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Some aspects of the antibacterial action of diphenyliodonium chloride

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Some aspects of the antibacterial action of diphenyliodonium chloride.

by

MARYAM GERAMI-NEJAD

A thesis submitted in fulfilment of the requirement for the award of Master of Science of Loughborough University of Technology.

Supervisor: Dr. R.J. Stretton.

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I would like to thank Mrs. M. Hardy for company during my stay at Loughborough.

I would like to thank Mr. K. Moore for his part in proof reading my thesis.

Finally, but by no means least, I would like to thank my husband for his patience, help and encouragement.

DEDICATION

This thesis is respectfully dedicated to

Mr & Mrs Riseam

for without their kind assistance during the period of my study, none of this could ever have been achieved.

SYNOPSIS

Some aspects of the antibacterial action of diphenyliodonium chloride.

The antibacterial activity of diphenyliodonium chloride was investigated against five Gram-positive and three Gram-negative bacteria. The minimum inhibitory concentrations were determined for these organisms and diphenyliodonium chloride was more active against bacteria growing under aerobic conditions.

Diphenyliodonium chloride is a bacteriostatic agent when its actions on the survival of proliferating and non-proliferating cells are considered. No synergistic or antagonistic action was observed with sixteen common antibacterial agents.

There was a loss of ribonucleic acid from the cell when in contact with the drug, suggesting that the permeability of the cell membrane is altered.

Diphenyliodonium chloride was rapidly taken up by the cell and adsorption followed a Langmuirian isotherm. The adsorption of the drug increased linearly with pH and antibacterial activity also showed an increase with increase in pH value.

The compound also interfered with respiration and impaired membrane function as judged by loss of intracellular K^+.

Diphenyliodonium chloride is primarily a bacteriostatic agent which acts by impairing membrane function and so interfering with cellular respiration.

SECTION 1

Introduction

Microbes are exposed to chemical stress by man and also in the everchanging natural environment. There is a widespread requirement in medicine and industry for substances which are used outside the body as disinfectants, antiseptics and biocides.

The term disinfectant, describes products intended for use in the presence of dirt and dense bacterial populations.

Biocide is used more particularly for preservatives that prevent bacterial and fungal attack on wood, paper, textiles and other organic materials.

The term antiseptic is usually reserved for a substance that can be safely applied to the skin, with the aim of reducing the chances of infection by killing the surface bacteria.

Many compounds which are successfully used against bacterial infections are not highly bactericidal but merely prevent their multiplication, most are inactive against non-growing bacteria. A distinction is often made between bacteriostatic and bactericidal compounds, but the division is by no means clear cut.

Interaction of antimicrobial agents with the cell.

Whole cell

The first apparent interaction with the whole cell is when a compound is adsorbed onto the cell surface, and this adsorption is important in the disinfection process. The uptake of drugs by cells has been measured by many workers as an initial study of their mode of action.

Antiseptics are readily taken up by bacteria, the amount absorbed increasing with an increasing concentration. The adsorption isotherm sometimes shows a point of inflection which corresponds to the minimum bactericidal concentration, higher concentrations lead to a much greater adsorption of the compound.

The extent of killing of the bacteria is governed by three principal factors: namely, concentration of the compound, bacterial cell density and time of contact.

The uptake of a given amount of the compound per cell leads to the killing of a definite fraction of the bacterial population in a chosen time interval. The technique usually involves adding a solution of the drug to a cell suspension of known density and after a suitable time interval the cells are removed by centrifugation and the residual amount of drug in the cell free supernatant solution is determined. The concentration of the drug in the supernatant fluid is reduced by adsorption onto the cell surface. Hence, the total amount of drug taken up by the cells may be measured.

Giles et al (1960) considered that four distinct patterns of adsorption can occur. These are S, L, H, C, shapes. The S (S shaped) pattern is found when the solute molecule is mono-functional and has moderate intermolecular attraction, causing it to orientate vertically. It meets strong competition for substrate sites from molecules of the solvent or by another adsorbed species. Monohydric phenols when adsorbed on a polar substrate from water usually give this pattern.

L(Langmuir) pattern; in this pattern as more sites are filled it becomes increasingly difficult for a bombarding solute molecule to find a vacant site. The adsorbed solute molecule is either not orientated vertically or there is strong competition from the solvent. If vertical orientation does occur there is a strong intramolecular attraction between the adsorbed molecules. Amongst the phenols, resorcinol shows this type of behaviour.

The 'H (high affinity) pattern' is obtained when the solute is almost completely adsorbed. Sometimes the process is accompanied by ion exchange, as in many bacteriological staining procedures.

The 'C (constant partition) pattern' is obtained when the solutes penetrate more readily into the adsorbate than does the solvent. It has been shown to occur when aqueous solutions of phenols are adsorbed by synthetic polypeptides. It might also be expected to occur when phenols are adsorbed from an aqueous solution by bacteria containing a high proportion of lipid in their cell wall.

More recent studies on the adsorption of antibacterial substances by micro-organisms include the adsorption of dequalinium by Escherichia coli and Staphylococcus aureus (Hugo & Frier, 1969). They found that dequalinium acetate penetrates quite rapidly into the cytoplasm where its effect seems to be exerted. For adsorption of fentichlor by E. coli and S. aureus Hugo & Bloomfield (1971a) suggested that the drug was taken up by the cell wall and cell membrane, the latter probably being the main site of adsorption and main site of action. They mentioned that although both whole cells and cell walls of E. coli have a higher affinity for fentichlor than those of S. aureus the former is less susceptible to its antibacterial action. The adsorption of chlorhexidine by Clostridium perfringens was studied by Hugo & Daltrey (1974) who found that a low concentration of chlorhexidine caused a drop in the turbidity of cell suspensions, but higher concentrations caused a rise in turbidity and precipitation of the cytoplasm.

Information of the site of adsorption may be obtained by studying the process at different pH values but it should be borne in mind that the ionization of the disinfectant as well as receptor sites on the cell surface may be affected by changes in pH (Salton, 1957;, Hugo & Longworth, 1964).

Cell wall

The cell wall is a prime target for a number of important antibiotics eg. penicillins, cephalosporins, cycloserine, vancomycin and ristocetin. Here the precise biochemical target has been identified. The antibiotics moenomycin, enduracidin, prasinomycin, antibiotic 11.837 R.P. and novobiocin likewise act on the cell wall however the site of action is not precisely determined.

