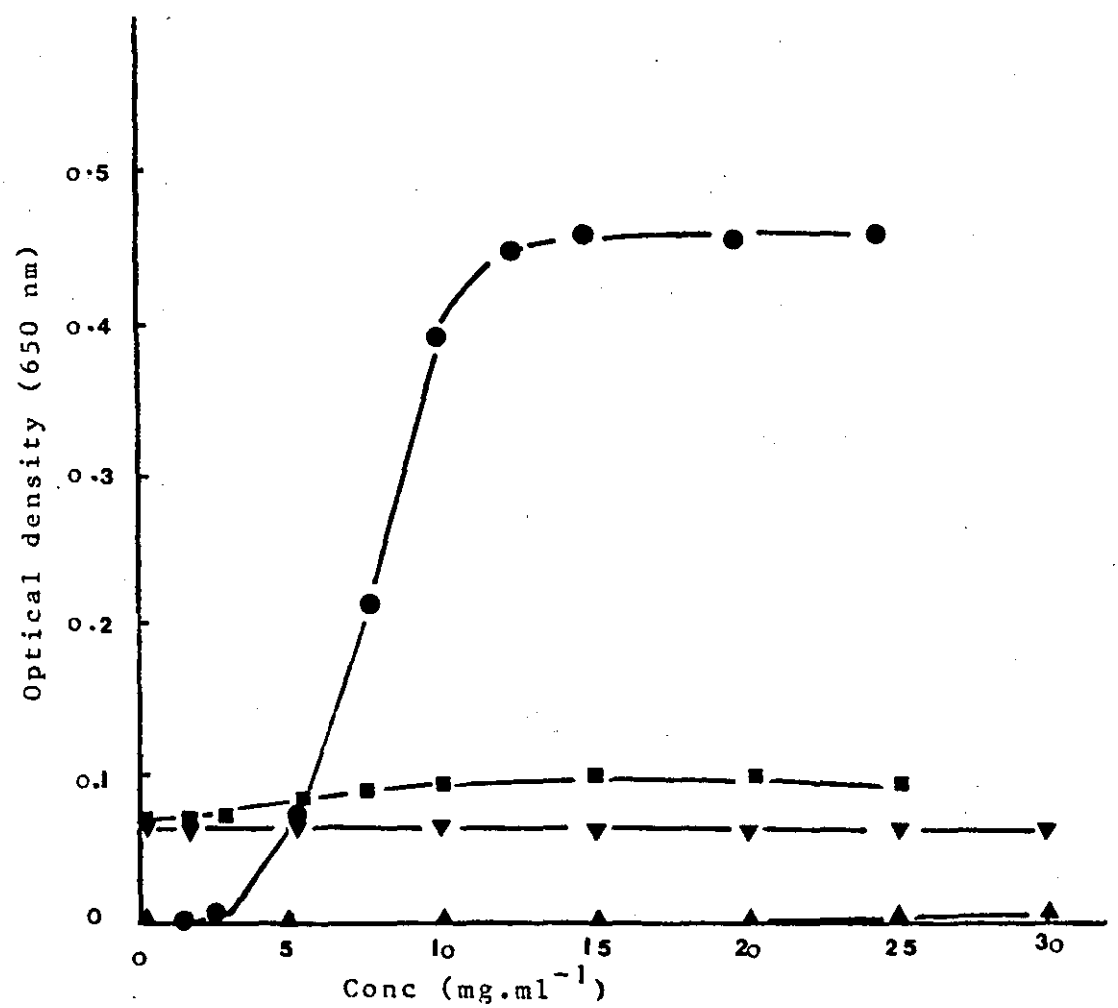
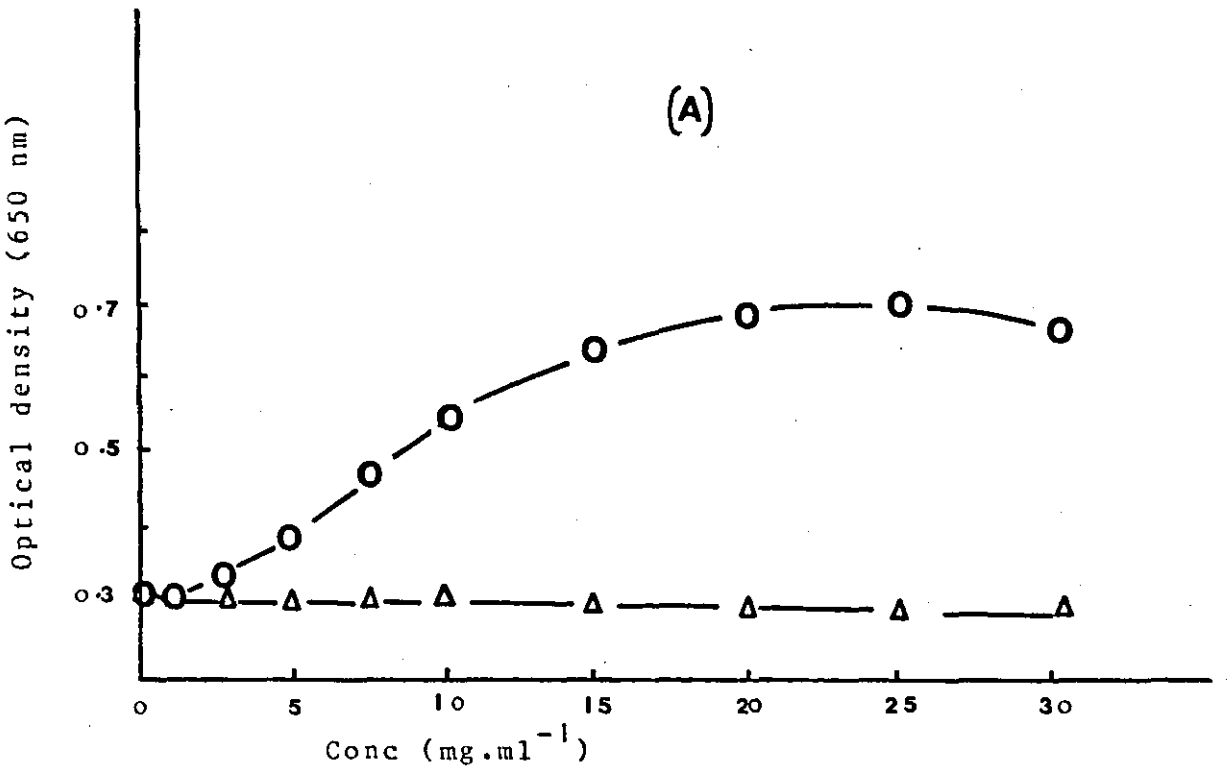

Optical density (650 nm)
0·5
0·4
0·3
0·2
0·1
0
0
5
10
15
20
25
30
Conc (mg.ml^{-1})

Fig 3.65 Turbidity changes (650 nm) of lipid depleted cells of E.coli suspended in CFM as a function of LA concentration. Contact time 10 min, Temp 22°C.

(A) o——o Amethocaine, Δ——Δ Procaine

(B) ●——● Cinchocaine, ▲——▲ Lignocaine


(A)
Optical density (650 nm)
0·7
0·5
0·3
0
5
10
15
20
25
30
Conc (mg.ml⁻¹)

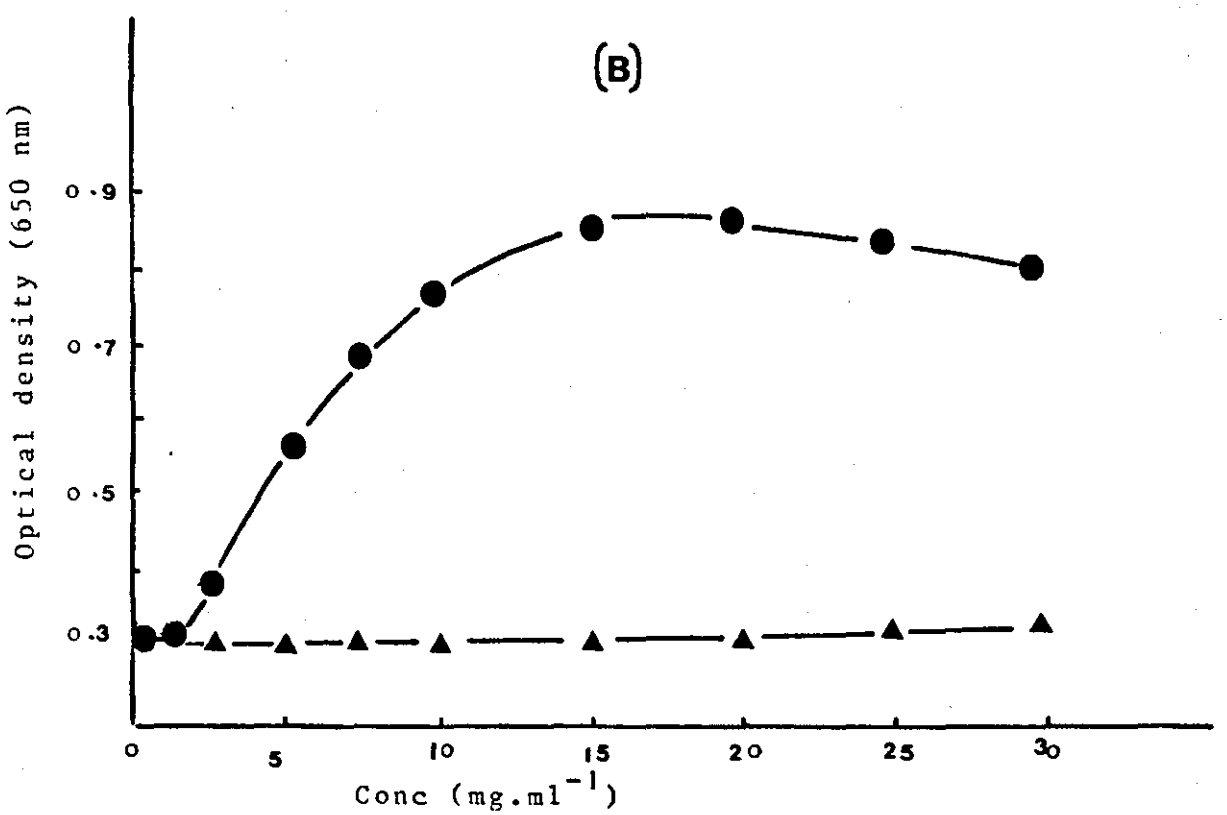
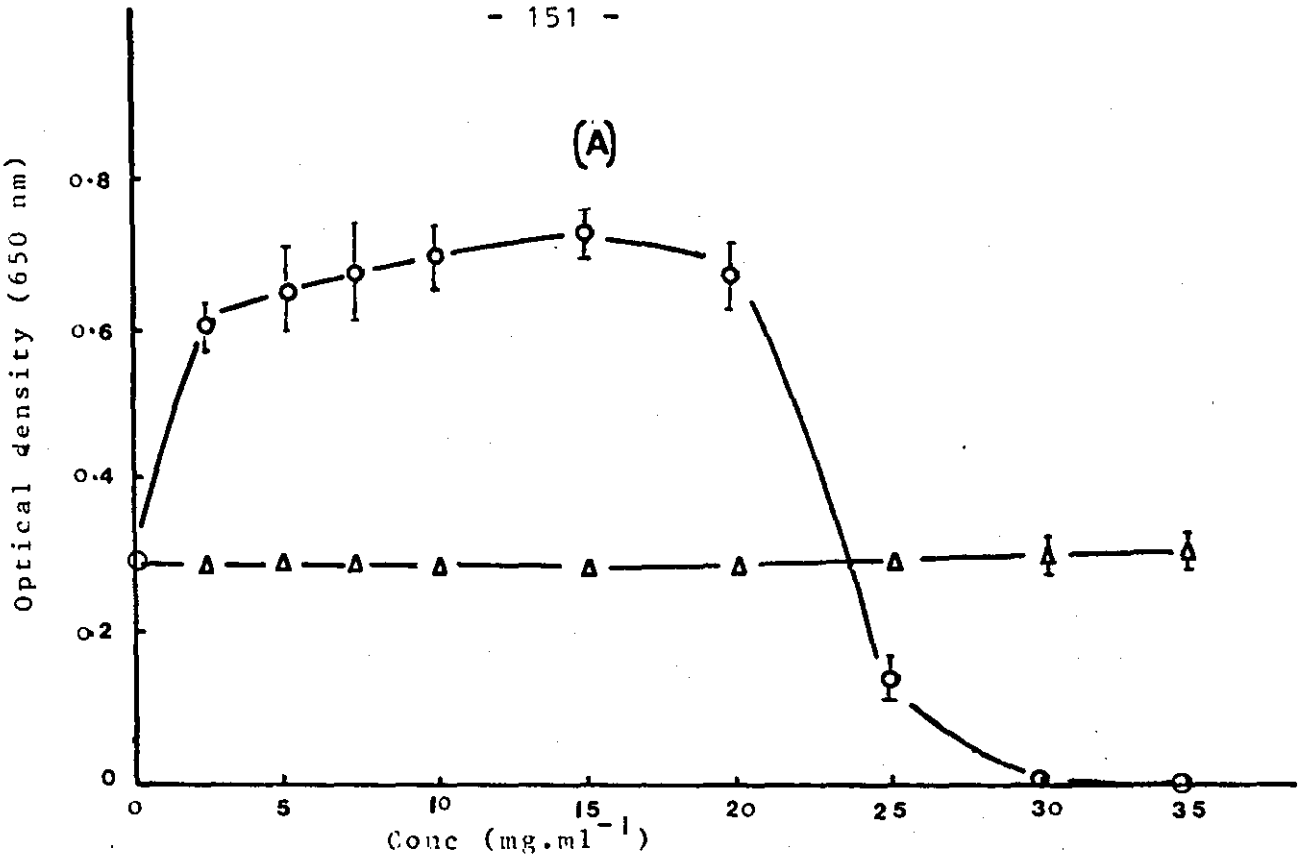

(B)
Optical density (650 nm)
0·9
0·7
0·5
0·3
0
5
10
15
20
25
30
Conc (mg.ml⁻¹)

Fig 3.66 Turbidity changes (650 nm) of lipids, extracted from E.coli cells, and dispersed in CFM as a function of LA concentration. Contact time 10 min, Temp 22°C.

(A) o—o Amethocaine, Δ—Δ Procaine

(B) ●—● Cinchocaine, ▲—▲ Lignocaine


(A)
Optical density (650 nm)
0·8
0·6
0·4
0·2
0
0
5
10
15
20
25
30
35
Conc (mg.ml⁻¹)

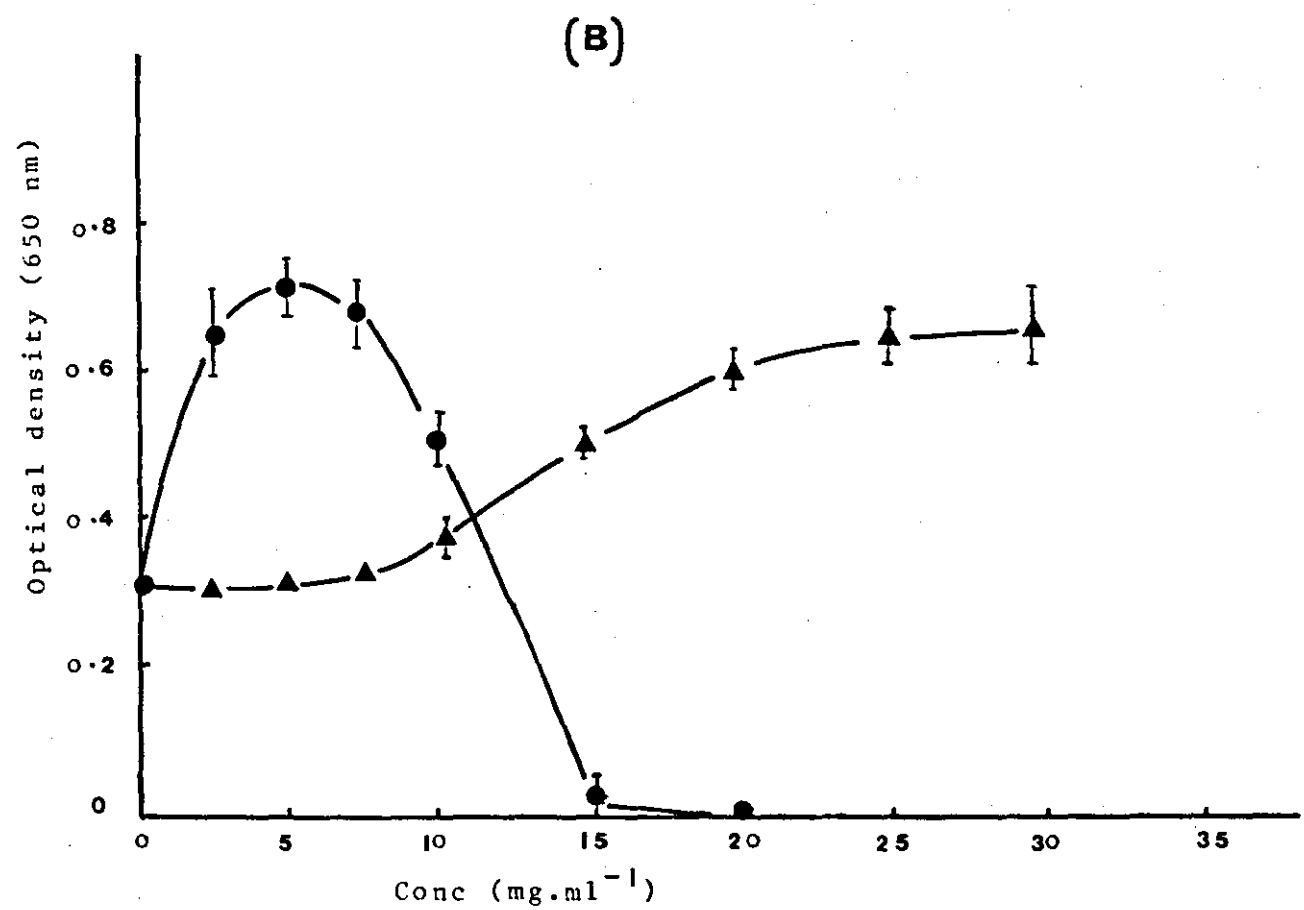
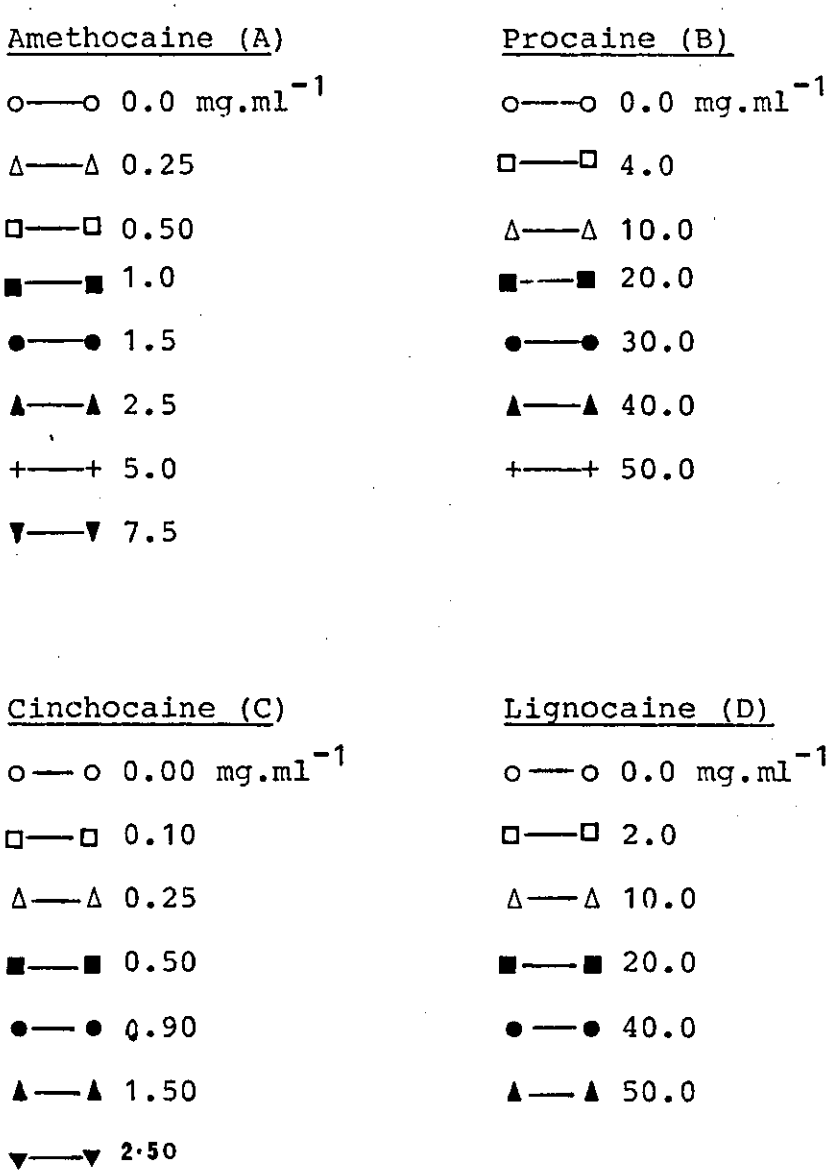
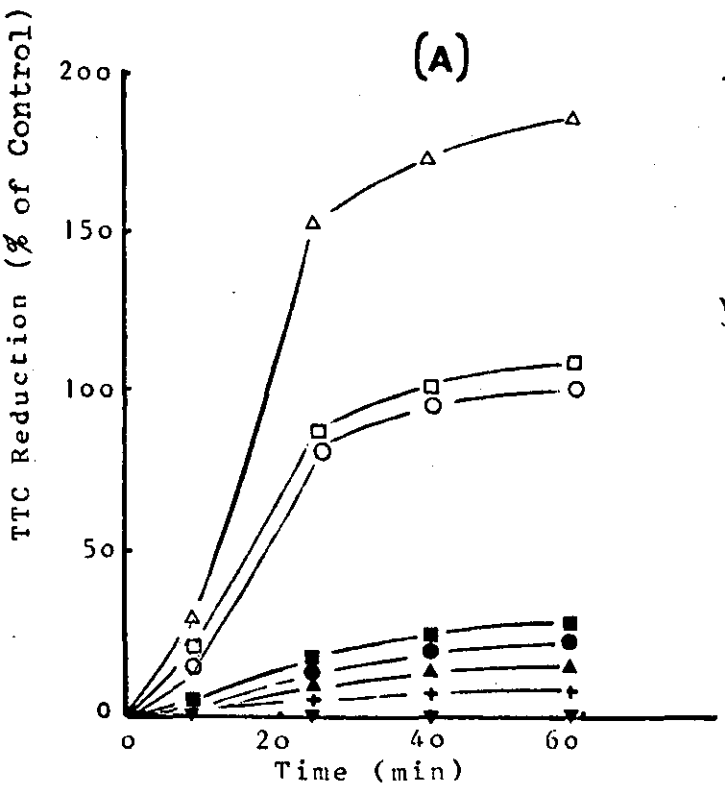
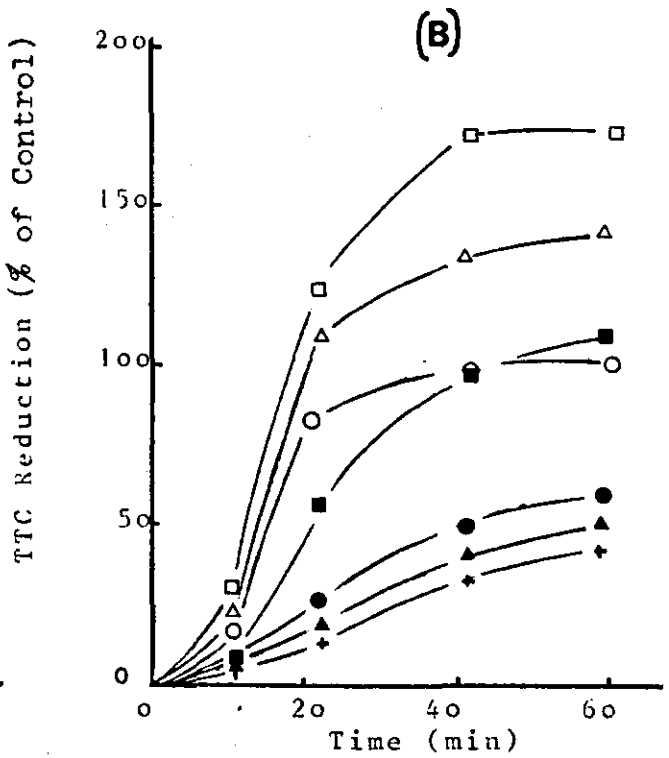
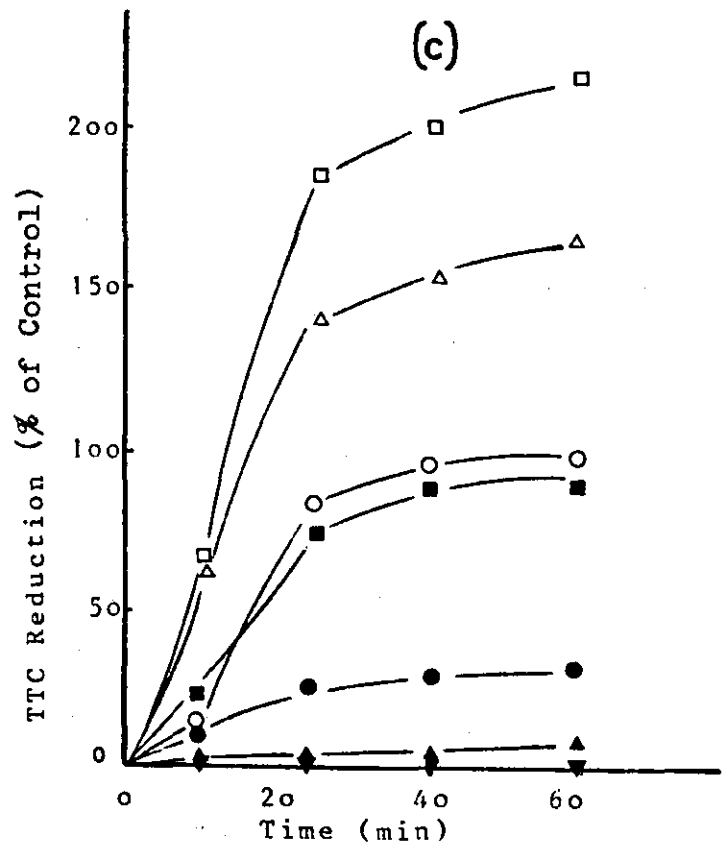