Pulvertaft & Lumb (1948) showed that E. coli,Streptococci and Staphylococci lysed almost completely when rapidly growing cultures were exposed to low concentrations of antiseptics such as formalin, phenol, mercuric chloride, sodium hypochlorite and merthiolate. The authors thought that the action was due to metabolic disturbance followed by uncontrolled action of lytic enzymes which function normally in cell wall synthesis during growth and cell division. Other examples of lysis induced by chemical agents and possibly having the same basic mechanism are given in the work of Smith et al (1975).

Cytoplasmic membrane

Early work in this area was concerned with drug-induced leakage of material and there is little doubt that this contributes to stasis or death according to the time and intensity of exposure to the chemical stress concerned. More recently reactions at the molecular level have been revealed and it is these reactions which are currently the most exciting. They include uncoupling of oxidative phosphorylation and inhibition of energy-dependent transport.

Cytoplasm

There are four targets for antibacterial drugs and antibiotics; the cytoplasm itself, cytoplasmic enzymes, the nucleic acids and the ribosomes.

1) Irreversible coagulation of cytoplasmic constituents

This drastic lesion is usually seen at drug concentrations far higher than those causing general lysis or leakage. The cytoplasmic components most likely to be coagulated or denatured are proteins and nucleic acids. Meyer (1901) showed that the degree of antibacterial action of phenols was proportional to their distribution between water and protein, thus suggesting that protein was a prime target. Cooper (1912) concluded that phenols destroy the protein structure within the cell.

2) Effect on metabolism and enzymes

The result of many experiments by many workers have shown that enzyme inactivation is only one of many events caused by chemical stress and is not likely to be a prime mechanism for death. In some cases it may be a cause of bacteriostatic action.

3) The nucleic acids

There are a number of antibiotics which affect the biosynthesis and functioning of nucleic acids. Actinomycin impairs the template function of DNA and binds to it. Antibiotics chromomycin, mithramycin and olivomycin also have similar effect on nucleic acids. Amongst the non-antibiotic antibacterial drugs, only three main compounds affecting these targets have been identified: these are the acridine dyes, formaldehyde and phenylethanol. Ferguson & Thorne (1946) after studying the effect of a series of acridine compounds on the growth and respiration of E. coli concluded that they inhibited reactions closely connected with synthetic processes.

Ribonucleic acid polymerase has also been identified as a target for acridines in cell-free preparations from E. coli. Dyes such as proflavin fit, or intercalate, into the double-stranded DNA helix and thereby prevent its functioning.

Grossman et al (1961) showed that the amino group of the purine and pyrimidine rings have been cited as likely sites for the interaction of formaldehyde.

Phenylethanol, is not only a membrane-active compound but it has also been shown to inhibit initiation of replication at high concentrations (Lark & Lark, 1966).

4) Ribosomes

Ribosomes are associated with the formation of peptides from amino acids ordered by messenger RNA and assembled by transfer RNA. This process is a singular target for streptomycin, neomycin, gentamycin, tetracyclines, chloramphenicol, lincomycin, puromycin and many others.

Ethylenediaminetetra acetic acid (EDTA) is a specific chelator of certain metals, including Mg^{++}, necessary for the integrity of the 50S and 30S ribosome units in prokaryotes. EDTA is used in conjunction with certain antiseptics to enhance their activity, specially against gram-negative organisms. (Russell 1971; Leive 1974). Its possible action on ribosome structure must be borne in min when it is present in antibacterial systems.

Effect of antimicrobial agents on the cell membrane.

Three main lesions may be induced in the cytoplasmic membrane by chemical disinfectants:

1) Leakage of cell constituents and modification of cell permeability

The modern notion of cell membrane structure is of a phospholipid bi-layer in which protein molecules are embedded. This is thought to be common to a variety of membranes ranging from the cytoplasmic membrane of prokaryotes, to the erythocytes and to mitochondrial membranes. (Singer & Micholson, 1972) (Fig 1 and 2).

The earliest experiments were those of Hotchkiss (1944) on the action of antibiotic tyrocidin on S. aureus. He showed that the addition of tyrocidin to a suspension of cells in the presence of glucose was followed almost immediately by a rapid fall in the rate of respiration. More detailed investigation showed that cells incubated in the presence of tyrocidin lost nitrogen and phosphate much faster than cells in the absence of the antibiotic. He also noted that similar effects were given by a variety of cationic and anionic surface-active agents.

Surface-active agents are divided into three classes according to the charge carried by the substance in solution:

1) Cationic surface-active agents possess a hydrophobic group, such as a hydrocarbon chain or alkyl-substituted benzene ring, together with a positively charged hydrophilic group e.g. a quarternary ammonium, sulphonium, or phosphonium group. Examples commonly used in studies of mode of action have been cetyltrimethyl ammonium bromide (CTAB), cetylpyridinium chloride (CPC) and the polypeptide antibiotics, tyrocidin

FIG 1. The lipid-gloubular protein mosaic model of membrane structure; schematic cross-sectional view. The phospholipids are arranged as a discontinuous bilayer with their ionic and polar heads in contact with water.
The integral proteins, are shown as globular molecules partially embedded in, and partially protruding from, the membrane. The protruding parts have on their surfaces the ionic residues (- and +) of the protein, while the nonpolar residues are likely in the embedded parts, accordingly, the protein molecules are amphipathic. The degree to which the integral proteins are embedded and, in particular, whether they span the entire membrane thickness depend on the size and structure of the molecules.

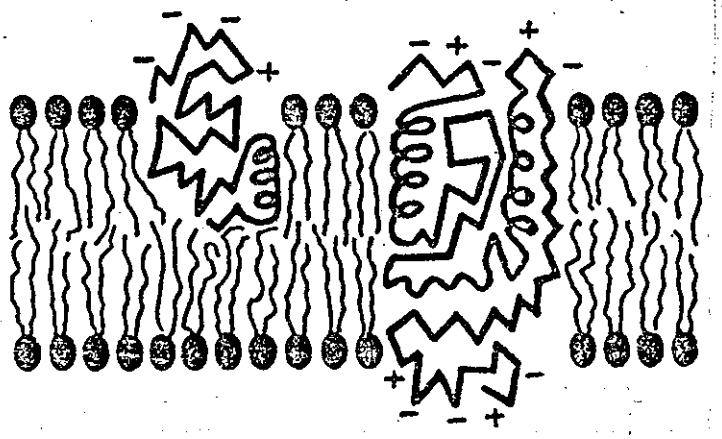
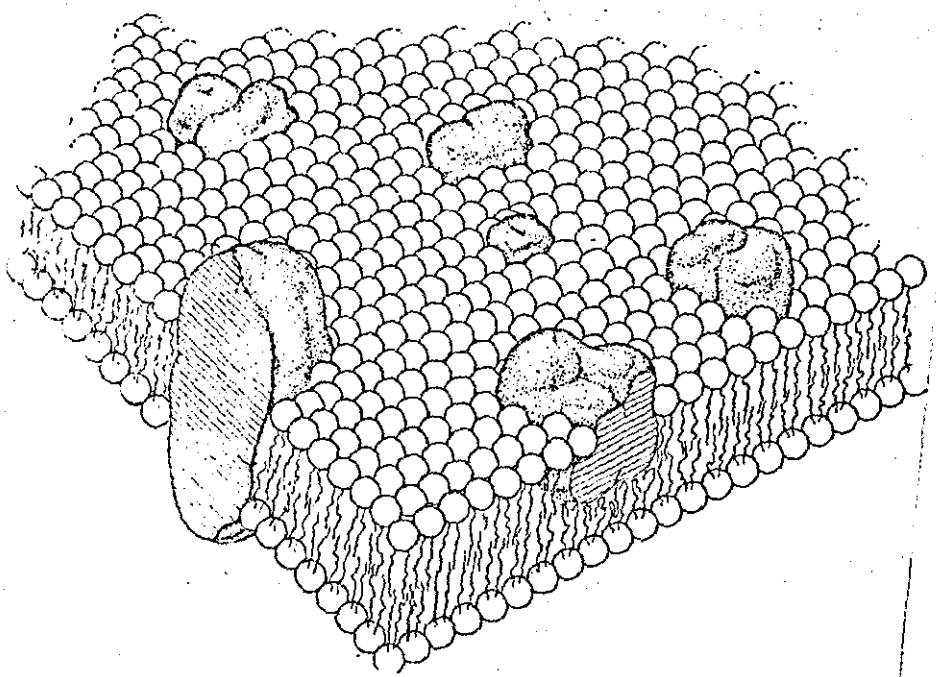


FIG 2. The lipid-globular protein mosaic model with a lipid matrix (The fluid mosaic model); schematic three-dimensional and cross-sectional views. The solid bodies with stippled surfaces represent the globular integral proteins, which at long range are randomly distributed in the plane of the membrane. At short range, some may form specific aggregates, as shown.



gramicidin S and subtilin. The activity of such substances increase as the pH is raised. Salton (1950, 1951) demonstrated that the concentration of CTAB producing a 99.99 per cent kill was related to the amount of purine or pyrimidine containing components leaking from the cell in the first 5 minutes of contact. Hamilton (1970) concluded that CTAB caused such a generalised effect on the membrane that it became permeable to all the ions.

Chlorhexidine is another cationic antiseptic, which is much less surface active than CTAB and has little detergent action. Hugo & Longworth (1964) investigated the ability of chlorhexidine to promote leakage of intracellular material from E. coli and S. aureus and found a diphasic leakage/concentration pattern.

2) Anionic surface-active agents posses a hydrophobic group similar to that in cationic agents and a negatively charged, hydrophilic group which may be a carboxyl, sulphate, sulphonate or phosphate group. Commonly used examples are sodium dodecyl sulphate (SDS), fatty acids and phenols. Anionic detergents such as SDS are much less toxic to bacteria although their target is also the cytoplasmic membrane (Gilby & Few 1960). This may be due to the fact that in anionic detergents the active ion is negatively charged and may be repelled by the negatively charged bacterial surface.

3) Non-ionic surface-active agents are practically non-toxic to bacteria, e.g. nonidet.

Certain solvents also cause leakage of ions, this has been by the detection of ions, labile nucleic acids and their components, purines, pyrimidines, pentoses and inorganic phosphorus.

Salton (1968) proposed that the sequence of events following contact of the antimicrobial with the cell is:

1) Adsorption onto and penetration into the porous cell wall.

2) Interaction with lipid-protein complexes involving reaction with hydrophobic portions of proteins and orientated lipids, leading to disorganization of the membrane.

3) Leakage of small molecular weight metabolites from inside the cell.

4) Degradation of proteins and nucleic acids.

5) Eventual lysis by the action of wall-degrading enzymes.

2) The inhibition of membrane bound enzymes

Membrane-bound adenosine triphosphatase has been implicated in the utilization of energy-dependent transport in microbial cells. Harold et al (1969 b) showed that chlorhexidine inhibits both membrane-bound and soluble adenosine triphosphatase (ATPase) and also net K^+ uptake in Streptococcus faecalis. They deduced that ATPase is coupled with K^+ transport in membrane processes in this organism. Daltrey & Hugo (1974) showed a similar inhibition of ATPase in Clostridium perfringens.

Dicyclohexylcarbodiimide (DCCD) was also shown to inhibit the growth of S. faecalis at 10^{-4}M (Harold et al 1969a). Energy-dependent transport of alanine and phosphate and the energy-dependent exchange of K^+ for Na^+ or H^+ were inhibited; glycolysis was decreased as a result of decreased ATP breakdown but ATP synthesis was unaffected. Membrane-bound ATPase was found to be sensitive whereas the solubilized enzyme preparation was resistant.

3) Attenuation of membrane electrochemical potentials

The membrane poses an osmotic barrier between the cytoplasm and the external milieu. The barrier is permeable to water. Other substances pass across the membrane as a result of specific mechanisms and the nature of the mechanism for a specific substance may differ in different types of cell. Transport of any substance across a membrane involves three considerations: namely how the substance passes through a hydrophobic barrier, how the internal concentration is related to that outside the cell and if the substance moves up a concentration gradient, how this is achieved.