(B)
Optical density (650 nm)
0·8
0·6
0·4
0·2
0
0
5
10
15
20
25
30
35
Conc (mg.ml⁻¹)

Fig 3.67 The effect of amethocaine (A), procaine (B), cinchocaine (C) and lignocaine (D) on the reduction of TTC by a suspension of E.coli cells in CFM using glucose as substrate. Cell concentration 3.8×10^9 cells.ml^{-1}. Temp 37°C.




(A)
TTC Reduction (% of Control)
200
150
100
50
0
0
20
40
60
Time (min)


(B)
TTC Reduction (% of Control)
200
150
100
50
0
0
20
40
60
Time (min)


(C)
TTC Reduction (% of Control)
200
150
100
50
0
0
20
40
60
Time (min)

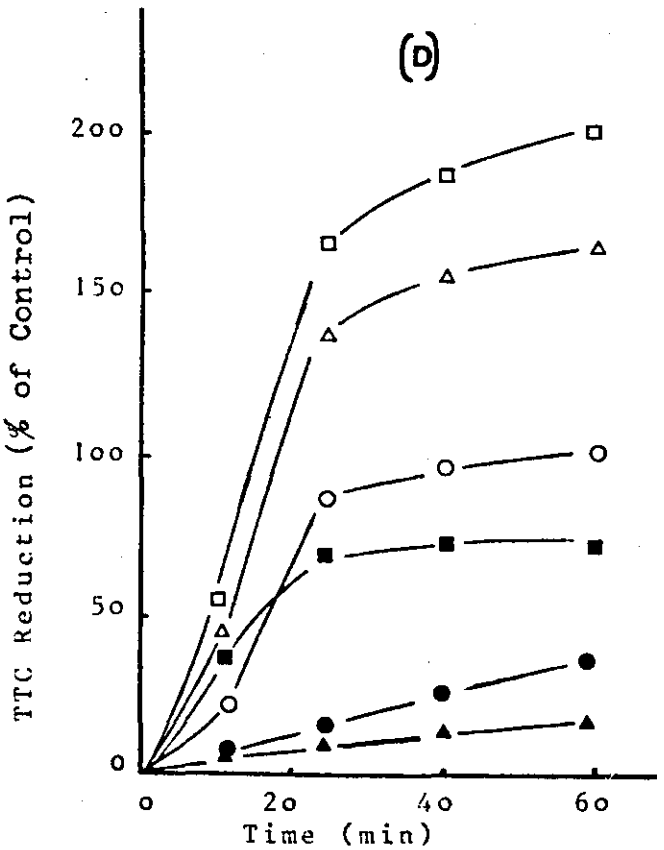
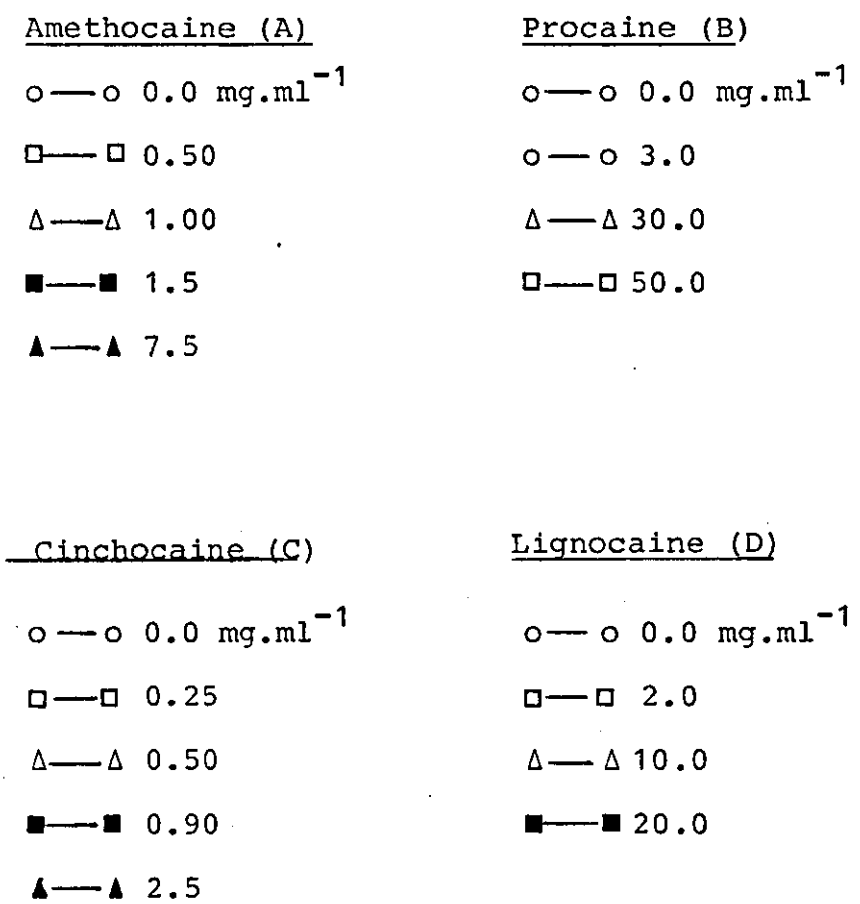
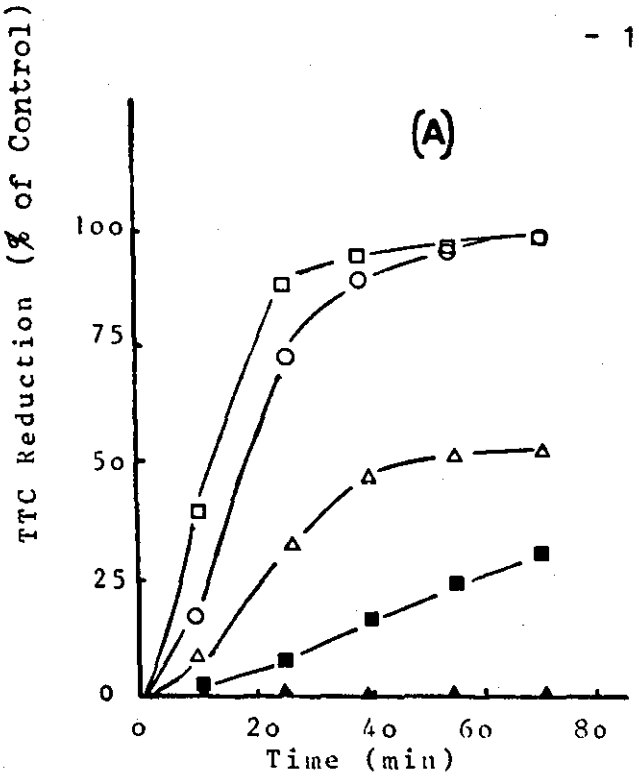
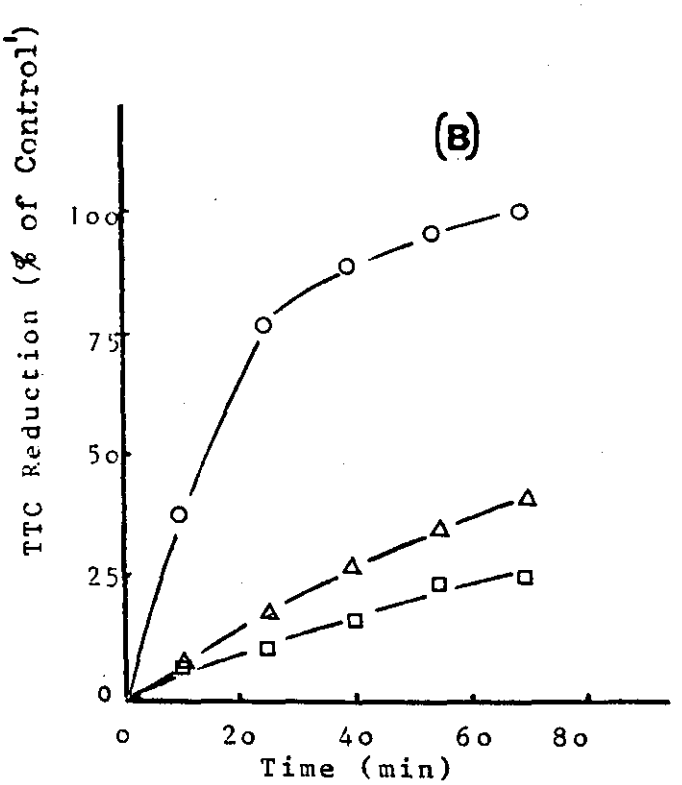
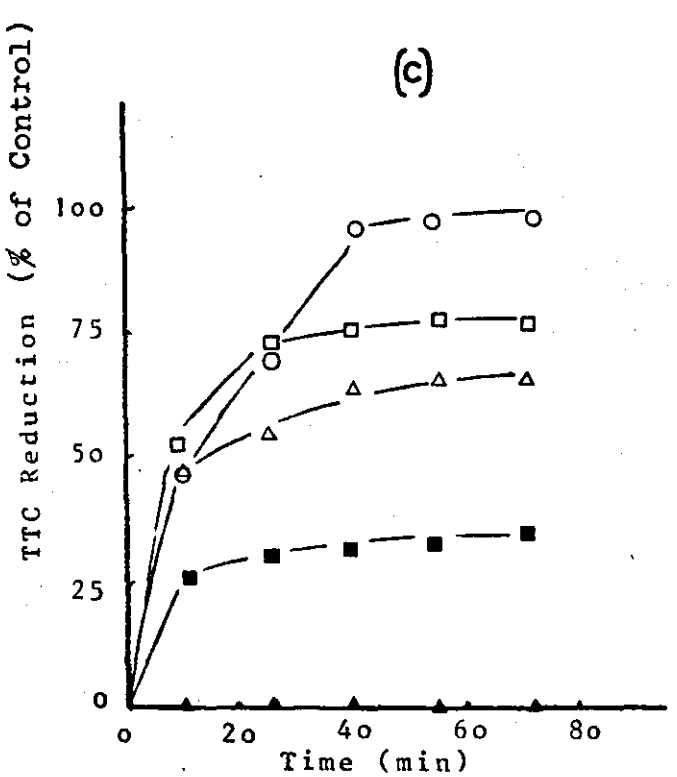

(D)
TTC Reduction (% of Control)
200
150
100
50
0
0
20
40
60
Time (min)

Fig 3.68 The effect of amethocaine (A), procaine (B), cinchocaine (C) and lignocaine (D) on the reduction of TTC by a suspension of E.coli cells in phosphate buffer (pH 6.4, 0.20M) using glucose as substrate. Cell concentration 3.8×10^9 cells.ml^{-1}. Temp 37°C.




(A)
TTC Reduction (% of Control)
100
75
50
25
0
0
20
40
60
80
Time (min)


(B)
TTC Reduction (% of Control)
100
75
50
25
0
0
20
40
60
80
Time (min)


(C)
TTC Reduction (% of Control)
100
75
50
25
0
0
20
40
60
80
Time (min)

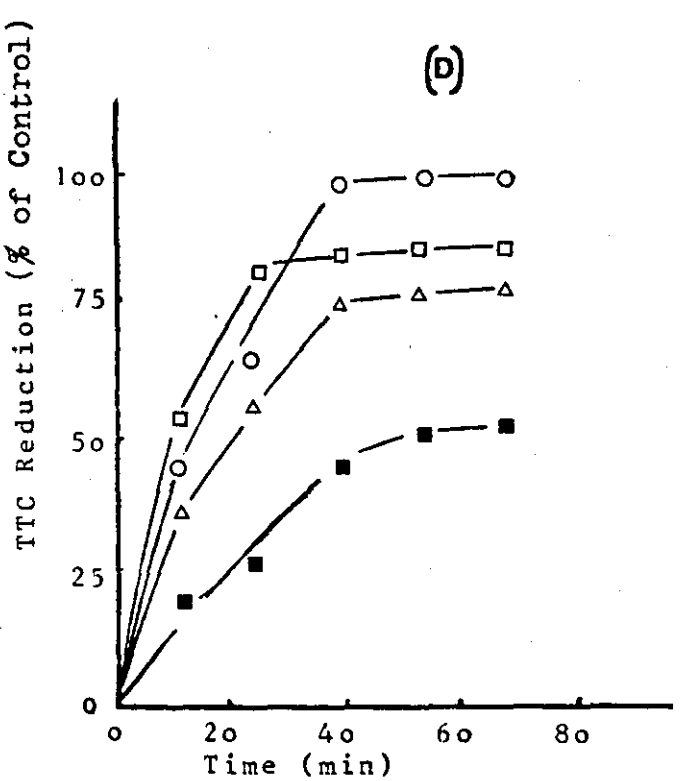
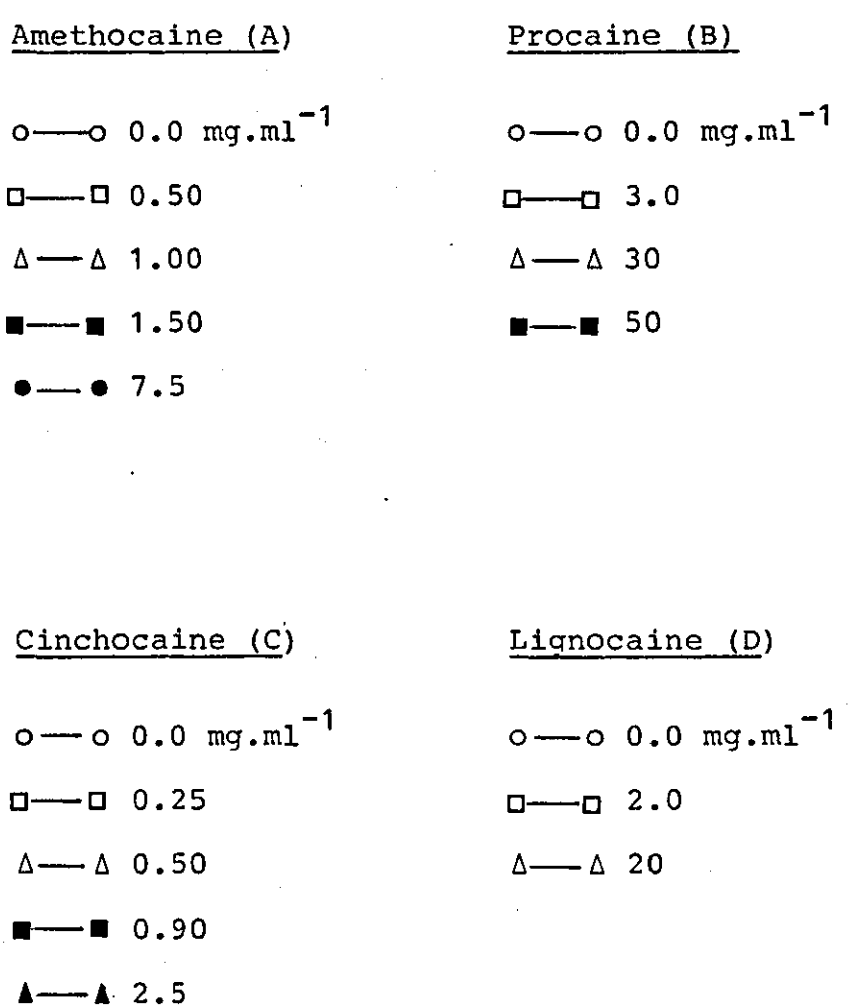
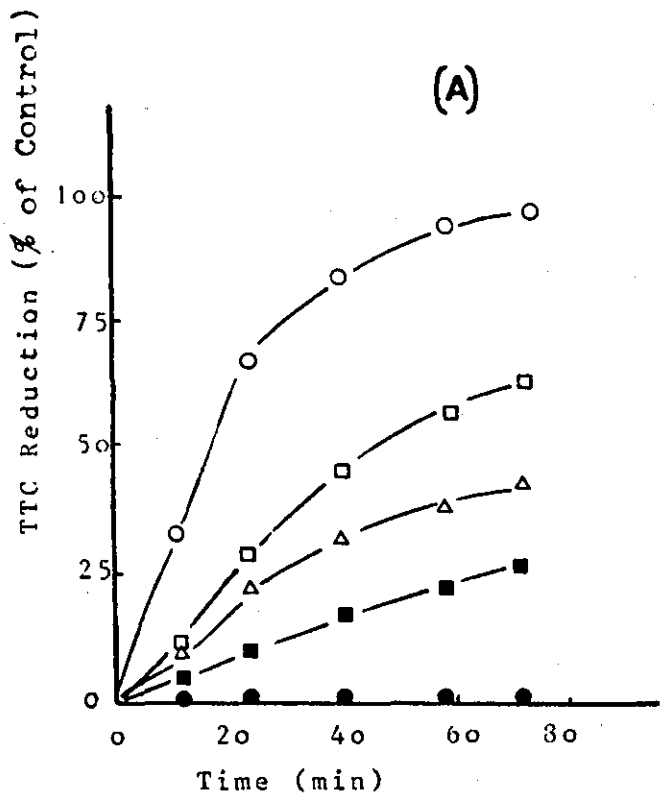
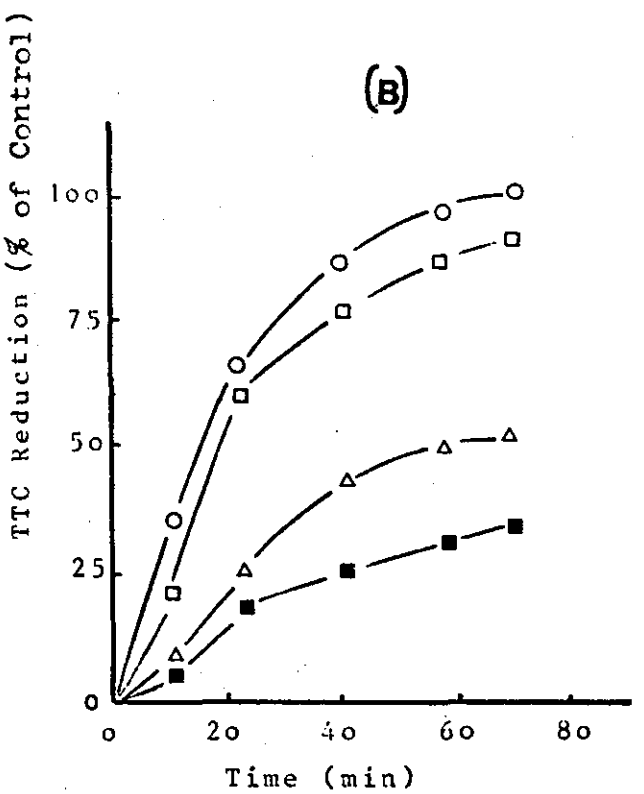
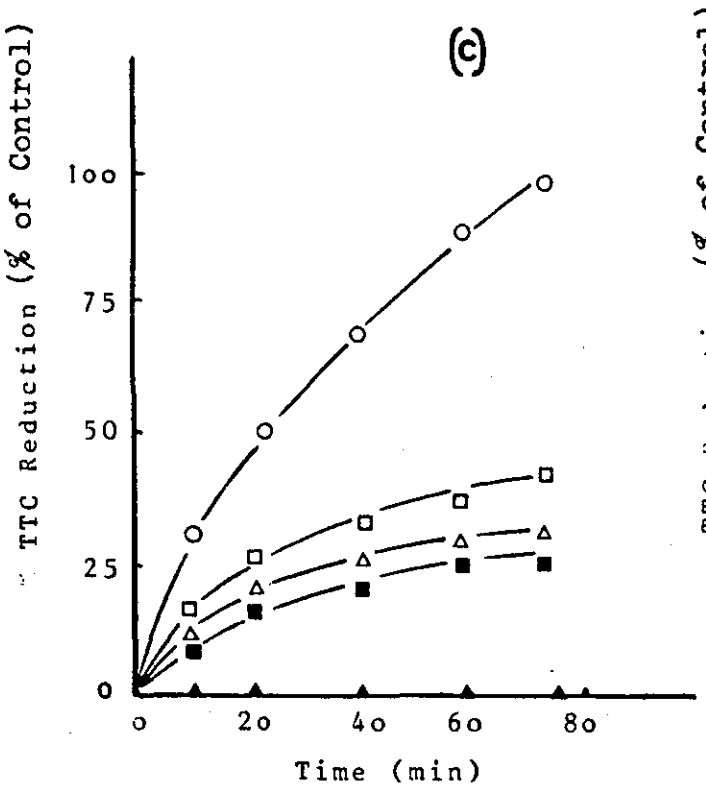

(D)
TTC Reduction (% of Control)
100
75
50
25
0
0
20
40
60
80
Time (min)

Fig 3.69 The effect of amethocaine (A), procaine (B), cinchocaine (C) and lignocaine (D) on the reduction of TTC by a suspension of E.coli cells in phosphate buffer (pH 6.4, 0.20M) using succinate as substrate. Cell concentration 3.8 x 10^9 cells.ml^{-1}. Temp 37°C.




(A)
TTC Reduction (% of Control)
100
75
50
25
0
0
20
40
60
80
Time (min)


(B)
TTC Reduction (% of Control)
100
75
50
25
0
0
20
40
60
80
Time (min)


(C)
TTC Reduction (% of Control)
100
75
50
25
0
0
20
40
60
80
Time (min)

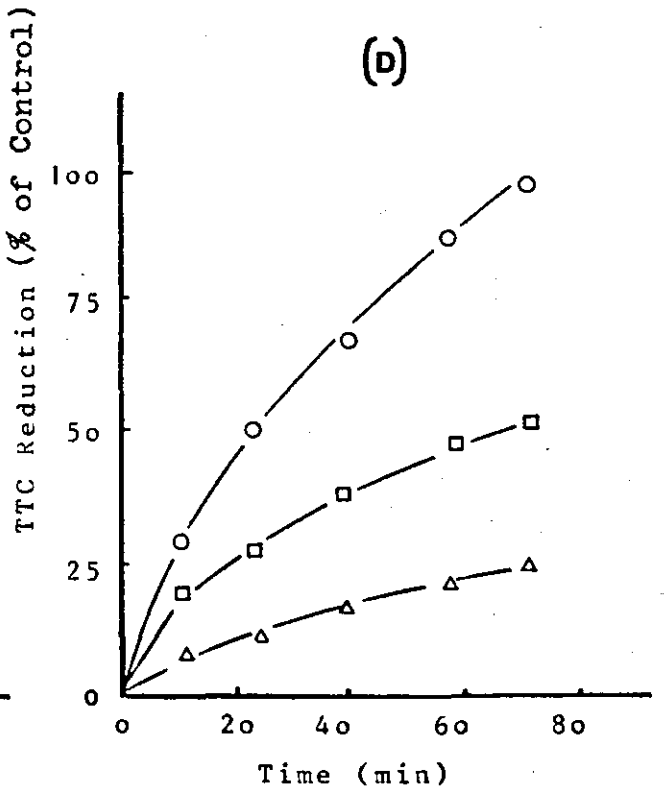
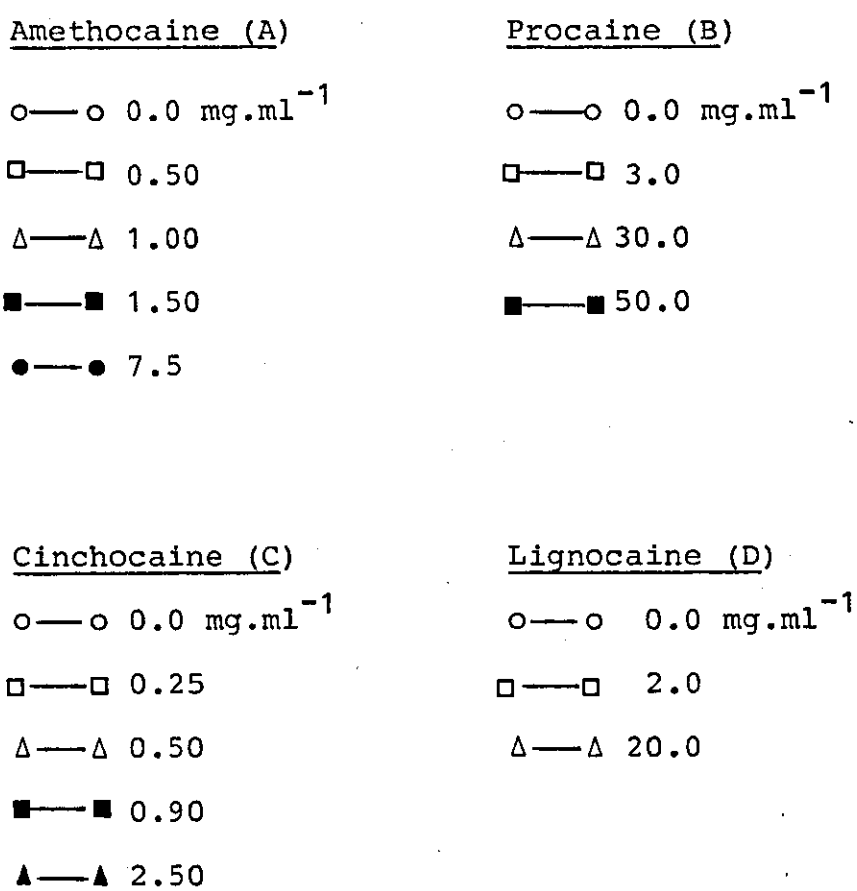

(D)
TTC Reduction (% of Control)
100
75
50
25
0
0
20
40
60
80
Time (min)

Fig 3.70 The effect of amethocaine (A), procaine (B), cinchocaine (C) and lignocaine (D) on the reduction of TTC by a suspension of E.coli cells in phosphate buffer (pH 6.4, 0.20M) using malate as substrate. Cell concentration 3.8×10^9 cells.ml^{-1}. Temp 37°C.



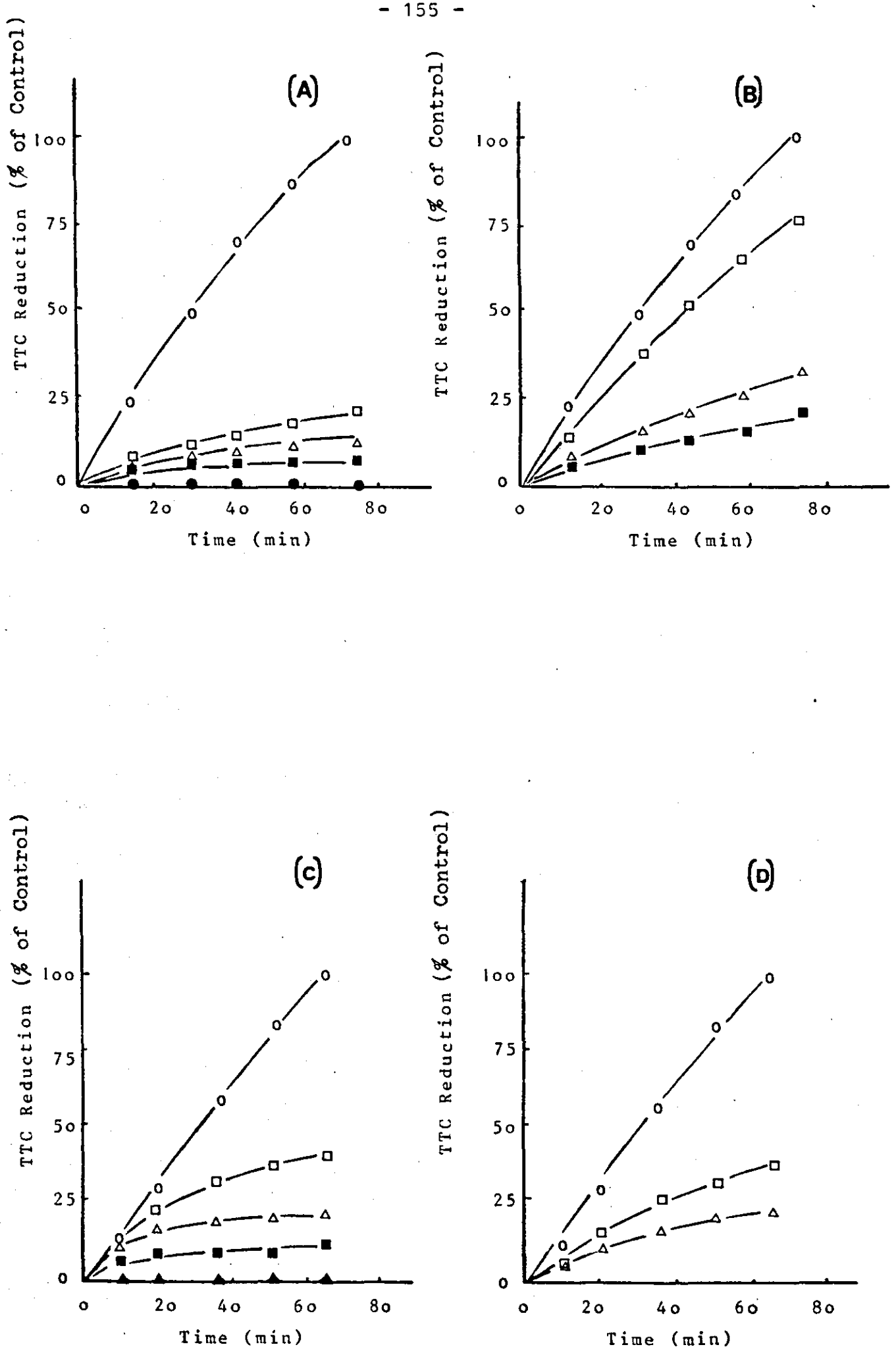
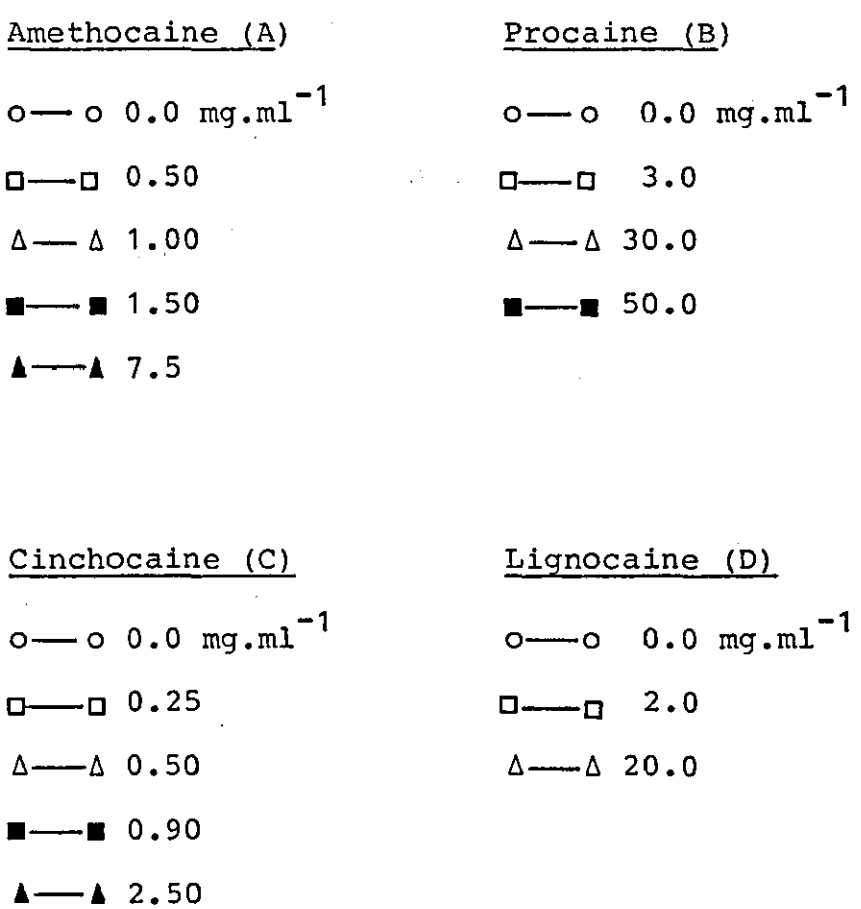
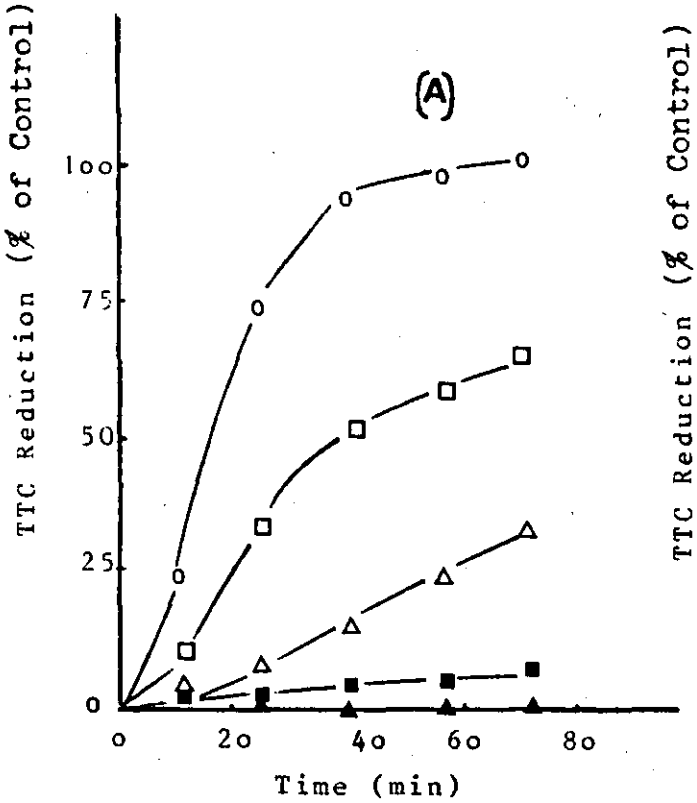
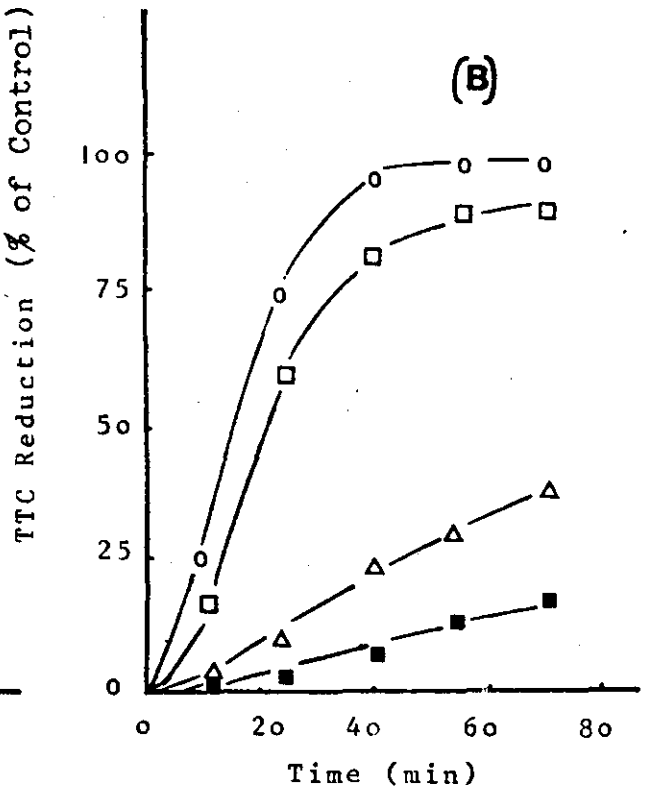
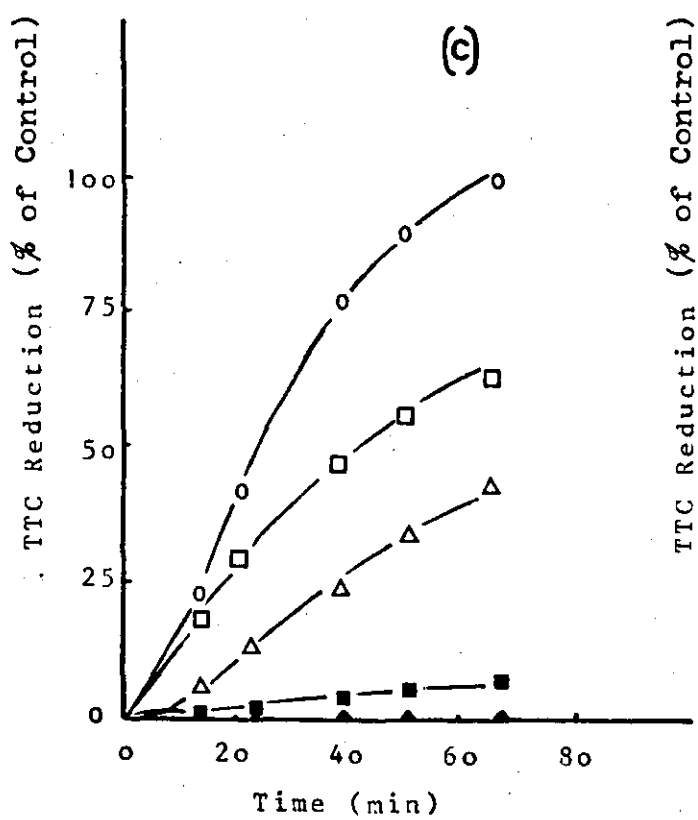