Transport processes are usually considered to fall into three classes:

A) Passive diffusion

The substance diffuses through the membrane passing down a concentration or electrical gradient. The substance must be sufficiently soluble in the hydrophobic medium of the membrane to allow it to move across the barrier. For an uncharged molecule the concentration inside a cell cannot exceed that outside but the concentration distribution of a charged molecule will be determined by any electrical gradient across the membrane.

With bacteria in a medium at pH 7, the proteins within the cell will carry a negative charge and set up an electrical gradient across the membrane; passive diffusion of a positively charged ions can then give rise to a concentration gradient determined by the Donnan equilibrium.

B) Facilitated diffusion

The substance is insoluble in the hydrophobic barrier but reacts with a carrier within the barrier to form a carrier-substance complex which can move across the membrane releasing the free substance in the hydrophobic outer layers. Kabak (1968) suggested that the accumulation of certain sugars by bacteria represents a form of facilitated diffusion.

C) Energy-dependent transport

The substance cannot pass across the membranes. It requires a source of energy for movement across the membrane. Carrier-mediated transport against a concentration gradient can thus take place. Transport of this type may result in the build-up of free substance in the cell at a concentration two or three times higher than that present in the external medium.

Effect of antimicrobial agents on membrane transport

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

1) The Coupling hypothesis

The coupling factor theory which proposes that high-energy intermediates are formed in the electron-transport system and these are also involved in transport (Slater, 1966).

2) Conformational Coupling

The conformation theory which proposes that conformational changes in membrane proteins are mediated by ATP and are responsible for driving translocation processes (Green et al, 1968).

3) Chemiosmotic hypothesis

The chemiosmotic theory of Mitchell (1967, 1968, 1970) which proposes that membrane is impermeable to H^+ and OH^-, that the electron-transport system in the membrane sets up a proton gradient across that membrane, and that the proton gradient then provides the source of energy to drive transport processes. Where the transport of an ion or other substance is coupled to the co - or countertransport of another ion, it may be that the carrier is common to the two processes. Mitchell(1970), who uses the word 'porter' to describe the carrier, distinguishes three types:

1) Uniporter involved in the non-coupled equilibration across the membrane of a single solute, as in facilitated diffusion, e.g. transport of D-glucose or leucine in erythrocytes.

2) Symporter involved in the equilibration of two solutes, the translocation of one being coupled to the translocation in the same direction of the other, e.g. transport of phosphate coupled with H^+ in mitochondria.

3) Antiporter involved in the equilibration of two solutes where the translocation of one is coupled to the translocation of the other in the opposite direction, e.g. countertransport of H^+ and Na^+ or K^+ in mitochondria or S. faecalis (Fig 3).

Mitchell proposed that oxidative phosphorylation, ATP synthesis, active transport are powered by a protonmotive force, generated by metabolic oxido-reduction, which is apparent as a chemical and electrical gradient or potential difference across the cytoplasmic membrane. It may be expressed thus:

$$\Delta P = \Delta \Psi - Z \Delta pH$$

Where ΔP is the protonmotive force, $\Delta \Psi$ is the membrane electrical potential in mV and Δ pH is the trans-membrane pH gradient.
Z is a factor converting pH values to mV, and at $37^{o}C$ has a value of 62.

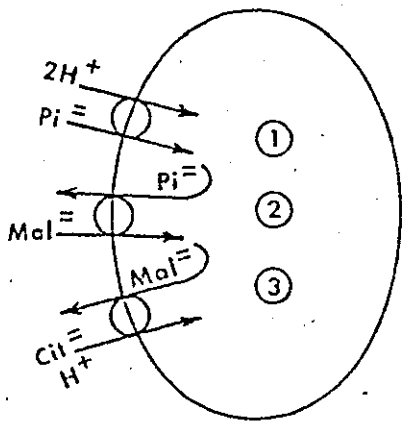


FIG 3 Interlocking systems for anion transport in mitochondria.

(1) phosphate-protein symport

(2) malate-phosphate antiport

(3) citrate-malate antiport

The expression-Z Δ pH, therefore, is a pH difference expressed in mV.

The gradient of pH and of electrical potential generated by the respiratory chain reverses the direction of an ATPase so as to bring about net synthesis of ATP. (Fig 4).

Several workers have shown that a group of chemicals such as uncoupling agent; dinitrophenol (DNP) and tetrachlorosalicylanilide will attenuate the membrane electrochemical potential generated by metabolism and inhibit reactions dependent on it.

Oxidative phosphorylation and its uncoupling

In the bacterial respiratory chains, as in that of mitochondria, reducing equivalents originating in NADH, succinate, or other electron donors pass over a cascade of carriers to oxygen.
The respiratory chains of bacteria differ from each other and from those of mitochondria. Bacterial respiratory chains oxidize a wider range of substrates than mitochondria do.
Important as such differences are, there is no reason to doubt that the coupling of respiration to phosphorylation is in principle the same in bacteria as in mitochondria. (Brodie & Gutnick 1972). (Fig 5).

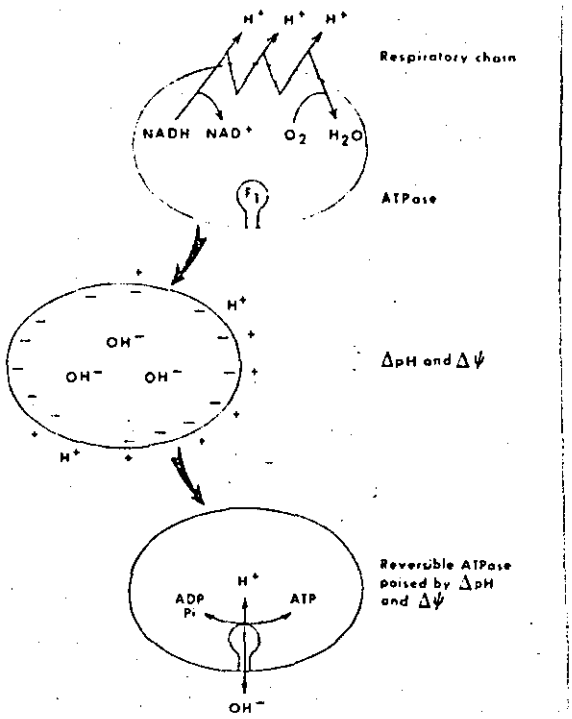


FIG 4 Chemiosmotic hypothesis in principle extrusion of protons by the respiratory chain, generation of Δ pH and $\Delta\Psi$ and the poising of ATPase by the proton-motive force.