(A)
(B)
(C)
(D)
TTC Reduction (% of Control)
Time (min)
0
25
50
75
100
0
20
40
60
80

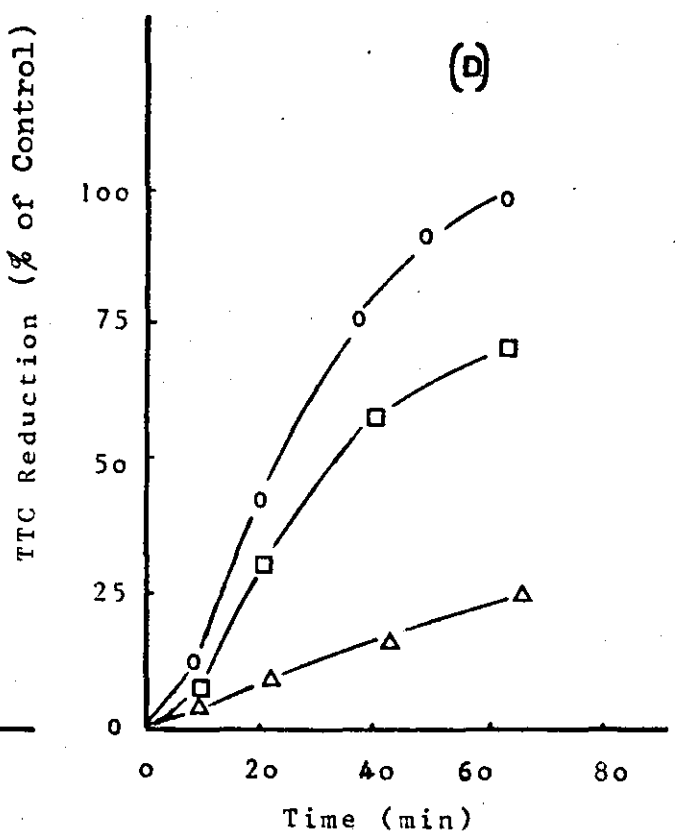
Fig 3.71 The effect of amethocaine (A), procaine (B), cinchocaine (C) and lignocaine (D) on the reduction of TTC by a suspension of E.coli cells in phosphate buffer (pH 6.4, 0.20M) using lactate as substrate. Cell concentration 3.8×10^9 cells.ml^{-1}. Temp $37^{o}C$.



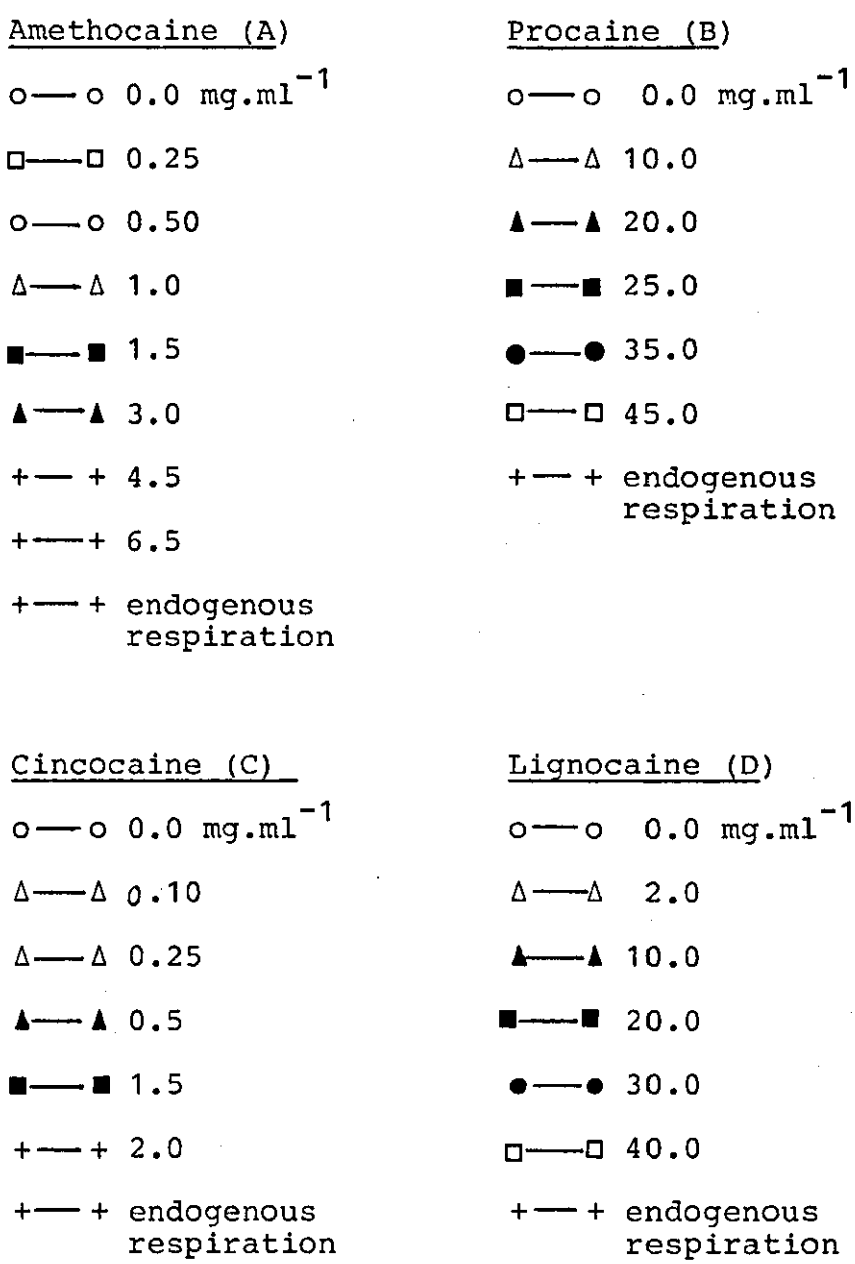

(A)
TTC Reduction (% of Control)
100
75
50
25
0
0
20
40
60
80
Time (min)

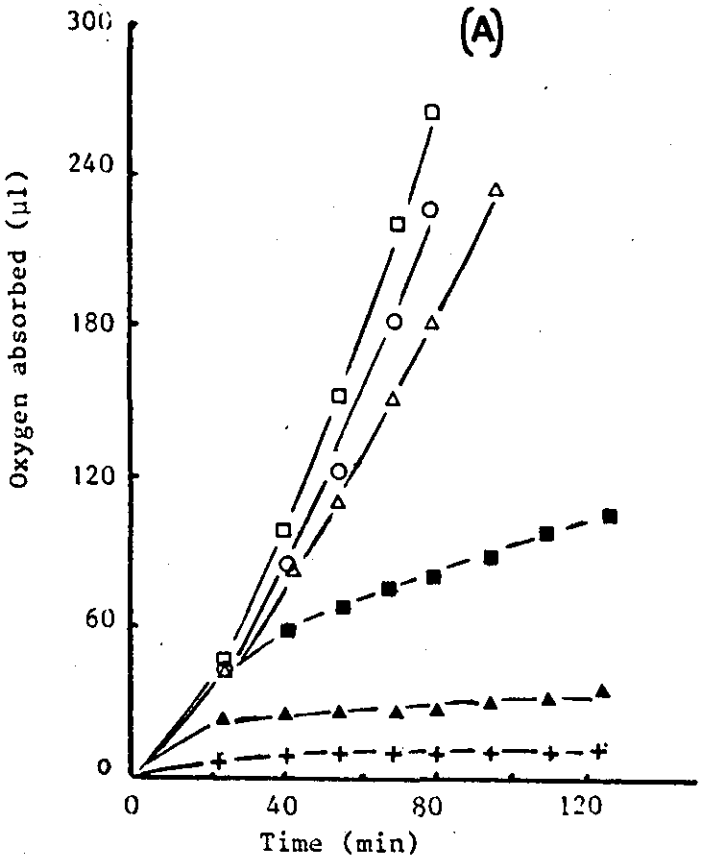

(B)
TTC Reduction (% of Control)
100
75
50
25
0
0
20
40
60
80
Time (min)

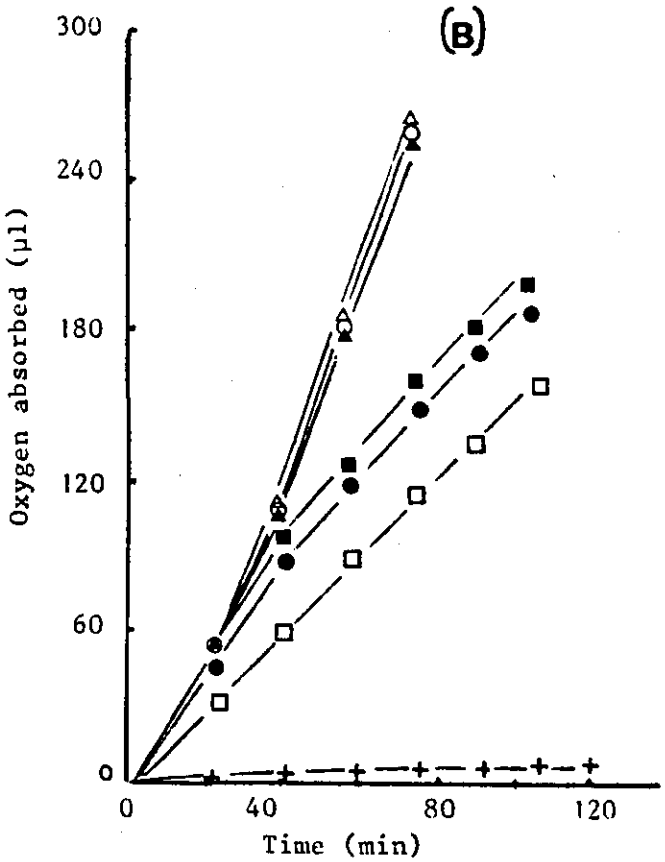

(C)
TTC Reduction (% of Control)
100
75
50
25
0
0
20
40
60
80
Time (min)

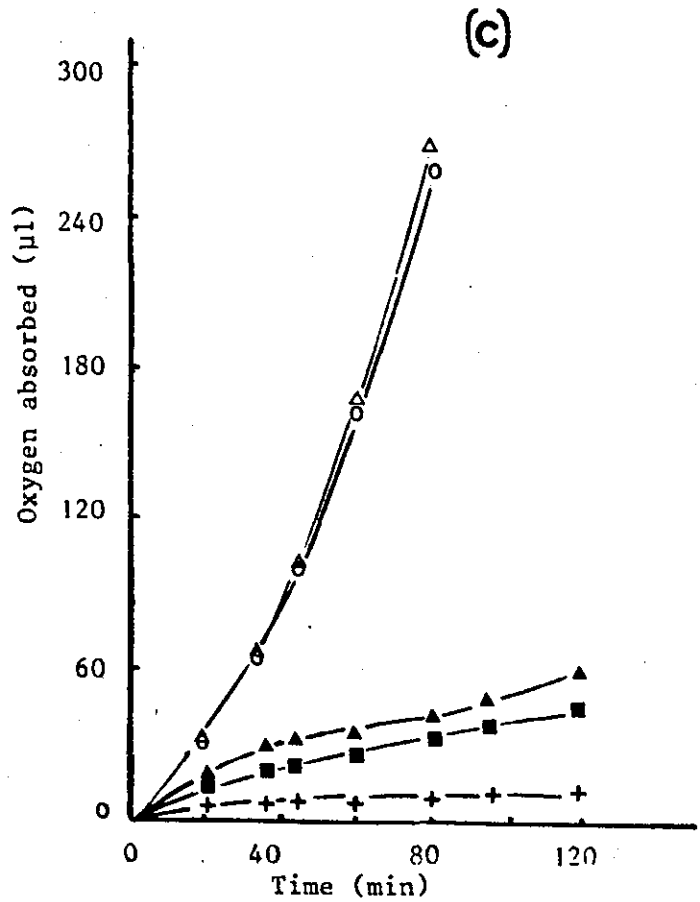

(D)
TTC Reduction (% of Control)
100
75
50
25
0
0
20
40
60
80
Time (min)

3.72 The effect of amethocaine (A), procaine (B), cinchocaine (C) and lignocaine (D) on the rate of oxygen uptake of E.coli suspended in CFM using glucose as substrate. Cell concentration 3.8×10^8 cells.ml^{-1}. Temp 37°C.




(A)
Oxygen absorbed (µl)
300
240
180
120
60
0
0
40
80
120
Time (min)


(B)
Oxygen absorbed (µl)
300
240
180
120
60
0
0
40
80
120
Time (min)


(C)
Oxygen absorbed (µl)
300
240
180
120
60
0
0
40
80
120
Time (min)

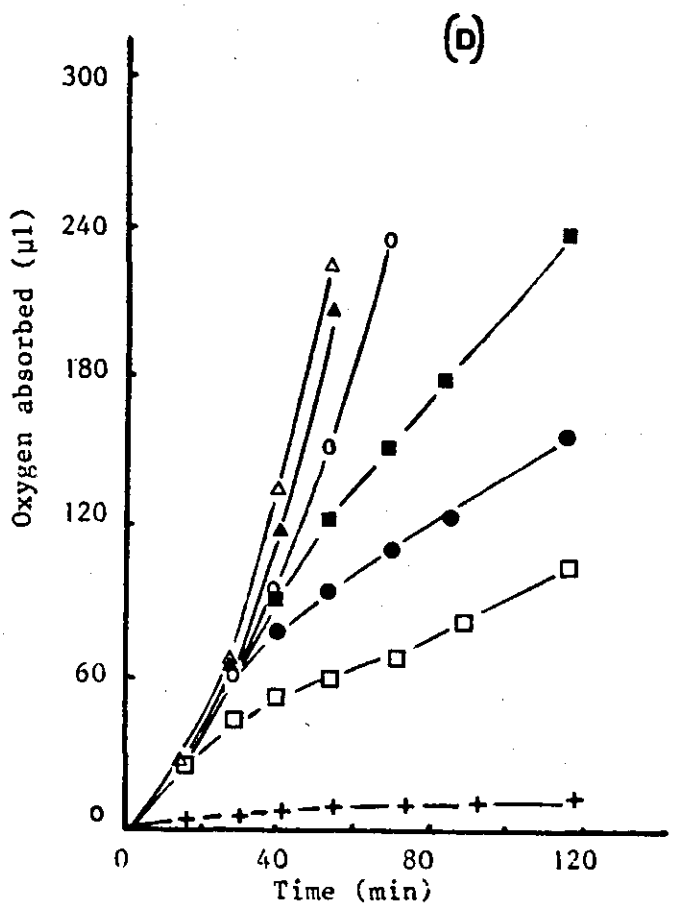
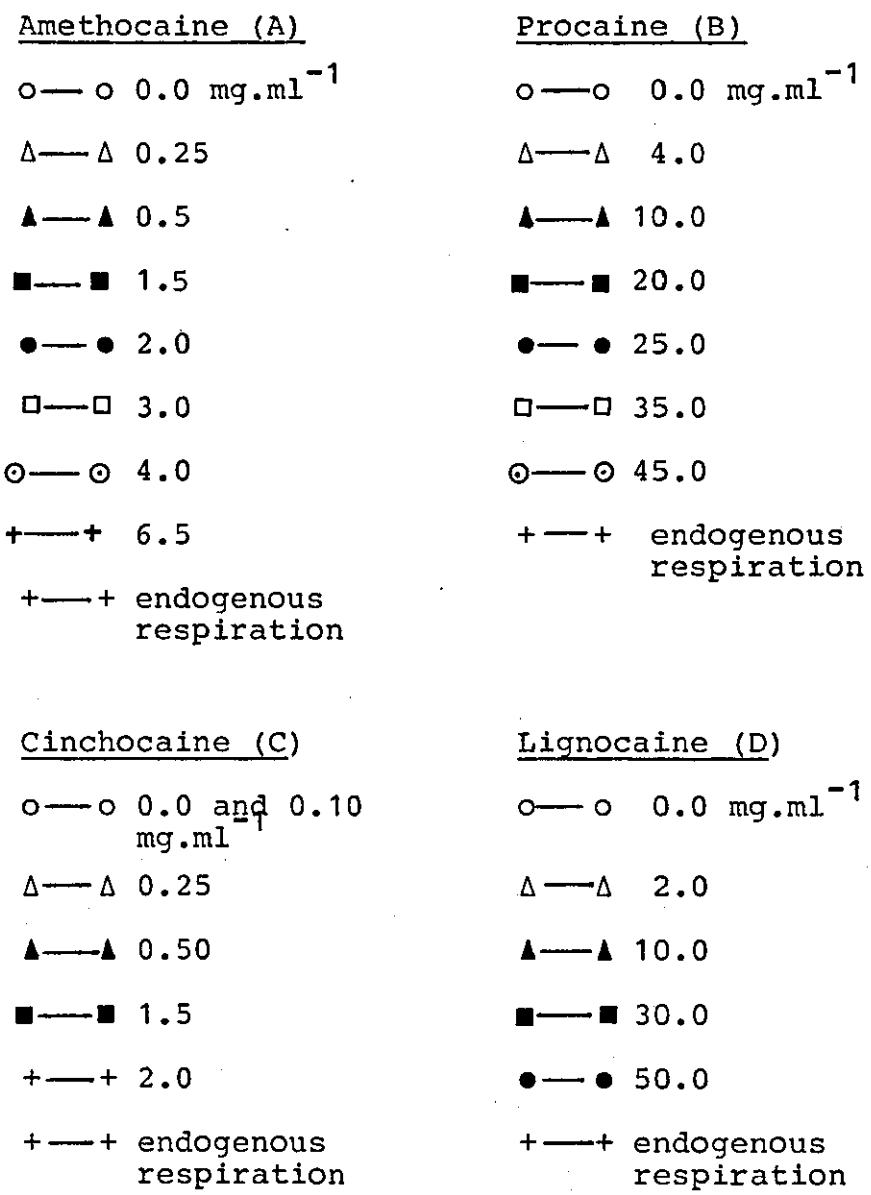
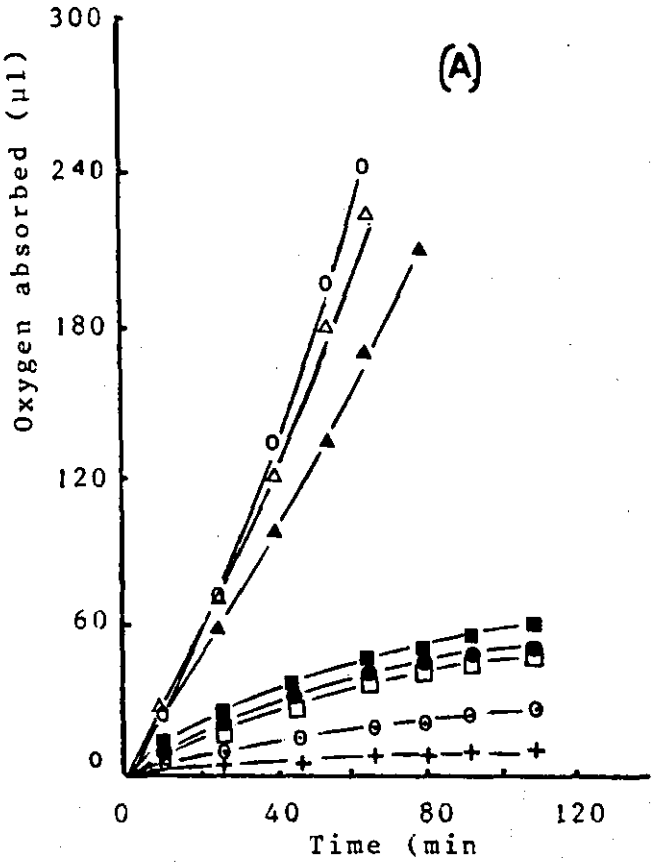
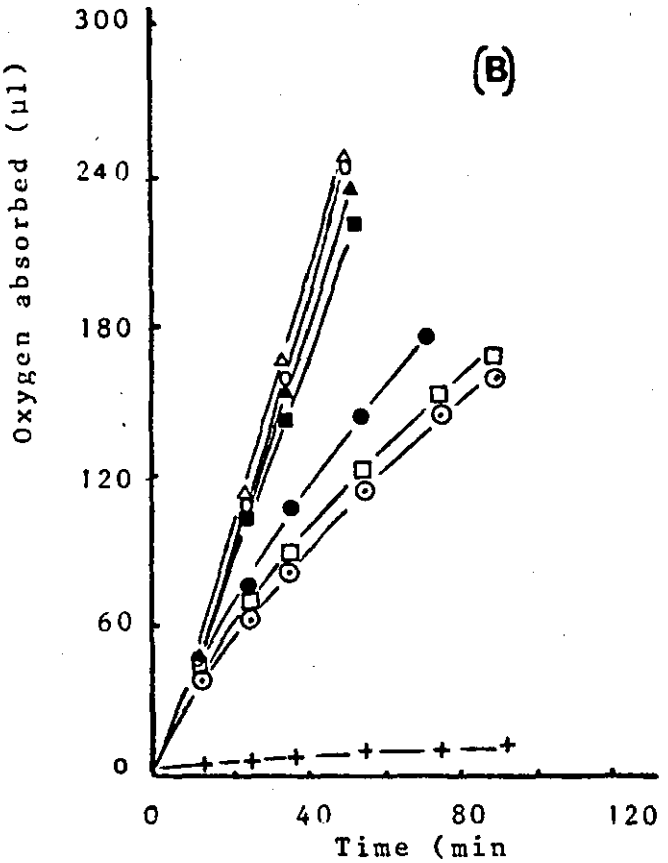
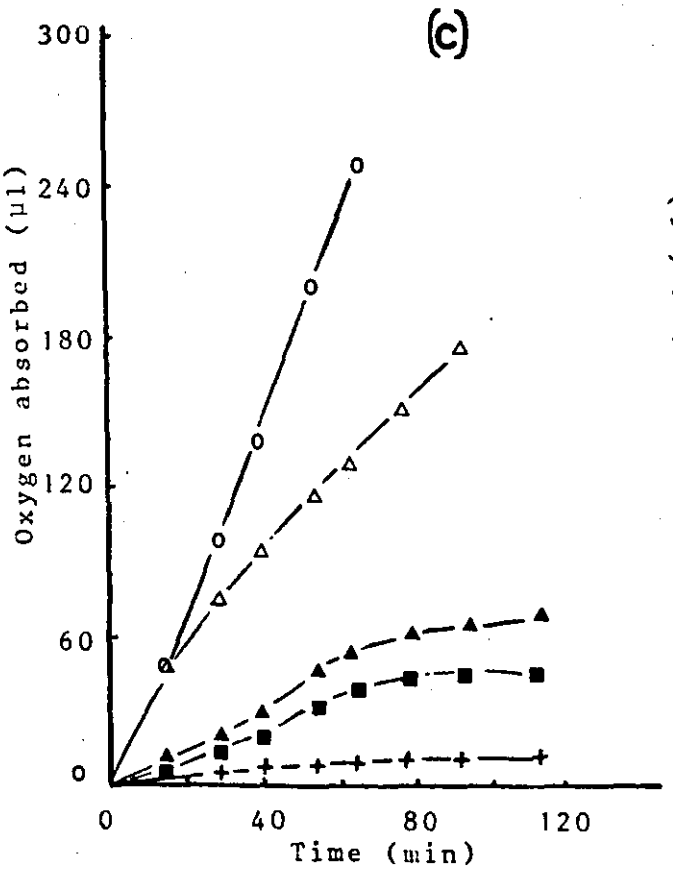