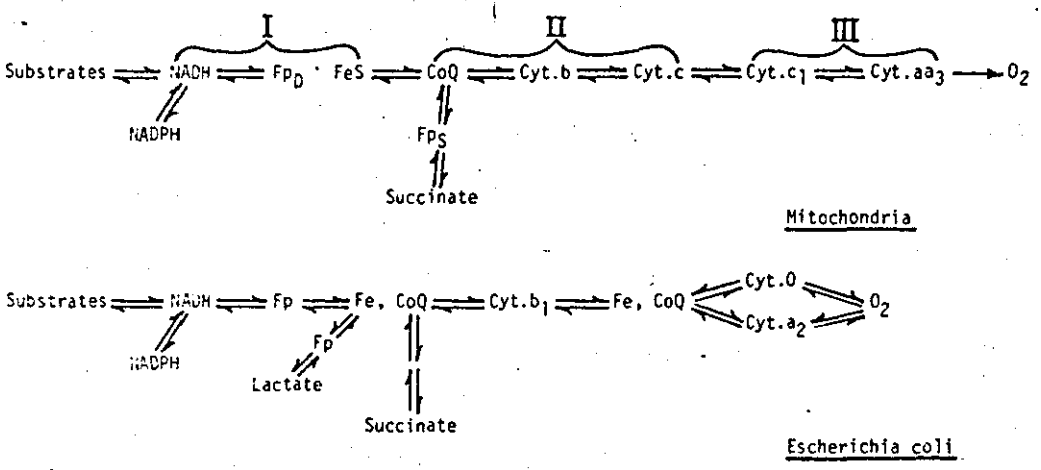


FIG 5 Respiratory chains of mitochondria and *E. coli*. Fp, flavoproteins; CoQ, coenzyme Q; FeS nonhaem iron.

Uncoupling agents (Mitchell, 1961) act as specific conductors of protons across bacterial, mitochondrial and artificial membranes, thereby modifying part of the protonmotive force across the membrane. He pointed out that dinitrophenol can sit in the hydrophobic portion of the membrane and act as a proton conductor and destroy the proton gradient which provides the energy for translocation, thus any proton symporter or antiporter system will be brought to a halt.

Harold & Baarda (1968) showed that tetrachlorosalicylanilide (TCS) uncouples the metabolic energy involved in translocation processes and they investigated its action on S. faecalis, an organism that lacks cytochromes and relies upon glycolysis for ATP generation.

The existence of a proton gradient in obligate anaerobic micro-organisms was demonstrated in Clostridium perfringens by Daltrey & Hugo (1974) (Fig 6a). TCS in combination with valinomycin partially discharged it (Fig 6c) and DNP did so completely (Fig 6d). Chlorhexidine and CTAB had no effect at 10 $\mu g/ml^{-1}$, although with CTAB at 100 $\mu g/ml^{-1}$ a further slight drop in pH was observed. (Fig 6b).

Harold and Papineau (1972) have shown that the active accumulation of dibenzyldimethyl ammonium ion (DDA^+) by metabolizing S. faecalis ceases upon adding TCS and the accumulated DDA^+ leaves the cells.

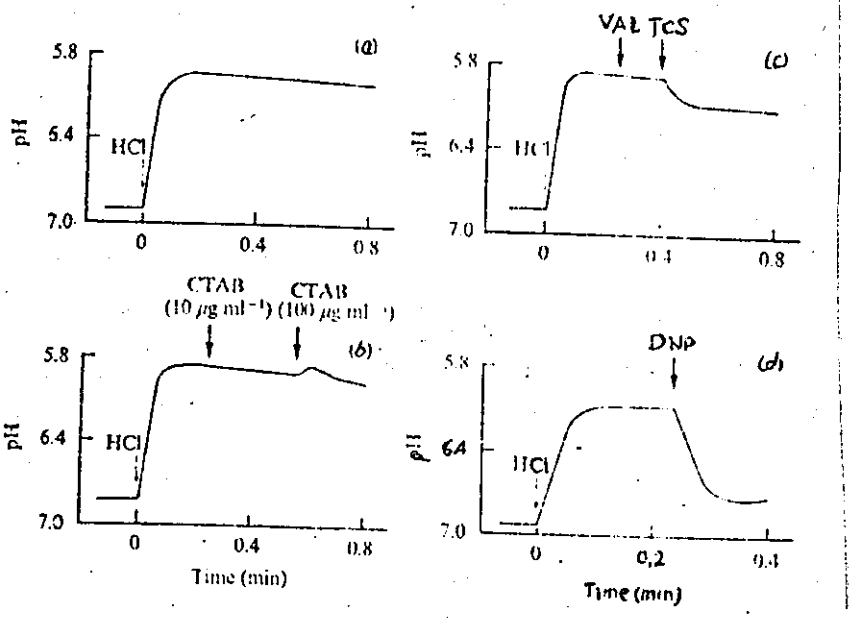


FIG 6 The effect of three antibacterial agents (CTAB, val and TCS) and one phenol (DNP) on proton flux across the membrane of Clostridium perfringens.

(a) Control suspension.

(b) Additions were made as indicated. CTAB, Cetyltrimethylammonium bromide.

(c) Additions were made as indicated. VAL, Valinomycin (0.5 $\mu g\ ml^{-1}$ final concentration) TCS, tetrachlorosalicylanilide (3×10^{-6}M concentration).

(d) DNP was added as indicated to a final concentration of 5×10^{-5}M. H Cl was added at 0 min.

(Reproduced with permission from Daltrey & Hugo 1974).

Recently Gatley and Sherratt (1976) studied diphenyleneiodonium and trialkyltin compounds,They found that these compounds catalyse a linked exchange of OH^- and of Cl^- ions across biological membranes and inhibit mitochondrial oxidations in Cl^- containing media as a consequence of this exchange. These compounds lowered the amount of succinate, citrate and glutamate accumulated in the matrix of mitochondria in the presence of Cl^-, also inhibit the oxidation of NADH-linked substrates by isolated mitochondria in a time dependent manner in the absence of Cl^-.

Inhibition of energy dependent transport.

Another postulate of the chemiosmotic theory is that it provides an explanation of the source of energy for the transport of metabolites into the cell. Several workers have shown that chemicals which act as uncoupling agents, are able to discharge transport processes. Thus, Hamilton (1968) showed that glutamic acid uptake in S. aureus was completely inhibited by TCS and DNP.

Table 1 shows some antibiotics and reagents which affect membrane processes.

Table 1 Inhibition of membrane processes (after Harold, 1972)

Metabolic region	Inhibitor	Mode of action
Respiratory chain	Cyanide	Inhibits cytochrome oxidase
	Azide	Inhibits cytochrome oxidase, often ATPase as well; conducts protons.
	Rotenone; piericidin	Specific inhibitors of first coupling site, probably on oxygen side of coenzyme Q
	Antimycin HOQ-NO	Specific inhibitors of second coupling site, between cytochromes b and c.
ATPase	Oligomycin	Typically inhibits mitochondrial, but not bacterial ATPase; site of action, the "oligomycin-sensitivity conferring-protein".
	DCCD	Inhibits both mitochondrial and bacterial ATPase;reacts covalently with a protein component of the membrane.
	Dio9	Inhibits mitochondrial bacterial ATPase; apparently binds to the ATPase itself.
Ionophores H^+	Dinitrophenol	Conduct H^+ very specifically across artificial and biological membranes; uncouple oxidative phosphorylation; H^+ movement is electrogenic.
K^+	Valinomycin monactin	Conduct K^+ very specifically across artificial and biological membranes. K^+ movement is electrogenic; do not always uncouple oxidative phosphorylation.
K^+, Na^+, H^+	Gramicidin	Relatively non-specific for mono-valent cations, uncouple oxidative phosphorylation.
K^+/H^+	Nigericin	Mediates electrically neutral exchange of K^+ for H^+ not usually an uncoupler.
Na^+/H^+	Monensin	Mediates electrically neutral exchange of Na^+ for H^+ not usually an uncoupler.
Lipid-soluble ions.	DDA^+	Lipid-soluble cations, accumulated by, and uncouple, intact mitochondria.

Iodonium Compounds

The iodonium compounds are members of a class of compounds in which iodine is an integral part of their structure and are present as positive ions. Hartman & Meyer (1893) synthesised the first iodonium compounds and Freedlander & French (1946) reported high inhibitory powers for many iodonium compounds including diphenyliodonium chloride against various organisms. The bacteriostatic and bactericidal activity of diphenyliodonium chloride and related compounds were tested by Gershenfeld & Witlin (1948 b), against a wide range of Gram-negative and Gram-positive organisms. They also found that diphenyliodonium chloride appeared as the most generally effective substance, with the chloride having a greater bacteriostatic efficiency than the iodide in general except against S. aureus and Eberthella typhosa.

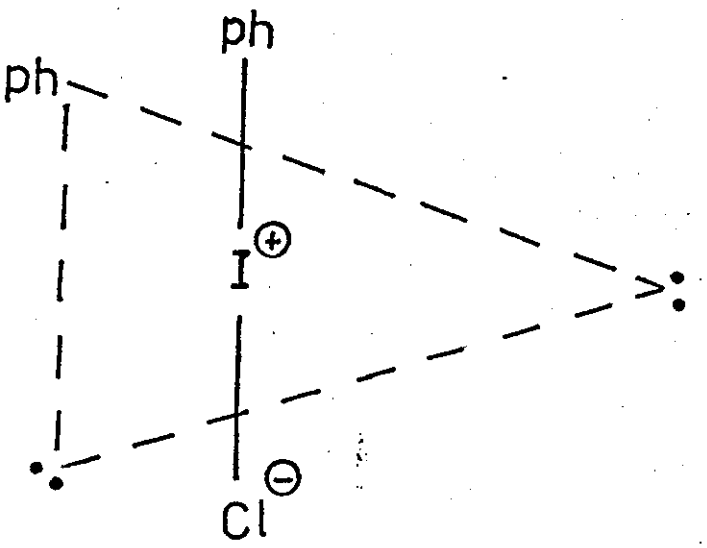
Engelhard & Worton (1956) investigated the fungistatic and bacteriostatic efficiency of bis (dichlorophenyl) iodonium chloride and indicated that this compound can be used in dermatological infections because it markedly reduced the transient and resident microflora of the skin.

The bacteriostatic and bactericidal efficiency of diphenyliodonium chloride with terpiniol and ethylalcohol and saponified mustard oil was studied by Zahir et al (1969) against many bacteria. Moyle (1975) compared a series of related iodonium compounds against some bacteria and he found that p.chlorophenyl (phenyliodonium) - chloride was one of the most effective antimicrobial agents of the diphenyliodonium type.

Freedlander & French (1946) suggested that certain compounds in this group are powerful cationic detergents but they are not notably surface-active. Gershenfeld (1948 a) compared results of the antibacterial action of diphenyliodonium chloride and iodide with those of bis-p-chlorophenyliodonium sulphate and iodide and concluded that the anion had an effect on the activity of the compound. He also showed the addition of sodium thiosulphate did not affect the bacteriostatic or bactericidal efficiency of iodonium compounds. Moyle (1975) found that bis (3,4 dichlorophenyl) iodonium chloride has greater activity than bis (4 chlorophenyliodonium) chloride and 4, chloro, diphenyliodonium chloride which have more activity than the unsubstituted diphenyliodonium chloride against S. aureus.

0.02 gm - 0.03 gm of diphenyliodonium chloride paralysed the muscles of frogs and dogs. (Gottlieb 1894). He also found that toxicology was analogous to metallic poisoning specially as found in lead or thalium poisoning. Gershenfeld (1948 b) found that intraperitoneal injections of diphenyliodonium chloride in white mice revealed increased excitability, increased respiration and paralysis of the hind legs. He also showed that the lethal dose for these mice was 20 mg/kg of body weight. Moyle (1975) studied the level of toxicity of different diphenyliodonium compounds on mice and found that the oral medium dose (LD_{50}) was 56.2 mg/kg of body weight for diphenyliodonium chloride.