(D)
Oxygen absorbed (µl)
300
240
180
120
60
0
0
40
80
120
Time (min)

Fig 3.73 The effect of amethocaine (A), procaine (B), cinchocaine (C) and lignocaine (D) on the rate of oxygen uptake of E.coli cells suspended in CFM using succinate as substrate. Cell concentration 3.8×10^8 cells.ml^{-1}. Temp 37°C.




(A)
Oxygen absorbed (µl)
300
240
180
120
60
0
0
40
80
120
Time (min


(B)
Oxygen absorbed (µl)
300
240
180
120
60
0
0
40
80
120
Time (min


(C)
Oxygen absorbed (µl)
300
240
180
120
60
0
0
40
80
120
Time (min)

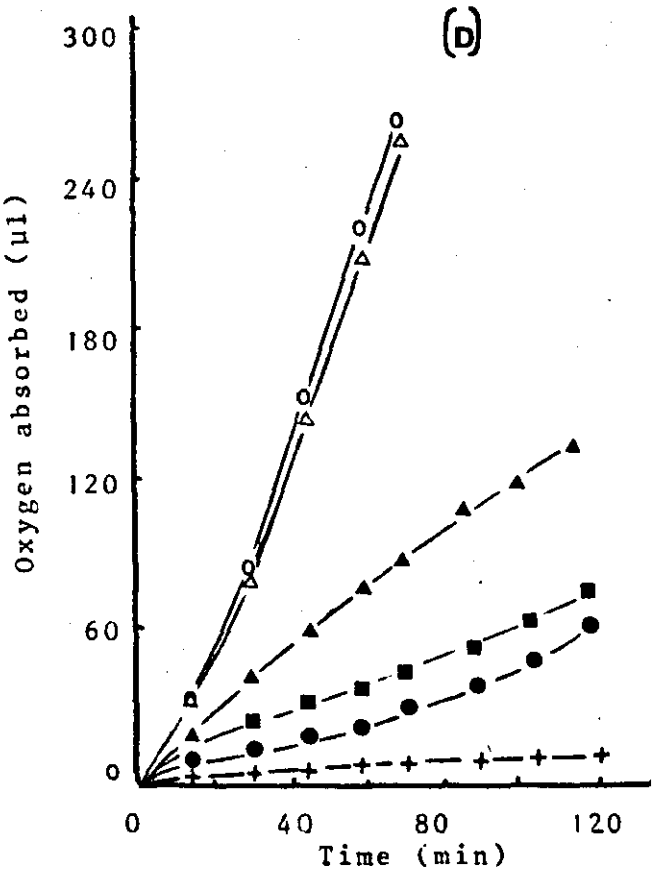

(D)
Oxygen absorbed (µl)
300
240
180
120
60
0
0
40
80
120
Time (min)

Plate 3.5 Scanning electron micrograph of E.coli shake-incubated in complete growth medium at 37°C. Bar indicates 1.0 µm.

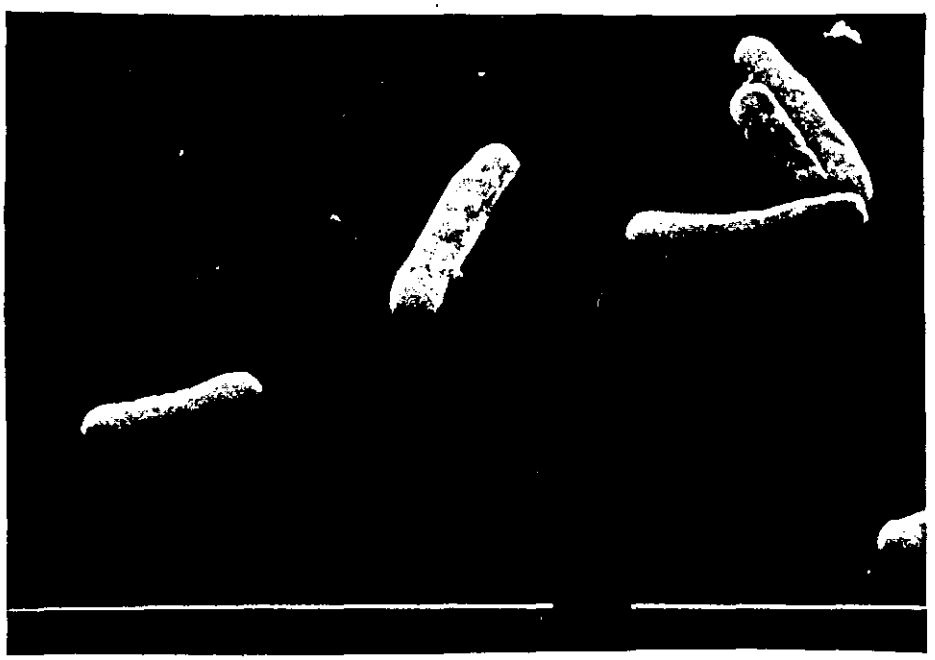
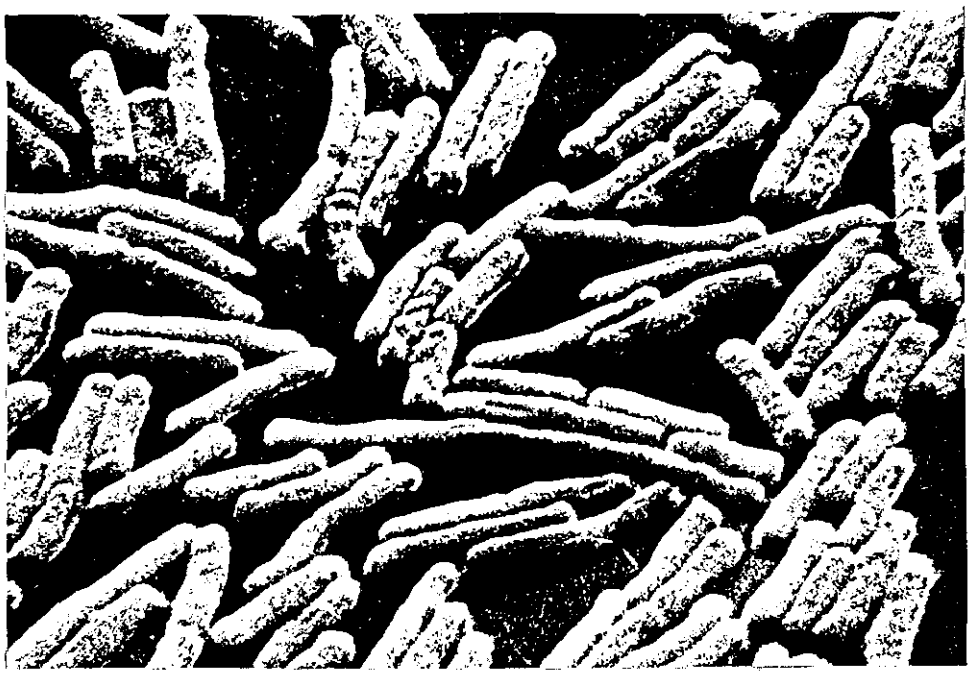


Plate 3.6 Scanning electron micrograph of E.coli shake-incubated for 15 min in the presence of 0.5 $mg.ml^{-1}$ amethocaine (ca ½ MIC) at 37°C, showing some filamentous forms with smooth surface X10,000.

Plate 3.7 Scanning electron micrograph of E.coli shake shake-incubated for 15 min in the presence of 1.0 $mg.ml^{-1}$ amethocaine (ca MIC) at 37°C, showing cells similar to the untreated one. However, clumps of cells in some parts can be observed. Bar indicates 1.0 µm.



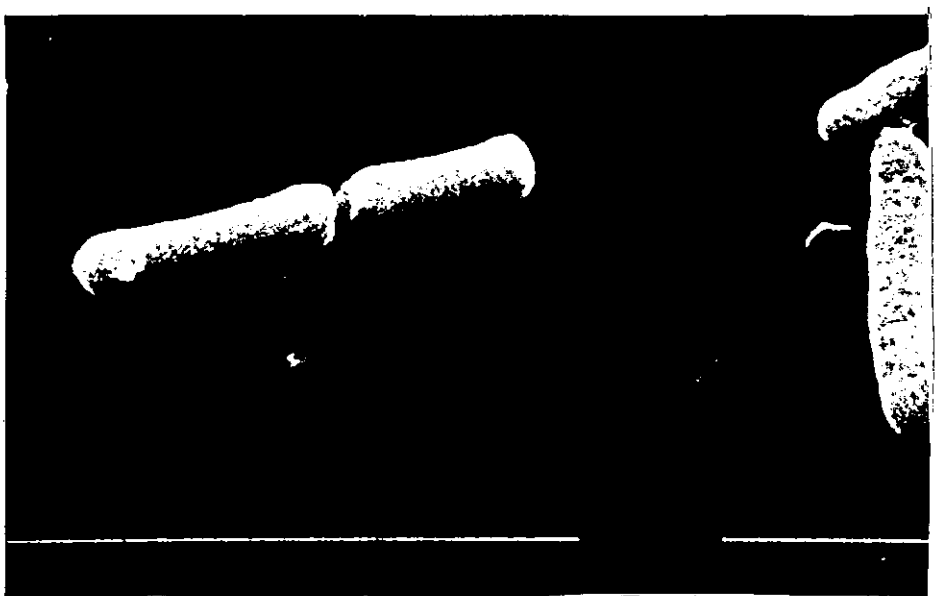


Plate 3.8 Scanning electron micrograph of E.coli shake-incubated for 15 min in the presence of 7.5 $mg.ml^{-1}$ amethocaine (MBC), at $37^{o}C$. Showing surface damages without any filamentous forms. Bar indicates 1.0 µm.

Plate 3.9 Scanning electron micrograph of E.coli shake-incubated for 15 min in the presence of 12 $mg.ml^{-1}$ amethocaine at $37^{o}C$. Showing surface damages without any filamentous forms. Bar indicates 1.0 µm.

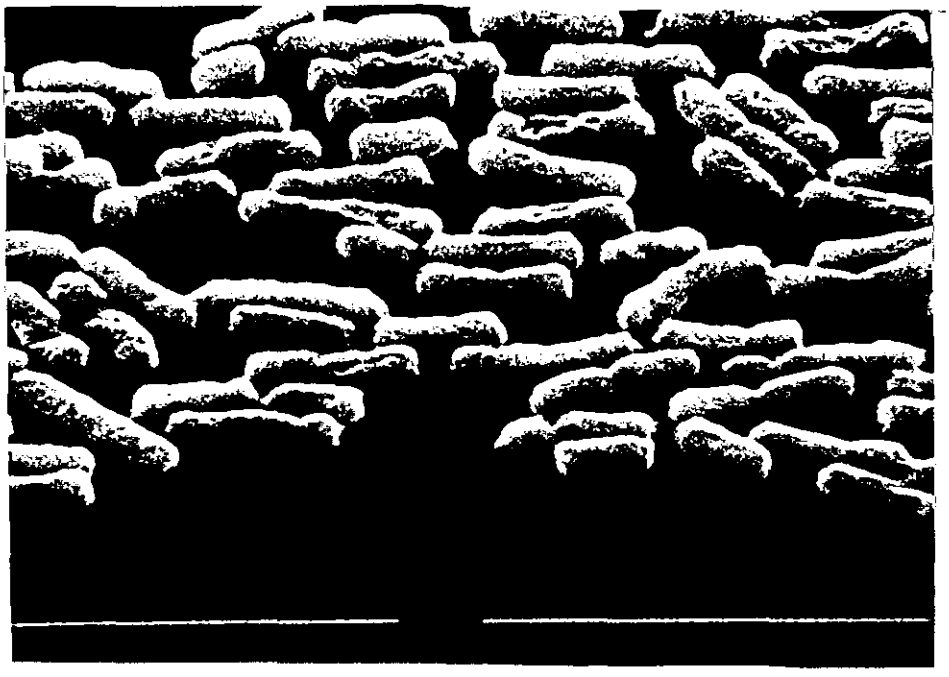
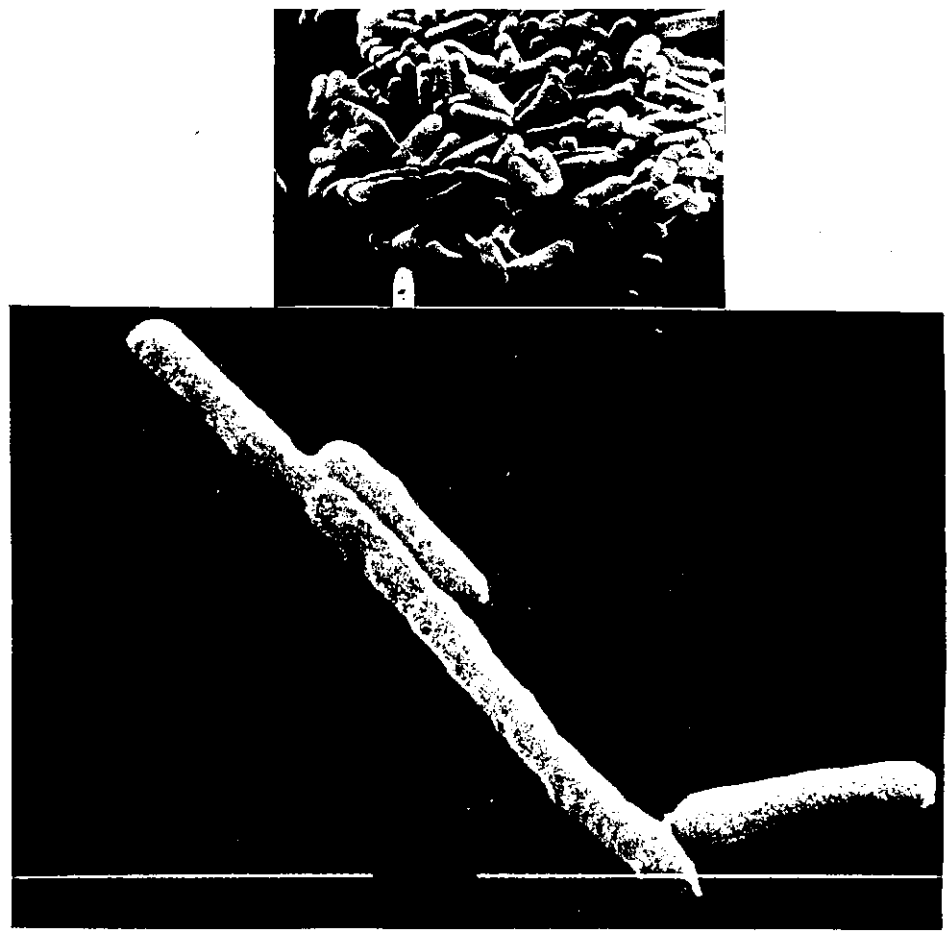


Plate 3.10 Scanning electron micrograph of E.coli shake-incubated for 15 min in the presence of 30 $mg.ml^{-1}$ procaine (ca MIC) at 37°C, showing some filamentous forms and some clumps of cells. Bar indicates 1.0 µm.

Plate 3.11 Scanning electron micrograph of E.coli shake-incubated for 15 min in the presence of 50 $mg.ml^{-1}$ procaine at 37°C. The cells surface are smooth and they appear as untreated cells. Bar indicates 1.0 µm.



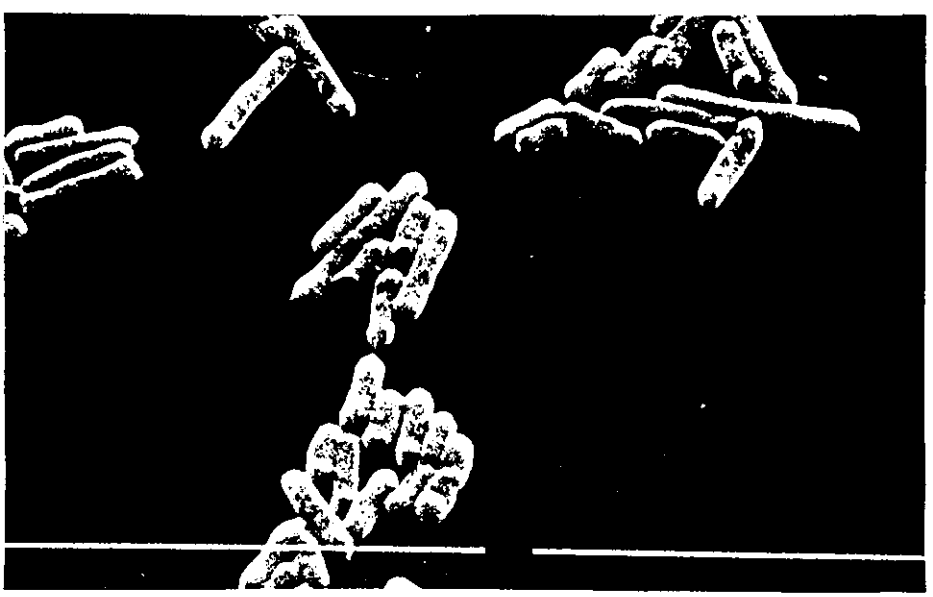


Plate 3.12 Scanning electron micrograph of E.coli shake-incubated for 15 min in the presence of 1.0 $mg.ml^{-1}$ cinchocaine (ca MIC) at $37^{o}C$. Showing smooth surface. Bar indicates 1.0 µm.

Plate 3.13 Scanning electron micrograph of E.coli shake-incubated for 15 min in the presence of 5.0 $mg.ml^{-1}$ cinchocaine (ca 2 x MBC) at $37^{o}C$. Surface damages can be observed. Bar indicates 1.0 µm.

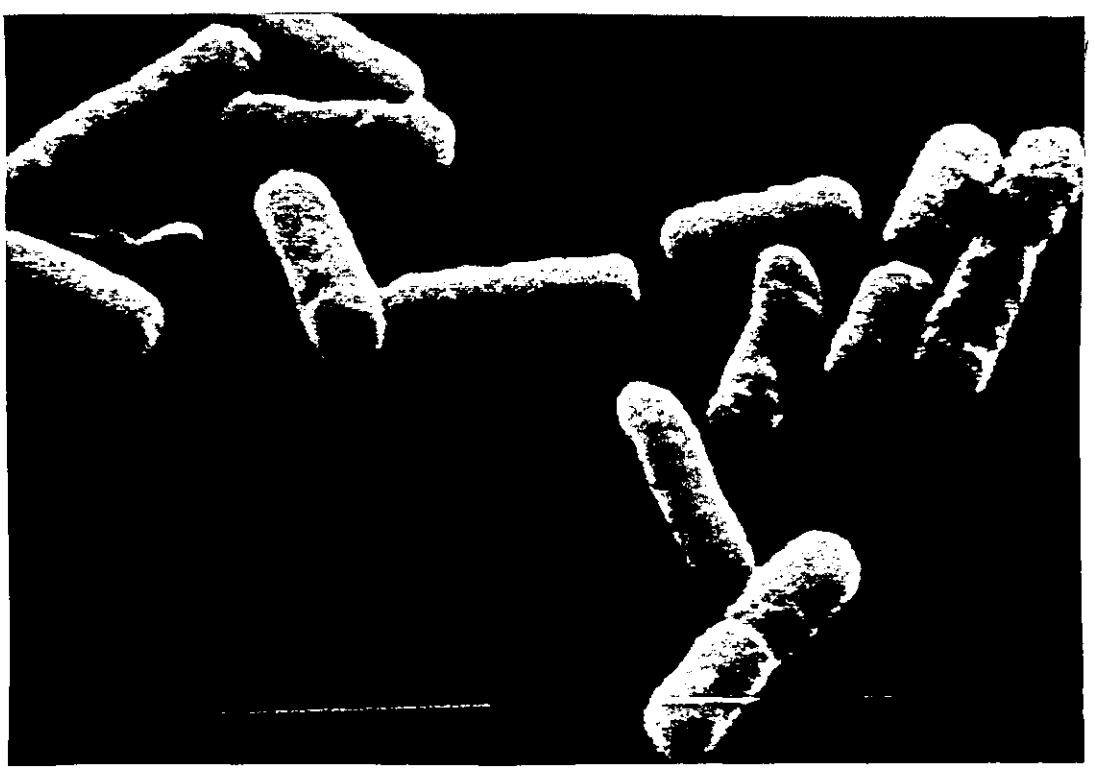


Plate 3.14 Scanning electron micrograph of E.coli shake-incubated for 15 min in the presence of 10 $mg.ml^{-1}$ lignocaine (sub-inhibitory concentration) at 37°C, showing some filamentous cells. X10,000.

Plate 3.15 Scanning electron micrograph of E.coli shake-incubated for 15 min in the presence of 30 $mg.ml^{-1}$ lignocaine (ca MIC) at 37°C, showing some filamentous cells. Bar indicates 1.0 μm.

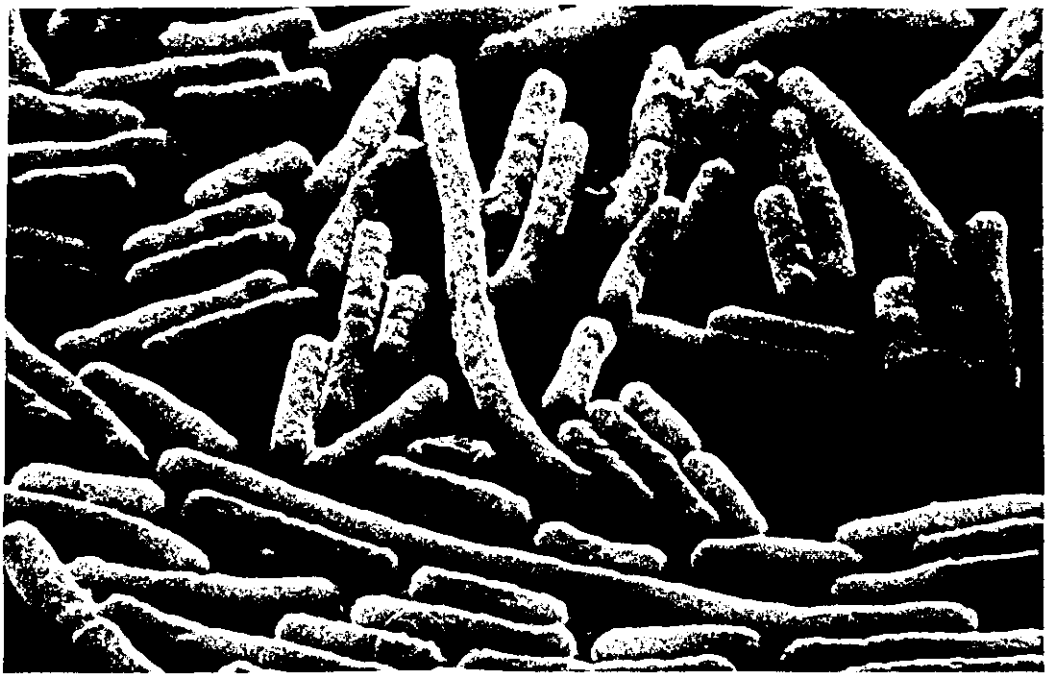
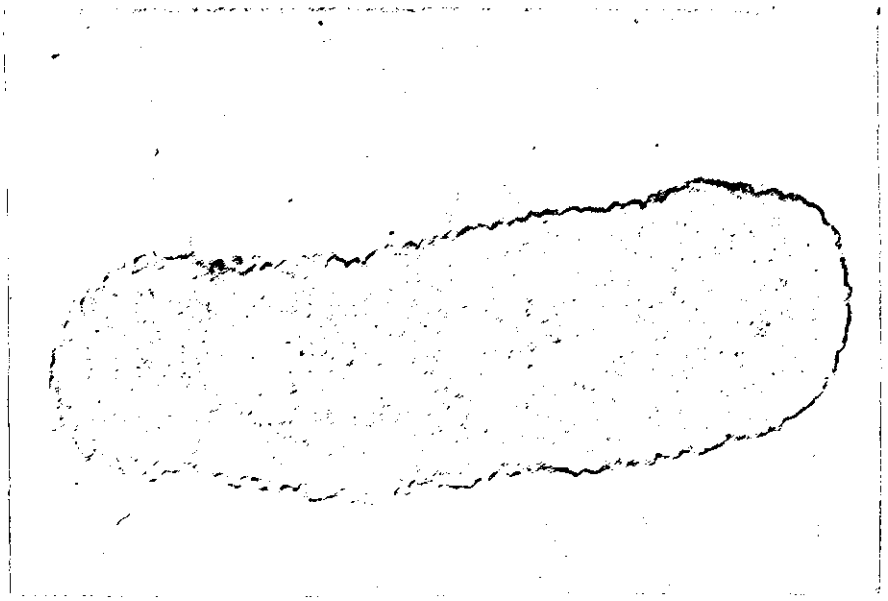


Plate 3.16 Electron micrograph of an ultra thin section of E.coli suspended in CFM for 15 min at 22°C, showing the usual feature of a bacterial cell. X60,000.

Plate 3.17 Electron micrograph of an ultra thin section of E.coli suspended in CFM in the presence of 50 $mg.ml^{-1}$ procaine for 15 min at 22°C. The section does not show any visible change comparing with control. X37,500.



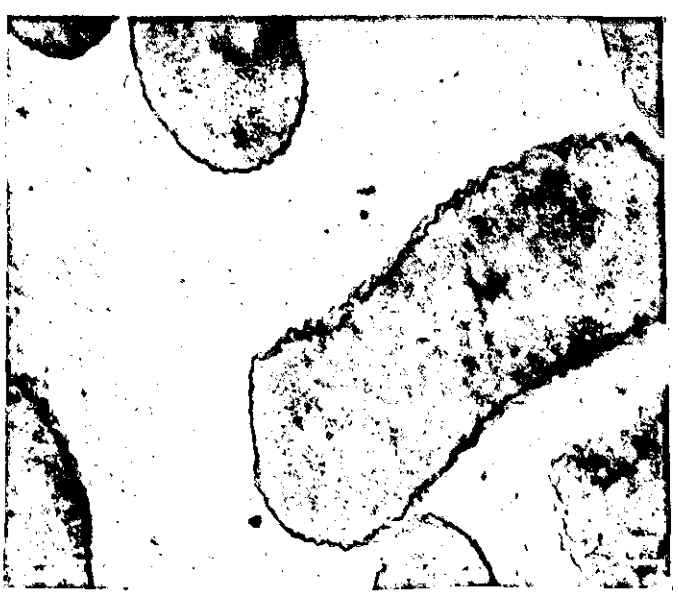
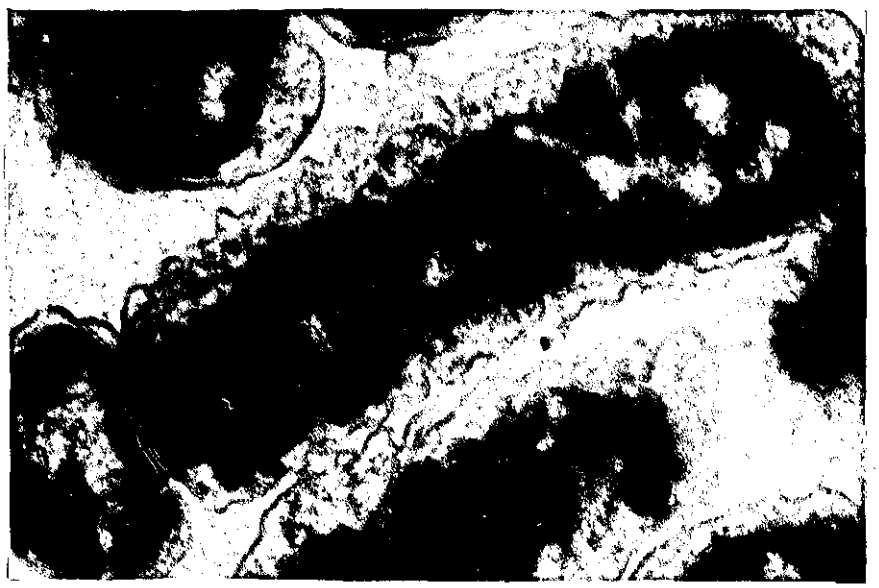
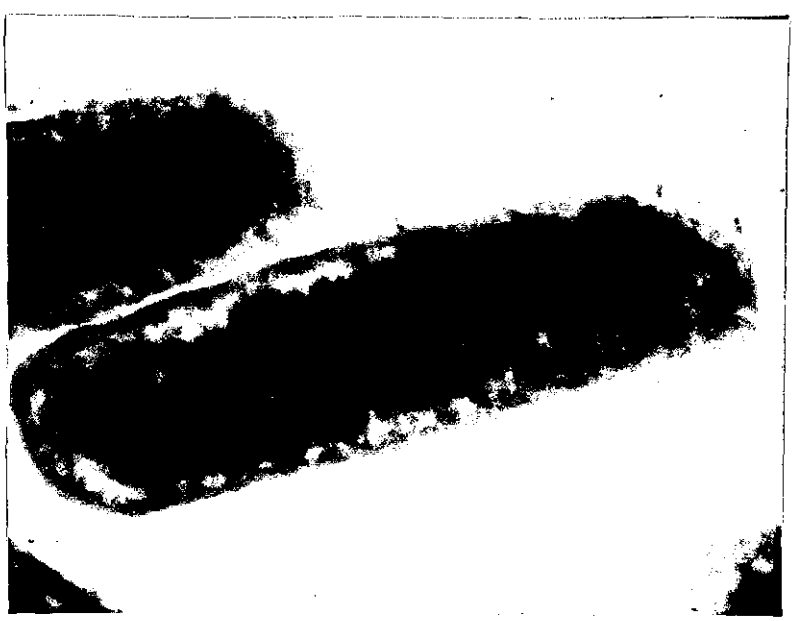


Plate 3.18 Electron micrograph of an ultra thin section of E.coli suspended in CFM in the presence of amethocaine (10 $mg.ml^{-1}$) for 15 min at 22°C. The section shows heavily and non-uniformly stained cytoplasm which in some parts is contracted away from the cell envelope. The outer membrane appear less well defined. X67,500.

Plate 3.19 Electron micrograph of an ultra thin section of E.coli suspended in CFM in the presence of amethocaine (20 $mg.ml^{-1}$) for 15 min at 22°C. The section shows heavily and non-uniformly stained cytoplasm which in some parts is contracted away from the cell envelope. The outer membrane appears less well defined. X45,000.





CHAPTER 4

DISCUSSION

Local anaesthetics (LAs) affect bacterial cells in many different ways. Their effects range from growth inhibition and stimulation/depression of respiration to leakage of cellular constituents, gross cytological changes and cell death. Not all the anaesthetics studied, however, gave rise to the same effects. For example procaine had little effect on cell viability.

LAs are generally more effective against Gram-positive cells than Gram-negative in terms of minimum inhibitory concentrations (Fig 3.1 to 3.16 and Table 3.1). This variation is probably due to combinations of (a) the differences in the lipid content of the cell envelope, (b) the presence of a capsule or (c) the production of a slime layer. Indeed a quantitative comparison of the LA concentrations required to produce a 50% inhibition of growth of the different Gram-negative organisms (Table 3.4) with those producing a similar effect with B.megaterium produced ratios greater than one. This indicates the significance of the "outer membrane" of the Gram-negative cells in the protection of the cell from the influx of the drug (Chopra and Shales, 1981). The local anaesthetics are not equally effective as antibacterial agents and considering them as esters or amides, amethocaine is more effective than procaine and cinchocaine is more effective than lignocaine. These differences probably reflect structural differences in these LAs (Figs 2.1) which though only slight for amethocaine and procaine gives rise to a marked increase in the lipophilicity of amethocaine. Similarly with cinchocaine and lignocaine.

Slightly different values were obtained for MICs

using the tube dilution or the shaken culture techniques indicating the significance of adequate aeration during growth. The amount of capsule or slime formation is influenced by oxygen concentration (Meadow, 1978) and of course there is a marked effect on the cellular growth rate which approaches the maximum possible under the shaken conditions. The formation of clumps of cells (Sykes, 1965) was also noted with LA treated cells (Plates 3.7 and 3.10) and this would be more likely to occur under the static conditions of the tube dilution method.

Growth of bacteria in the presence of LAs was followed as mass increase (absorbance, 650 nm). This technique was suggested for bacterial cells which increase in size rather than numerically during the incubation in the presence of drug (Lang and Rye, 1972). As the presence of subinhibitory concentration of LA in the growth medium caused some changes in cell size for E.coli cells (Plates 3.6, 3.10, 3.14 and 3.15), the measurement of mass increase rather than cell counts was adopted.

When the bacterial cultures were grown in the presence of different concentrations of LA using the shake culture method, the reduced growth rate depended on the drug concentration. The extent of the reduction produced by equal increments in LA concentration became progressively larger at higher concentrations, and complete inhibition was observed at higher concentrations still. Even higher concentrations caused a reduction in turbidity. The inhibitory pattern of LA which also is shown in Fig 3.18 are unlike those which have been reported by Garrett and Miller (1965) and Garrett et al (1966) for inhibitors of protein synthesis such as chloramphenicol and tetracycline

and by Mielck and Garrett (1970) for spectinomycin on E.coli cells. With these agents cell growth is slowly inhibited to a new steady state with a new rate constant which is linearly dependent on drug concentration. In these situations they suggested that only a small fraction of the receptor sites are required to be blocked before totally inhibition of bacterial growth occurs. The inhibitory patterns reported for erythromycin (Garrett et al, 1970) and for lincomycin (Mielck and Garrett , 1969) with E.coli cells, however, show that the growth rate decreases on increasing the inhibitor concentration and that the rate constant, approaches zero asymtotically at higher concentrations. Mielck and Garrett (1969) suggested that this shape of curve, indicates the presence of some saturable processes such as binding of drug to a limited number of essential receptor sites and the generation rate is proportional to or dependent upon the number of free receptor sites. The plots of rate constants against concentration of LAs (Fig 3.18) are similar to the growth rate patterns of benzyl and phenethyl alcohols reported by Lang and Rye (1972), who showed that these compounds altered cellular permeability in a non-specific reversible manner.

The observed restriction of growth of bacteria produced by many compounds could be due to either a partial uniform inhibition of growth or to a non-uniform inhibition of the cells. Garrett and Miller (1965) in the kinetic studies of the effect of chloramphenicol and tetracycline on partially inhibited culture of E.coli found that the total and viable cell counts coincided and

they suggested that this resulted from a general inhibition of growth of all of the cells rather than only some of the cells. Rye and Wiseman (1968) suggested that this approach was not entirely satisfactory as organisms which are not multiplying under the conditions of the experiment may do so under the changed conditions used for detecting viability. They suggested another method based on the effect of subinhibitory concentration of drugs on E.coli cells and the measurement of the cell-size distribution, after growth had occurred in the presence of sufficient ampicillin to suppress cellular division. In the culture treated with amethocaine, however, the size distribution, in terms of threshold units, (Fig 3.20) shows a single distribution of cell sizes developing in the presence of the agent which is similar in shape to that of the untreated cultures. The subinhibitory concentration of the LA used, however, causes no loss in cell viability (Fig 3.40) and the observed restriction of growth in the partially inhibited cultures is probably due to a general and uniform inhibition of growth rather than to a selective inhibition of some of the cells. A uniform inhibition of growth which is due to an even distribution of a highly effective agent amongst the cells can be achieved if only a small proportion of the antibacterial agent is adsorbed by the cells at the concentration used (Rye and Wiseman, 1968). Indeed as the uptake of LAs by E.coli cells was so low, the number of bacterial cells was increased about a hundred times to get a measurable uptake.

To investigate the reversibility of action of LAs on the growth of E.coli, two different methods were used (Figs 3.21 to 3.25). Cultures treated with sub-MIC levels

of LA, (for example up to 1.0, 2 0, 0.4 and 10 $mg.ml^{-1}$ for amethocaine, procaine, cinchocaine and lignocaine, respectively, which cause about 85, 30, 85 and 32% growth inhibition) on resuspension or dilution with fresh growth medium, grow rapidly at a rate very similar to that of the untreated culture or the relevant new concentration of drug. However, higher concentration showed, on resuspension or dilution with the fresh growth medium, an initial lag period, after which the growth continued with the growth rate similar to the corresponding new concentration of LA. This lag phase may be attributed to a possible need for the cell to recover or undergo repair before restablishment of the new growth rate, or to a consequence of the shock of dilution (Garrett et al 1970). However, the results do show that the inhibition of growth of the bacteria in the presence of varying concentration of LA up to the MIC is reversible.

The extent of loss in viability of the bacteria is governed by three principal factors: drug concentration, bacterial cell density and time of contact (Figs 3.40 to 3.43 and Tables 3.2 and 3.6). The results of these determinations show that, with the exception of the effect of procaine on the Gram-negative organisms, the number of viable organism was reduced by these LAs within a short period of time (10 min). While amethocaine and cinchocaine have significant bactericidal capacity, lignocaine has much less, and procaine is even less effective. The order of bactericidal activity was found to be cinchocaine > amethocaine > lignocaine > procaine. Also comparing the bacteria affected by LAs, B.megaterium

(Gram-positive) was the most sensitive organism. The viability curves (Figs 3.40 to 3.43) show threshold concentrations about the MIC for the system, below which cell viability is virtually unaltered. Above this threshold as the LA concentration increases, so does the bactericidal activity. Both amethocaine and cinchocaine have similar MBCs for each of the different Gram-negative organisms. Table 3.6 also shows that the bactericidal action of amethocaine and cinchocaine is rapid, as there is an appreciable drop in viable number of E.coli cells almost immediately after the addition of the LAs. Furthermore the results show that increasing the contact time of E.coli with amethocaine and cinchocaine decreases the number of viable cells considerably (Table 3.6). Surprisingly increasing the bacterial cell density over the range of 2.66×10^8 to 4.05×10^9, did not have a significant effect on the MBC of amethocaine on E.coli (Table 3.2).