The crystal structure of diphenyliodonium chloride was studied by Khotsyanova (1975). He showed that the molecule is T-shaped. If the two nonbonded pairs of electrons on iodine are taken into account, the molecule should have a trigonal bipyramidal structure.



Diphenyliodonium chloride

pH phenyl

•• electrons

SECTION 2

MATERIALS

Organisms

The following organisms were used throughout; Streptococcus faecalis NCIB 6783; Staphylococcus aureus NCIB 8625; S. aureus type Oxford; Bacillus subtilis NCIB 3610; B. megaterium NCIB 8291; Escherichia coli NCTC 9001; E. coli Texas NCTB 10097; Pseudomonas aeruginosa NCIB 8295; Clostridium sporogenes NCIB 532; Candida albicans A39. Unless otherwise specified E. coli 9001 and Staphylococcus aureus NCIB 8625 were used.

Media

The following media were used and were sterilised at 15 lb/in^2 for 15 minutes (unless otherwise specified). All media were prepared from "Oxoid" products unless stated otherwise.

A nutrient broth containing (g/l) Lab-lemco 10; neutralised bacteriological peptone 10; sodium chloride 5; was used for the maintenance of the bacteria. The pH was adjusted to 7.5 before use.

For Candida albicans a medium containing (g/l) maltose 38; yeast extract 2.5; mycological peptone 8; malt extract 2; agar No. 3;20. The pH was adjusted to 4.5 - 5 before use.

For the growth of Clostridium sporogenes, Reinforced Clostridial Medium and Reinforced Clostridial Agar were used.

For the growth of Streptococcus faecalis the following media were used:

1) medium NATY (Zarlengo & Schultz 1966) was used which contained the following; tryptone, 1.0%, yeast extract, 0.5%; glucose, 56 mM; Na_2H PO4 7 H_2O, 60 mM. The pH was adjusted to 7.6 before use.
2) Plate - count Agar.
3) medium which contains: 1.0% glucose; 1% tryptic digest of casein; 1.0% K_2H PO_4 and 0.5% yeast extract. The pH was 7.8 before use.

When solidified media were required agar No. 3;1.5% (w/v) was added.

The purity of cultures was checked periodically by plating out onto Mac Conkey medium for E. coli and Staphylococcus medium 110 (Oxoid CM145) for S. aureus. All cultures were incubated for 18 hours at 37°C unless otherwise indicated.

Chemicals

Aldrich Chemical Co. Ltd, supplied diphenyliodonium chloride, dicyclohexylcarbodiimide and dipicrylamine;

I.C.I. chlorhexidine;

Merck Sharp & Dohm, fosfonomycin;

Eli Lilly & Company Ltd D-cycloserine; and

British Schering Limited, gentamycin.

All other chemicals were of AR grade and obtained from B.D.H. Ltd Poole.

METHODS

Determination of Minimum Inhibitory Concentration (MIC) Values

Tube Methods:

1) Various concentrations of diphenyliodonium chloride were prepared in 5 ml of sterile nutrient broth and inoculated with a loopful of ca.4 x 10^4 cells/ml culture. The presence and absence of growth was noted after 24 hours at 37°C. An approximate Minimum Bactericidal Concentration (MBC) was determined by subculturing onto a nutrient agar plate, a loopful of broth from tubes showing no visible growth.

2) A range of diphenyliodonium chloride concentrations was prepared in 5 ml of sterile double strength nutrient broth. An overnight culture of the test organisms was diluted 1:10 with sterile Ringers solution. One ml of this inoculum was then added to the diphenyliodonium chloride solutions, which were then made up to 10 ml with sterile distilled water. The presence and absence of growth was noted after 24 hours at 37°C. Each test was carried out four times, on separate days.

Plate methods

Predried plates of nutrient agar containing a range of diphenyliodonium chloride (10 µg - 100 µg/ml) were prepared. A loopful of *E. coli* or *S. aureus* was then streaked out onto these plates, the presence and absence of growth was noted after 24 hours at 37°C. Plates were incubated both aerobically (normal incubator) and anaerobically (Gaspak CO_2/N_2 atmosphere) (Brewer & Allgeier 1966). In both cases the absence

or presence of growth was noted. The sensitivity of E. coli and S. aureus was also estimated using filter paper discs (AA disc Whatman) impregnated with 200 µg/disc of diphenyliodonium chloride. They were placed onto seeded plates and the diameter of the zones of inhibition measured.

The MIC value for C. albicans was determined at pH 5 and pH 7 by the plate method.

Effect of cell number on the MIC value

A range of diphenyliodonium chloride concentrations were prepared in sterile nutrient broth. These were inoculated with 1 ml of E. coli cell suspension to obtain a final cell concentration ranging from ca. 10^1 to 10^7 cells per ml. The MIC value was noted after 24 hours at 37°C. Each test was carried out in triplicate on successive days.

Preparation of cell suspensions

The organisms were grown for 24 hours in sterile nutrient broth and then cells were harvested by centrifugation at 5000 g at 4°C. The cells were then washed three times with ¼ strength Ringers solution and resuspended when required in ¼ strength Ringers solution (unless otherwise mentioned).

Determination of the dry weight of organisms

E. coli was grown in a Roux flask for 24 hours at 37°C on nutrient agar slants. The cells were then washed off and any small lumps of agar remaining were removed by centrifugation at 130g. The cells were then harvested at 5000g, washed and resuspended in water. Serial dilutions were prepared with water. The optical density of these suspension was determined at 420nm. 10 ml of each dilution was dried over calcium chloride under a partial vacuum. From this data a calibration curve of dry weight versus optical density was drawn.

Effect of diphenyliodonium chloride on growing cultures

E. coli was incubated in nutrient broth to give a final concentration of ca. 10^7 cells/ml and shaken in a water bath at 37°C. At appropriate time intervals the required concentration of diphenyliodonium chloride was added to the inoculated broths. Growth and turbidity were measured as described above. This procedure was also repeated using S. aureus. The experiment was carried out in triplicate on successive days.

Effect of diphenyliodonium chloride on non-proliferating cells.