Neither procaine nor lignocaine are effective bactericidal agents especially against Gram-negative organisms. This may reflect the inability of the active agent to achieve an effective concentration at its site(s) of action, since the drug must first penetrate the various permeability and structural barriers that separate the target site from the external environment before exerting its affect. In the case of bacterial cells the difference in the properties of these permeability barriers among the various genera and species play an important role in determining the antibacterial spectrum of a drug (Brown, 1975). Target sites e.g. membranes, ribosomes, DNA, enzymes, etc. isolated from different

groups of bacteria, are frequently found to have comparable sensitivities to an antibacterial agent and yet the intact cells have very different sensitivities. In many cases it is the differences in the composition of the bacterial cell envelopes which affect the ease of penetration of drugs to the intra-or peri-cellular target sites.

In the viable count method of assessing bactericidal activity, the method of recovery of microorganism is very important. There is a possibility that cells, although not killed within the period of drug treatment, are damaged during the dilution stages to such an extent that recovery becomes impossible. The experiments on the reversibility of growth inhibition (Figs 3.21 to 3.25) suggest that this is unlikely with local anaesthetics. Morever experiments in which the recovery media contained 1% Tween 80, gave no detectable improvement in the % recovery from amethocaine treatment. This does not, however, mean that any degree of non-lethal damage to cell can not be reversed by other methods.

The MIC/MBC data obtained in this study, confirms the findings reported by previous authors (Murphy et al., 1955; Kleinfeld and Ellis, 1966 and 1967; Schmidt and Rosenkranz, 1970; Weinstein et al, 1975; Zaidi and Healy, 1977; Leung and Rawal, 1977; Salt and Traynor, 1979). Significant antibacterial activity of amethocaine and cinchocaine, but not procaine and lignocaine against E.coli, K.aerogenes, P.aeruginosa and B.megaterium was observed.

There are some observations, however, that are at variance with previously reported data. Schmidt and

Rosenkranz (1970) found that P.aeruginosa was the only Gram-negative organism resistance to lignocaine and procaine, even at 20 $mg.ml^{-1}$. While in this study it was found that these LAs have nearly the same effect on E.coli, K.aerogenes and P.aeruginosa, and lignocaine was more effective than procaine. This difference could be due to differences in the bacterial strains and the techniques used. Leung and Rawal (1977) reported the MBC of amethocaine for P.aeruginosa to be 1.5 $mg.ml^{-1}$, while in this work it was found to be 7.5 $mg.ml^{-1}$. The difference is due to different contact times, inoculum levels and the media. Zaidi and Healy (1977) reported much lower MBCs of procaine against E.coli and P.aeruginosa. In addition to the variation in media and inoculum level etc., their results would certainly be influenced by the presence of 0.2% chlorocresol as preservative in the procaine containing ointment which they evaluated.

Results so far mentioned on the evaluation of both bacteriostatic and bactericidal activity of LAs in conjunction with other authors' results, confirm that LAs are active against a wide range of bacteria. This perhaps, suggest that the mode of action of LAs might involve a system or systems that is or are common in, or to, a wide variety of microorganisms, and (or) that LAs might have more than one mode of action and, therefore, may affect many different microorganisms but in different ways.

The result of present study shows that LAs are capable of reducing dehydrogenase activity as interpreted via oxygen uptake and TTC reduction depending on the

concentration and the structure of the compound. Amethocaine and cinchocaine were found to be more effective than procaine and lignocaine (Figs 3.67 to 3.73) and LA effectiveness was influenced by medium composition. For example, with glucose as substrate, TTC reduction was affected to a greater extent when the experimental medium was phosphate buffer rather than CFM. This indicates the significance of the ions in the environment stablizing the bacterial cell membrane and or its enzymes (McQuillen, 1960; Morris and Russell, 1970; Salt and Wiseman, 1970) and competing with the LA so that a higher concentration of the drug is required to produce the same effect.

Very low concentrations of LAs either stimulated or had no effect on the cellular respiration. This was more detectable when glucose was used as substrate. For example concentration of 0.25, 10, 0.25 and 10 $mg.ml^{-1}$, amethocaine, procaine, cinchocaine and lignocaine respectively stimulated the oxygen consumption when glucose was utilized by E.coli cells (Fig 3.72), while these concentrations had an inhibitory effect when succinate was metabolised (Fig 3.73). Such a stimulation has been reported by Hugo (1956), Hugo and Bloomfield (1971b)and Bloomfield (1974) with other antibacterial agents. Hugo and Bloomfield (1971b)deduced that an increase in the total oxygen comsumption with low drug concentrations was typical of the action of energy uncouplers. Whether the LAs acts as uncoupler or not requires further investigation, particular as others have reported no stimulation of oxygen consumption by LAs in other cell systems (Fink et al, 1969).

Hugo and Street (1952) however suggested that this

stimulation could be due to an increase in the permeability of the cell, thus facilitating the access of substrate to the enzymes which are localized within the cells. The marked inhibition of lactate or malate or succinate activity could be interpreted if the enzymes responsible for their oxidation were located at or near the cell surface. Salton (1971) proposed that the mechanism of an increase in permeability must involve the "solubilization" or "opening up" of the membrane bound enzyme, so that its active site is available to substrate. Solubilization of lipophilic components of the membrane would be facilitated by the presence of drug micelles. This seems unlikely with LAs, as the concentration which stimulates the respiration is far less than the CMC value and unless localized concentration effects due to adsorption permit 'limited' micelle formation, colloidal aggregates would not be present.

Cellular respiration, as reflected by TTC reduction and oxygen uptake, is affected by all LAs studied at concentrations below the MIC suggesting that the inhibition of respiration is at least partially involved in growth inhibition.

If a critically sensitive enzyme exist, its activity is affected by small amounts of LA, it would be expected that increasing the concentration of LA would soon give an excess capable of altogether blocking metabolic activity. Results show that, once the concentration of the agent was high enough to inhibit respiration to a very low level, it did not reduce the oxygen consumption to zero. TTC reduction did, however, become effectively zero. The true meaning of this apparently total inhibition is difficult to

interpret as the technique involves an extraction procedure which is itself inevitably an equilibrium state. Therefore it seems unlikely that there is an enzyme of key importance that is very sensitive to LAs. So it seems reasonable to assume that LAs, at this level of concentration, disorganize not an enzyme protein but rather some component in the bacterial cell membrane, changes in which would then influence enzyme activity.

Since damage is presumably caused only by adsorbed drug, the first stage of antibacterial activity by any cytotic agent is its adsorption by bacterial cells. The study of drug uptake can yield information about the site of action of the drug but does not necessarily provide direct evidence for a proposed mode of action (Russell et al, 1973).

It is unlikely that adsorption of antibacterial drugs by a cell is, itself, a fatal event. After the adsorption process, however, secondary process can be shown to occur and these contribute to the inhibition of the reproductive and metabolic processes of the cell (bacteriostatic effect) or even to a rapid loss of viability (bactericidal effect).

As the uptake isotherms illustrate (Figs 3.50 to 3.52), at equilibrium in treated cell suspensions, the concentration of cinchocaine or amethocaine on or within the cell is much greater than the external medium. On the other hand lignocaine and procaine are taken up to a much lesser extent, indicating that the cells have a greater affinity for amethocaine and cinchocaine than for procaine and lignocaine. Since amethocaine and cinchocaine are

relatively lipophilic (partition data, Table 3.7, shows that o/w partition coefficient of amethocaine and cinchocaine is higher than procaine and lignocaine) it seems reasonable to assume that cellular LA is largely present in the lipid containing region of the cell, which in E.coli is the cell envelope.

A comparison of the relative activities of these LAs when present within the cell can be made by combining the uptake data of Figs 3.50 to 3.52 with the growth inhibitory patterns shown in Fig 3.17(A) or 3.18. Table 4.1 shows the total concentrations and calculated extracellular and intracellular concentrations of LAs which reduce the growth rate by 50%. It seems that approximately the same concentration of amethocaine and cinchocaine is present within the cell, although this value for the latter is a little less than for the former. Lignocaine and procaine are both present at much greater concentrations. The greater uptake of procaine and lignocaine required for 50% growth inhibition could be due to the presence of a significant proportion of the intracellular molecules in the aqueous biophase and consequently not at the (lipophilic) site of action. An approximate correction can be made for this by calculating the effective intracellular LA concentrations. It is assumed that the concentration of the compound in the aqueous biophase is the same as in the external medium (Lang and Rye, 1973) and the water content of E.coli to be 73-78% (Luria, 1960). The total amount of LA present in the aqueous regions of the cell can be calculated and substracted from the overall intracellular uptake to get

Table 4.1 The total, extracellular and intracellular LA concentrations required to reduce the growth rate of E.coli by 50%.

LA	Total Conc. ($mg.ml^{-1}$)	Conc. in medium ($mg.ml^{-1}$)	Overal intera-cellular conc. ($mg.ml^{-1}$)	Effective intracellular conc.	
				$mg.ml^{-1}$	% of total
Cinchocaine	0.37	0.025	0.345	0.31	86
Amethocaine	0.66	0.088	0.572	0.49	75
Lignocaine	14	6.4	7.7	2.82	20
Procaine	28	15.55	12.44	0.84	3

what would probably be the intracellular LA associated with the cell envelope. This effective intracellular concentration is shown as both the % of the total LA concentration and as $mg.ml^{-1}$ (Table 4.1). This shows a closer agreement between the amount of each LA in the cell which is effective in reducing growth rate by 50%. These results together with others suggest that the apparent differences in the antibacterial activities of these compounds are due not just to the differences in their uptake properties but additionally to their mode of intracellular distribution.

Although the uptake isotherms show the differences in the amount of different LAs taken up by the cells, it is difficult to specify the similarity of these isotherms to any one of the uptake isotherm reported by Giles et al (1960) and Giles and Smith (1974). The uptake isotherm shows a straight line, which is generally characteristic of the constant partition of LA between solution and substrate (bacteria), corresponding to the "C" curve of Giles et al (1960). However, some "L" uptake isotherms are linear over much of their range (Giles et al, 1960), and "C" and "L" isotherms can be difficult to differentiate.

LA molecules are amphipathic and it seems likely that they would be absorbed by cellular membranes, the lipophilic part penetrating by hydrophobic interactions within the phospholipid region, and the polar group remaining associate with the polar region of the membrane. A disturbance in the membrane phospholipids could be responsible for an alteration in the protein-lipid hydrophobic interactions with a consequent effect on

membrane bound enzymatic activity.

It is thus relevant to briefly outline the possible mechanisms for the interaction of LAs with the phospholipid membranes of other cells that have been proposed by Feinstein (1964); Ohki (1970); Papahadjopoulos (1972) and Papahadjopoulos et al (1975); common processes may be identified with cellular uptake. It is suggested that LAs interact with polar groups as well as the hydrocarbon phase of the nerve phospholipid membrane. The former may be both dipole-dipole interaction as well as electrostatic interaction (Fig 4.1). This would cause the LA to exhibit an effect similar to that of Ca^{++} which interacts with the polar groups on the surface of the membrane (Feinstein, 1964 and Papahadjopolous, 1968).

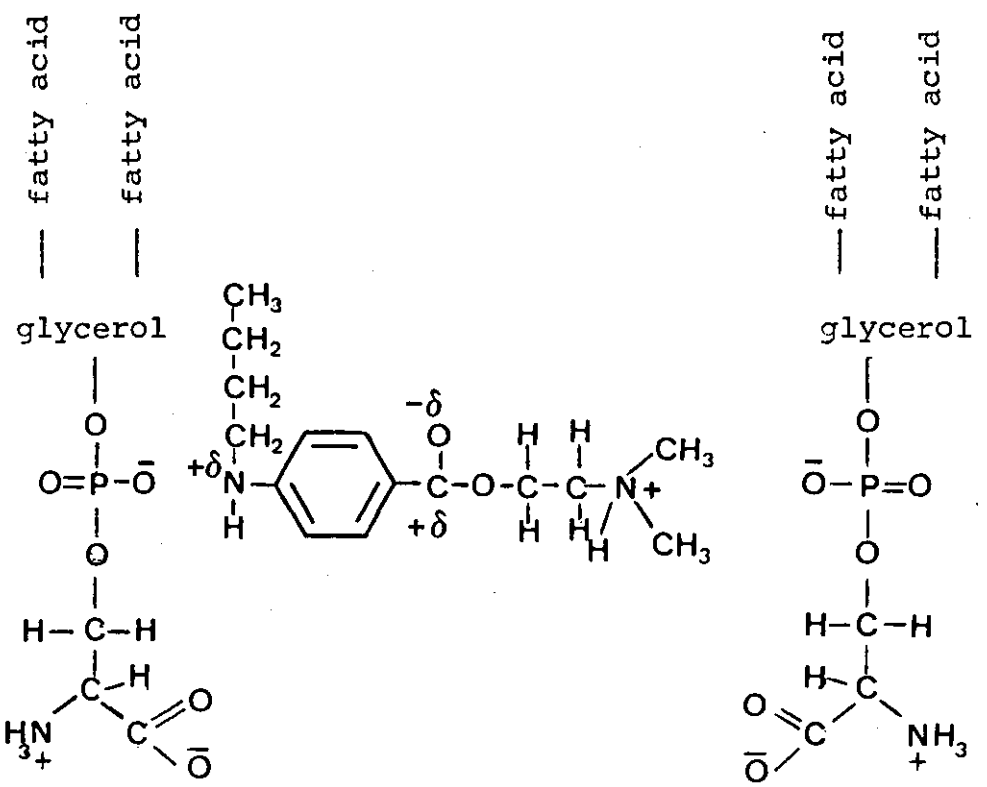
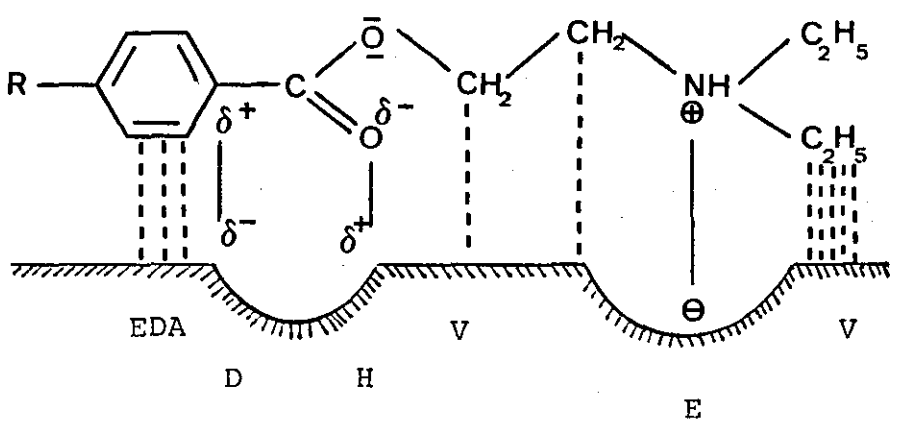


Fig 4.1 Proposed model for the mechanism of complex formation between acidic phospholipids and LAs. (After Feinstein, 1964).

The latter interaction would be hydrophobic interaction between non-polar groups of the LAs and of the phospholipids, which suggests that some part of the LA can penetrate into the hydrocarbon phase of the membrane. This would provide a greater degree of freedom for the lipid chains and to lessen van der Waals interaction forces (Feinstein et al, 1975). Any resulting increase in disorder of the lipid molecules would cause a conformational change in proteins intimately associated with the lipid (Johnson and Miller, 1970; Seeman, 1972 and Lenaz et al 1978).

Büchi and Perlia (1972) suggested that LAs are bound to the nerve membrane by few types of bonds, which are illustrated in Fig 4.2. They also pointed out that



EDA Electron donor-acceptor binding
V van der Waals binding
D Dipole attraction
E Electrostatic binding
H Hydrogen bond

Fig 4.2 Binding of LA to the site of action (After Büchi and Perlia, 1972)

the substituent R is an electron donating group, which causesan increase in the density of electrons at carboxyl oxygen with a consequent increase in the attachment of the anaesthetic to the receptor. Therefore amethocaine having H_9C_4-NH- instead of $-NH_2$ in procaine will have more binding power to attach to the receptor site, and will be more effective than procaine.

The spectrophotometric method for determination of uptake of LAs by bacteria used in this study is not very sensitive. As the overall extent of the uptake was very low, dense cell suspensions were used to get a detectable difference from the corresponding control concentration. Other experiments were performed using lower cell concentrations, thus making comparison difficult. The amount of absorbed drug is not measured directly, but by calculation from the differences between the drug concentration of the suspending medium before and after contact with the cells. The accuracy of values of K (the ratio of concentration of LA taken up by the cell to the concentration in the medium) depends on the accuracy of C (drug concentration in the supernatant liquids from the control mixture), C' (concentration of drug remaining in the supernatant after uptake has taken place), v (the bacterial cell volume) and V (the total volume of reaction mixture). The main source of error is in dilutions of C and C' in order to get a measurable OD, and the need for correction due to the leakage of intracellular material (260 nm absorbing material). Therefore, the use of radioactive LA, would be expected to give a more accurate result.

Experimental results so far mentioned, show the

higher antibacterial activity of amethocaine than procaine, and of cinchocaine than lignocaine. This reflects the structural differences between the members of each group, and is apparently related to a number of differences in physical properties, such as solubility, miscibility, micellisation, surface and interfacial tension. Indeed Seeman (1972) is of the opinion that the narcotic activity of anaesthetics correlates much better with the non-aqueous-aqueous partition coefficient than with any other parameter of the anaesthetic molecule. The partition coefficients of LAs used (Table 3.7) are in general agreement with the antibacterial activity of these LAs. It therefore seems likely that the difference in the antibacterial activity of the LAs is related to their lipid solubility, the more lipophilic cinchocaine and amethocaine showing a greater antibacterial effectiveness than lignocaine and procaine. Similarly in a homologous series the surface anaesthetic activity of these agents has been shown to be parallel to their lipid solubility, degree of ionization, diffusion coefficient, surface and interfacial activity (Büchi et al 1964).

The correlation of anaesthetic potency and antibacterial potency of LA to the non-aqueous/aqueous partition coefficient supports the idea that membrane anaesthetic interaction is lipophilic in nature (Schneider, 1968; Seeman et al 1971; Machleidt et al 1972 and Seeman, 1972). The hydrophobic region of a membrane may consists of 1) non-polar portions of lipid molecule, 2) the non-polar interfaces between lipid and protein molecules; or 3) the hydrophobic regions of protein

molecules. Amethocaine and cinchocaine being more lipophilic than procaine and lignocaine would be expected to have stronger hydrophobic binding with the membrane components and therefore exert a greater antibacterial activity.

There is a similarity between the action of the LAs and that of the cationic antibacterial agents (e.g. CTAB). Such compounds usually have a marked surface activity and form colloidal aggregates (Salt and Wiseman, 1970). Consequently the micellar formation of these agents was investigated. Amethocaine lowered the surface tension of both water and CFM whereas procaine did not have any significant effect. Any surface activity inherent in the LA will of course contribute to its antibacterial effectiveness by aiding its concentration at the bacterial cell surface and this again reflects the difference between amethocaine and procaine. Similarly in his adsorption expansion hypothesis Schneider (1968) reported that anaesthetics would adsorb to a cell membrane, altering the amount of water in contact with each side of the membrane, and consequently altering the interfacial tension at the membrane-water interface. The net result of these effects would be for membrane to undergo extension.

Such an effect would be difficult to imagine in a bacterial cell with its rigid/semi-rigid cell wall unless such extension occurred into the cytoplasm. Certainly intracellular changes do occur in LA treated cells (Plates 3.18 and 3.19) but direct membrane intrusion into the cytoplasm was not observed.

The formation of micelles causes abrupt changes

in many physical properties of a surface active agent provided that the aggregation number is fairly large (Grindley and Bury, 1929). Measurement of the concentration at which these changes take place is generally taken as indicative of coalescence of the monomers and CMC values obtained by different methods have been found to be comparable. The CMC's (dye solubilization) of amethocaine and cinchocaine (Fig 3.53) are in agreement with those reported by previous workers (Eckert et al, 1966; Farhadieh et al 1967; Salt and Traynor, 1979), and have values of 31.5 and 23.5 $mg.ml^{-1}$ for amethocaine and cinchocaine respectively in water. These values were lowered to 10.5 and 7.5$mg.ml^{-1}$ when CFM was used instead of water. This again favours a comparison with the cationic surfactants (Salt and Wiseman, 1970). In the concentration ranges studied, no detectable CMC values were observed for procaine or lignocaine. Farhadieh et al (1967), however, reported that procaine forms small ionic micelles, though they did not reported any value for the CMC of this compound.

Antibacterial activity increases with decrease in the CMC values. This is to be expected, the smaller the CMC value of the LA, the more hydrophobic is the compound. The uptake of the compound will thus occur more easily from aqueous solution, and most of it will accumulate in the lipid region of the cell.

The cytoplasmic membrane of the bacterial cell plays a dual role: it forms an osmotic barrier to the free diffusion of small molecules, and controls the internal concentration of metabolites by specific transport mechanism. Destroy either of these roles and the cell is no longer able

to function as an effective metabolic unit. The barrier action of the membrane lies in the organization of the lipoproteins that compose it. Distortion or dissociation of the lipoprotein structure leads to the leakage of intracellular material. The level of the leakage depends on the extent of damage to the membrane. However, although membrane damage and loss of cytoplasmic constituents might be expected to result in the inhibition of cellular growth, loss of viability may only occur when the membrane damage is irreversible.

Procaine and lignocaine did not cause any leakage of intracellular material from E.coli cells after 10 to 30 min contact (Figs 3.44 to 3.46 and 3.49, Plates 3.2 and 3.4). Also SEM (Plates 3.10, 3.11, 3.14 and 3.15) and TEM (Plate 3.17) show no surface or intracellular damage due to the action of procaine or lignocaine on E.coli cells. Amethocaine and cinchocaine, however, caused the leakage of considerable quantities of intracellular materials, including 260 nm absorbing material, pentoses and inorganic phosphate (Figs 3.44 to 3.49). Low concentrations of amethocaine and cinchocaine induced little or no leakage, but as the concentration increased, the leakage also increased reaching a maximum at about 10 and 5 $mg.ml^{-1}$ for amethocaine and cinchocaine respectively. SEM (Plates 3.8, 3.9 and 3.13) also show some surface damages which could be due to the effect of these LAs on the bacterial cell surface. Prolonged contact, however, between drug and cell (Fig 3.46 and 3.49) produced a different pattern in the leakage profile. After the maximum release was reached at about 10 $mg.ml^{-1}$ amethocaine

and 5 $mg.ml^{-1}$ cinchocaine, the extent of leakage was reduced as the LA concentration was increased. Furthermore when the rate of loss of pentoses was followed (Fig 3.47), concentrations of amethocaine and cinchocaine about those causing peak leakage showed very rapid initial release which was completed within 10 min. This was followed by a secondary slower release of material; with higher concentrations of LA, however, only the rapid initial release was observed.

Such a pattern of leakage was reported by Leung and Rawal (1977) for the action of amethocaine on P.aeruginosa and by Silva et al (1979) for chlorpromazine, nupercaine and amethocaine on B.cereus. This leakage profile is described as diphasic and can be compared to that of chlorhexidine (Rye and Wiseman, 1964 and 1965; Hugo and Longworth, 1964). This reduction in the leakage at higher drug concentration could be due to sealing of the cells by one of a number of mechanism. For example Hugo and Longworth (1964) suggested that high concentrations of chlorehexidine brought about the coagulation of protein which would prevent leakage by the simple mechanism of blockage. However, when the effect of LAs on the induced changes in the turbidity of cell-free cytoplasmic material was investigated (Figs 3.63 and 3.64), increase in LA concentration (amethocaine or cinchocaine) caused increase in turbidity which could be due to the precipitation of cytoplasmic material (proteins and nucleic acids). At higher drug concentrations no further increase in turbidity was observed, probably as all the cytoplasmic material had been coagulated at lower concentration of drug. These

results are also substantiated by the TEM (Plates 3.18 and 3.19), which show ultra thin sections of E.coli cells pretreated with 10 $mg.ml^{-1}$ amethocaine (Plate 3.18) which caused nearly maximum leakage (Figs 3.44 to 3.46 and 3.49). The cell have intensely stained interiors recalling the "dense blocks" reported by Silva et al (1979). Such sections were not subjected to post-sectioning staining and had only been pre-washed with uranyl acetate. Intracellular staining must therefore be due to the infiltration of stain through the cell membrane. Higher concentration (20 $mg.ml^{-1}$ amethocaine) give rise to similar micrographs (Plate 3.19) in which the contraction of cytoplasmic constituents from the cell envelope is more detected.

An alternative explanation for the reduction of leakage at high drug levels was put forward by Hugo and Longworth (1964). They suggested that cytoplasmic membrane was "sealed off" by a surface film of chlorhexidine. Büchi and Perlia (1972) suggested the obstruction of membrane surface by LA bases as one of the modes of action of these agents on the nerve membrane, though no evidence of this sealing effect can be seen in the electron micrographs of E.coli cells treated by high concentrations of amethocaine (Plates 3.18 and 3.19).

Salton (1951) pointed out, that a decrease in cellular leakage could be due to the readsorption of leaked material by the drug saturated cells. This possibility was not examined in this study.

Another possible explanation is that if the leakage is due, at least in part, to the activity of autolytic anzymes following cellular damage, this

enzymatic activity could be inhibited at higher drug concentrations (Hugo and Longworth, 1964; Rye and Wiseman, 1964 and 1965). The two phenomena may be distinguished by conducting the experiment at 1^{o}-4^{o}C, as the autolytic enzyme activity is inhibited and resulting leakage level represents only material from metabolic pool (Rye and Wiseman, 1965). Indeed when the pentose release was followed at 1^{o}C, increasing the contact time did not increase the amount of released material, i.e. this secondary release could be due to the break-down of large molecules (such as ribonucleic acids) by the action of autolytic enzymes which were inhibited at low temperature.

The amount of ^{32}P released by exposure of E.coli cells for 10 min to amethocaine and cinchocaine was comparable to that released when the cells were treated with cold TCA, which extracts the metabolic pool (Britten and McClure, 1962). The amount of 260 nm absorbing material or pentoses released, however, was for both amethocaine and cinchocaine less than the amount released when the cells were boiled for 10 min.

While the exposure of E.coli for 10 min to procaine and lignocaine did not cause any significant leakage, a longer exposure time (5 hr), did cause minor release of intracellular material (Fig 3.47). Similarly Silva et al (1979) reported that procaine (100 mM) caused a minor release of K^{+} from B.cereus.

Although the leakage of 260 nm absorbing material and pentoses by LAs occurred over a comparatively longer period of time than ^{32}P, a comparison of experimental

results suggest that it is the loss of intracellular materials in general that has an effect on the viability of the organism. These substances, together with some others, such as amino acids, have been shown to be important to osmoregulation (Tempest, Meers and Brown, 1970) and one of the effects of their loss is the reduction in internal osmotic pressure. Apart from osmoregulation, the small molecular weight substances leaked by LAs treatment form the basic materials from which complex molecules like structural proteins and nucleic acids are formed. The formation of these complex molecules required enzymatic activity but it is possible that some enzymes are also lost through the action of LA on the cell membrane. The results of the leakage of these materials therefore is that, the organism loses compounds necessary to manufacture complex molecules. This will bring about the cessation of growth, and if the materials lost are not replaced, lead to the death of the organism.

Gross leakage has been related both to cell death (Salton, 1951) and to the bacteriostasis action (Hugo and Longworth, 1964; Woodroffe and Wilkinson, 1966). Salton (1968) reported that in many cases with cationic detergents it is possible to show quantitative correspondence between killing and release of solutes, so that disruption of the membrane can safely be taken to be the lethal event. LA induced leakage appears to be related to the bactericidal rather than bacteriostatic activity. Thus bacteriostatic concentrations of LA induce little or no leakage of intracellular material whereas, as the

concentration increases, there is a relationship between % loss of viability and % of metabolic pool material released. Concentrations of about 7.5 $mg.ml^{-1}$ amethocaine and 5 $mg.ml^{-1}$ cinchocaine which produce considerable loss of metabolic pool material within 10 min, causes 99.9% loss of viability over the same period. The difference in the MBC and the concentration that cause maximum leakage might be due to a carry over of bactericide which continues to exert its cytotic effect during the dilution and incubation period necessary for plate count. Another possibility is that, as the most immediate effect of a membrane active compound on the cell is the leakage of K^+ (Hugo, 1977), therefore it might be possible if the K^+ leakage was investigated, a better relation to cell death could be observed. However, although the loss of viability and leakage are caused by similar LA concentrations, the autolytic release which occurs comparatively slowly probably accompanies and follows, rather than causes, the observed loss of viability.

The optical density of non-growing cell suspensions of E.coli, K.aerogenes, P.aeruginosa and B.megaterium was influenced by amethocaine and cinchocaine treatment, but not by procaine or lignocaine in the concentration range studied (Figs 3.55 to 3.58). While low concentration of amethocaine and cinchocaine did not change the turbidity, higher concentrations caused a rapid and significant increase in culture turbidity.

Increase in turbidity of bacterial cell suspension can be attributed either to an increase in effective

(reflecting) surface area of the cell or to an increase in refractive index of the cell or both (Hugo and Longworth, 1964). The change in the effective reflecting surface of the cell would be accompanied by a change in the size or shape of the cell. A comparison of drug adsorption (Figs 3.50 to 3.52) and turbidity of bacterial suspensions in the presence of LAs (Fig 3.55) suggests that the first increase in turbidity may be caused by changes in the reflecting surface of the cells due to the presence of the adsorbed drug, or to a drug induced physical alteration of the cell surface. Furthermore when the effect of amethocaine and cinchocaine on turbidity changes was followed by time (Fig 3.59), concentration of 10 $mg.ml^{-1}$ amethocaine and 5 $mg.ml^{-1}$ cinchocaine, which cause peak leakage, caused reduction in turbidity. This suggests that gross cytological damage is linked with a well-marked change in optical density of the cell, possibly caused by reduction in size of the cell. The rise or nearly no change in turbidity at higher LA concentrations, may suggest complete precipitation of cell constituents.

The effect of drugs on the cell wall, has been shown to be one of the factors contributing to the increase in optical density of bacterial cell suspension (Munton and Russell, 1970; Lamikanra and Allwood, 1977b). However, when the effect of LAs on the cell envelope preparation of E.coli was investigated (Figs 3.63 and 3.64), only minor OD increase in the presence of amethocaine and cinchocaine was observed. This possibly corresponded to the increase light scattering properties

of walls as caused by other adsorbed drug (Hugo and Longworth, 1966). Also Plates 3.8, 3.9 and 3.13 show that amethocaine and cinchocaine caused some surface damages on E.coli cells. This could be either due to the direct effect of drug, or perhaps because of vacuum collapse (indirect effect of drug, which makes the cell wall weak).

When the effect of LAs on the isolated cell contents was investigated (Figs 3.63 and 3.64), the turbidity increased, reaching a maximum at higher LA concentration, perhaps due to the complete coagulation of the cytoplasmic contents. Similar results were obtained when the effect of LAs (amethocaine and cinchocaine) on lipid depleted cells of E.coli investigated (Fig 3.65). Confirming that the integrity of the cell is not essential for these effects of the drug.

However, because of the lipophilic character of some of the drugs used in this study an attempt was made to correlate culture turbidity with the effect of these LAs on the turbidity of a cell-free dispersion of extracted lipids (Fig 3.66). Dispersion turbidity initially increased as LA concentration was increased. This OD increase could account for the small increase in OD of the isolated E.coli cell envelope preparation described earlier. However, this increase was followed at higher LA concentration, by a decrease in dispersion turbidity to zero optical density. This decrease is probably due to the solubilization of the lipids in the hydrophobic core of the LA micelles which formed at concentration above the CMC (Fig 3.53). The amount of

reduction in OD induced by these high amethocaine concentrations was less than that for cinchocaine. This is probably because the latter is a more effective solubilizing agent for the bacterial cell envelope lipids. Such a drastic decrease in turbidity was not observed for the effect of LAs on E.coli cells suspended in distilled water (Fig 3.60). As the CMC of LAs in distilled water is much higher than in CFM, no solubilization of lipid component of bacterial cell envelope had occurred in the concentration range studied. When the effect of procaine on the dispersed lipid was investigated no change in turbidity occurred (Fig 3.66). While with lignocaine concentrations in excess of 7.5 $mg.ml^{-1}$, turbidity increased, which suggests this drug is perhaps more lipophilic than procaine.

Salt (1976) suggested that changes in turbidity are indicative of post mortem changes in the cells following penetration of the cell membrane. Comparing LA concentrations having measurable effects on culture viability, cellular leakage and culture turbidity, confirms the above statement.. Concentrations of LA about and higher than the MIC, change the membrane permeability. This causes the release of cytoplasmic constituents which in turn causes cell death. The cytoplasmic constituents interact with the agent, perhaps both within and without the cell and are the significant cause of the turbidity increase.

Further evidence which support the view that, induced changes in turbidity might be the result of post mortem changes comes from a comparison of the changes in turbidity of E.coli cells suspended in distilled water with that of cells suspended in other media (Figs 3.60 to

3.62). The extent of the first region of little or no change in turbidity, is increased, probably due to the presence of ions in the suspending medium. As the metal ions will stabilize the membrane (McQuillen, 1960; Morris and Russell, 1970; Salt and Wiseman, 1970), and compete with the similarly charged drug during adsorption (Salt and Wiseman, 1970), a higher concentration of LA is necessary to increase membrane permeability. Leakage, cell death and increase in turbidity will thus occur at different concentrations in the various media.

Induced increases in turbidity of non-growing cells by LAs (amethocaine and cinchocaine) therefore seem to be due to the action of LA on the cytoplasmic constituents following penetration of the cell membrane and to a lesser extent to a direct effect on the cell envelope components. Higher concentrations, however, may cause a reduction in turbidity due to the partial solubilization (fluidizing effect) of the cell surface lipids which are present in Gram-negative bacteria.

Most previous investigations of the inhibition of protein synthesis by antibacterial agents have been carried out by studying the uptake of radiolabelled amino acids. Schmidt and Rosenkranz (1970) showed that procaine and lignocaine inhibit the synthesis of DNA, RNA and protein to the same extent. An alternative method was developed and used in this study.

Bacterial cells, when growing in a medium containing certain pairs of compounds as carbon sources, undergo diphasic growth, if the presence of one of the carbon compounds represses enzymes and/or permeases needed to

utilize the other(Monod,1949).Such growth curves consist of two exponential phases separated by a distinct lag phase, marking the switch over from one carbon source to the other. It is during this enforced lag period that de-repression occurs and the cells commence de-novo synthesis of the necessary enzymes and/or permeases specific for the second carbon source. Normally when sufficient protein has been synthesised, the cells return to logarithmic growth. Any inhibition of this specific protein synthesis would result in the cells having a reduced capacity to use or being unable to use the second (now sole) carbon source in which case the lag should extend into a pseudo-stationary phase.

In the glucose-galactose containing medium, as was used for this series of experiments the bacteria utilize glucose exclusively during the initial growth phase. Upon depletion of glucose, the cells utilize galactose giving rise to the second growth phase. As expected the growth of E.coli when glucose is being used as a carbon source shows a faster growth rate than when galactose is used (Figs 3.26 to 3.39).

During the studies of the effect of the addition of antibacterial agents to E.coli growing under diauxic conditions two approaches, differing only in the timing of the drug addition, were used. Drug added at the start of growth (Figs 3.26 to 3.32) was deemed to be not a very ideal method for the evaluation of antimicrobial agents by the diauxic growth technique, as the early presence of anti-bacterials at sub-MIC values influenced the cell growth rate sufficiently to make the prediction of the diauxic

lag and the interpretation of the curves complex. So mostly the results obtained from experiments in which the drugs were added just prior to the onset of the diauxic lag will therefore be discussed.

The antibacterial agents used were, chloramphenicol and puromycin as examples of inhibitors of protein synthesis (Franklin and Snow, 1975), ampicillin, an inhibitor of cell wall synthesis (Gale et al, 1972) and CPB which is membrane active agent (Sykes, 1965).

Both chloramphenicol and puromycin gave similar patterns of inhibition, not permitting the use of the secondary carbon source at concentrations approaching, but less than, the relevant MIC. Lower concentration of these agents, however, permitted growth to continue at a reduced rate; the extent of the diauxic lag phase, however, depended on the ability of the cells to synthesize protein de-novo, and this in turn will be affected by drug concentration. Both drugs caused a marked extension of the diauxic lag. For example it increased from 10 min (control) to ca 50 min (7.5μg.ml^{-1} chloramphenicol) and ca 60 min (600 μg.ml^{-1} puromycin) after which the cells continued to grow but at very low rate (Figs 3.36 and 3.37).

Ampicillin and CPB gave essentially similar curves at low concentration (Fig 3.38 and 3.39) and did not inhibit the appearance of the second logarithmic growth phases, though they gave rise to curves of various slope indicating inhibition probably not involving direct suppression of protein synthesis. With CPB the extent of the lag phase was from 10 (control) to 25 min (5μg.ml^{-1}) and after that the cells continued to grow. Ampicillin at

concentration more than 2.5μg.ml^{-1} ,after a time depending on drug concentration, caused reduction in cell turbidity due to the cell lysis (Rye and Wiseman, 1967). Similarly, higher concentration of CPB > 6 μg.ml^{-1}) caused a reduction in cell turbidity (Fig 3.32).

Diauxic growth of E.coli in the presence of LAs (Figs 3.27, 3.29, 3.33 and 3.35) showed a lag phase, the extent of which depended on the LA concentration, it increased from ca 10 min to 30 min (0.75 mg.ml^{-1} amethocaine), 30 min (0.3 mg.ml^{-1} cinchocaine), 50 min (25 mg.ml^{-1} procaine) and 55 min (10 mg.ml^{-1} lignocaine) and after which the cells continued to grow at a reduced rate as compared with the control. The overall shape of these curves indicates that LAs, especially amethocaine and cinchocaine have much more in common with CPB than with chloramphenicol or puromycin. The increase in the concentration of CPB or LA cause relatively small increase in the lag phase (although it is longer for LAs) perhaps reflecting an overall reduction in cell metabolism, after which the bacteria continue to utilize the second carbon source at a rate relating to the initial degree of growth inhibition. Specific inhibitors of protein synthesis do not normally cause cell lysis as an immediate part of their mode of action and though results indicate that LAs may inhibit protein synthesis to a small extent, it seems likely that this is not the prime mode of action. Such an inhibition is probably a result of a general reduction in the efficiency of the cell following structural changes in membrane causing a reduction in metabolic effectiveness.

Furthermore electron micrographs (Plates 3.6, 3.10,

3.14, 3.15) show that in the presence of low concentrations of LAs (up to about MIC) some E.coli cells in a growing state form filaments, indicating an interruption of the division process. The concentration range which induced filaments, is comparable with the concentrations that cause DNA synthesis inhibition (Schmidt and Rosenkranz, 1970). DNA replication and cell division are intimately inter-connected (Slater and Schaechter, 1974) and a wide variety of chemical treatments which inhibit DNA synthesis also inhibit cell division. Cells in which DNA synthesis has been inhibited often elongate without division, eventually forming very long cells (Stanier et al 1980).

The inhibition of protein or DNA synthesis could have been caused by loss of essential molecules through a damaged cytoplasmic membrane (Woodside, 1973). With LAs, however, this is not the case, as the concentration that inhibits protein or DNA synthesis is lower than the concentration that cause release of cytoplasmic constituents. Therefore, it seems reasonable to assume that inhibition of protein or DNA synthesis may well be secondary to a change in the association of DNA with the cell membrane during replication (Ryter, 1969; Garland and Highton, 1973) due to LA induced changes in membrane structure.

Thus local anaesthetics do possess some antibacterial properties. However, although all LAs studied affected respiration and inhibited cellular growth in a reversible manner, significant differences in effectiveness are apparent between the individual drugs. Neither lignocaine or procaine were bactericidal agents against Gram-negative organisms, nor did they induce turbidity increases in non-

growing cell suspensions, or increase the loss of cellular constituents. Both amethocaine and cinchocaine are active antibacterial agents and appear to have much in common with the cationic antibacterial surfactants, though in concentration terms they are much less effective. Their effects reflect gross changes in cellular structure and may therefore be related to the general toxicity of these drugs, suggesting the possible use of bacterial cell suspensions as an alternative in the assessment of tissue toxicity.

It must, however, be emphasized that all four drugs are effective local anaesthetics. Taking them as a single group it seems probable that the effects on growth inhibition give a more realistic indication of relative LA effectiveness than the induced turbidity changes or effects on cell viability, which may well be reflecting the potential tissue toxicity of the drugs concerned.

The physical properties of LAs are of great importance in their role both as local anaesthetics and as anti-microbial agents. The hydrophilic-lipophilic balance of an individual molecule will influence not only its cellular uptake but also its intracellular distribution and consequent location at or near its site of action.

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