E. coli was suspended in sterile 0.9% sodium chloride solution to give a final concentration of ca. 2×10^6 cells/ml and shaken in a water bath at 37°C. At the required time intervals, the inoculated saline was separated into two equal volumes. Diphenyliodonium chloride was added to one and an equal volume of water to the other. Decimal dilutions were

prepared. Viable counts were determined by the Miles & Misra (1938) method. Four replicates were carried out on each sample and control. The plates were incubated at 37°C for 18 hours. Following incubation plates with 20 to 200 colonies were counted. The test was carried out four times on separate days.

Effect of diphenyliodonium chloride on proliferating cells.

E. coli was inoculated into nutrient broth to give a final concentration of ca. 2×10^6 cells/ml and shaken in a reciprocal shaker at 37°C. After two hours the inoculated broth was separated into two equal volumes. The required concentration of diphenyliodonium chloride was added to one and an equal amount of water was added to the other. Decimal dilutions were prepared and viable counts carried out as above.

Effect of pH on the bacteriostatic action of diphenyliodonium chloride.

A pH gradient plate was prepared by a modification of the method of Szybalaski (1952). Two media of sterile nutrient agar were prepared at pH 5 and 9 respectively. Into a sloping petri dishes a layer of pH 5 nutrient agar was poured, allowed to set and placed on a level surface. Then the pH 9 nutrient agar was poured on top, thus the resulting plate on equilibration had a pH gradient from 5 to 9. The plate was then seeded with either B. megaterium or S. aureus. A strip of filter paper 0.8 x 4.0 cm (Whatman No. 1) impregnated with a 1000 µg/ml solution of diphenyliodonium chloride was placed along the pH gradient, and the plates incubated at 37°C overnight. The pH gradient was checked with narrow range indicator paper.

Interaction of diphenyliodonium chloride with other antibacterial agents

The technique of Maccararo (1961) was used to investigate the interaction of diphenyliodonium chloride with other antibacterial agents and antibiotics. Two strips of filter paper (Whatman No. 1) were placed at right angles on seeded nutrient agar plates. One of these filter papers was impregnated with diphenyliodonium cloride (1000 µg/ml) and others were impregnated with the following: penicillin (500 µg/ml); methicillin, (1000 µg/ml); ampicillin (1000 µg/ml); cloxacillin, (1000 µg/ml); D-cycloserine (1000 µg/ml); Vancomycin (200 µg/ml); fosfonomycin (1000 µg/ml); streptomycin (500, 1000 µg/ml); gentamicin (1000 µg/ml); tetracycline (500 µg/ml); lincomycin (2000 µg/ml); clindomycin (2000 µg/ml); ethidium bromide (1000 µg/ml); acriflavine (10000 µg/ml); phenol (1000 µg/ml); cetylpyridinium chloride (1000 µg/ml); cetyltrimethylammonium bromide, (cetrimide) (1000 µg/ml); dinitrophenol (1000 µg/ml); sodium lauryl sulphate (1000 µg/ml); thioglycolic acid (0.1M); thioglycerol (0.1M); mercaptoethanol (0.1M); tween 80 (25%); adenine (1000 µg/ml); thymine (1000 µg/ml); uracil (1000 µg/ml); This experiment was also repeated using 10,000 µg/ml of diphenyliodonium chloride.

Assay of diphenyliodonium chloride

A modified method of Bowd & Burns (1966) was used. Standard solution of diphenyliodonium chloride were prepared to give a range of concentration up to 500 µg/ml. 10 ml of these solutions, 5 ml of 0.1% dipicrylamine (dissolved in 0.1g Na_2Co_3); and 2 ml of 0.5% sodium carbonate were placed in a 100 ml separating funnel and the pH checked to ensure that it was > 10. Under alkaline conditions the

extract shows maximum absorption at 400 nm. Further sodium carbonate was then added to ensure that the pH was >10. 10 ml chloroform was then added and the solution shaken for two minutes in order to extract the water insoluble product of the reaction between diphenyliodonium chloride and dipicrylamine. The extract was run off through filter paper (Whatman No. 1) into a 50 ml flask. The chloroform extraction was then repeated twice more and the extracts combined and made up to 50 ml with chloroform. The absorbtion was determined at 400 nm against pure chloroform. Care was taken not to expose the organic extract to strong daylight.

Loss of intracellular constituents

An overnight broth culture of E. coli was centrifuged to remove the cells. The cells were then washed three times with ¼ strength Ringers solution and resuspended in 0.01M phosphate buffer containing graded concentrations of diphenyliodonium chloride. After 2 hours the cells were removed by centrifugation and the RNA concentration in the supernatant estimated by the method of Ogur & Rosen (1951).

Uptake of diphenyliodonium chloride by bacteria

A suspension of test organism was prepared by washing an 18 hours nutrient agar slant with ¼ Ringers solution. The suspension was centrifuged at 130g to remove agar fragments, washed twice by centrifuging at 8000g for 10 minutes and finally resuspended in phosphate buffer 0.05M pH 7.2.

Diphenyliodonium chloride concentration was determined by the method of Bowd & Burns (1966). Drug uptake (adsorption) was measured by allowing cells to remain in contact with the drug under varying conditions of time and pH. Following which the cells were centrifuged at 8000g for 10 minutes and the diphenyliodonium chloride determined in the supernatant liquid.

Inhibition of membrane-bound APTase and of cation transport in Streptococcus faecalis.

Streptococcus faecalis NCIB 6783 was grown on medium NaTy (Zarlengo & Schultz (1966). The cells were harvested by centrifugation at 8000g for 15 minutes, washed twice with 2mM $MgCl_2$ and resuspended in water. Cation transport was studied at room temperature.

Glycolysis was monitored continuously at pH 7.5 by means of a pH stat (Radiometer, Copenhagen) (Zarlengo & Schultz (1966) which automatically adds NaOH to neutralize the lactic acid produced by the cells. Inhibition of net K^+ uptake by diphenyliodonium chloride was then determined by the method of Harold et al (1967). The samples were filtered through 0.45 µmMillipore filters and washed with $MgCl_2$. The cations were extracted with trichloroacetic acid and estimated by flame photometry. Membrane-bound ATPase was prepared by osmotic lysis of protoplasts. The enzyme was dissociated from the membrane using lysozyme by the method of Abrams (1965). The effect of diphenyliodonium chloride was tested as follows: Native or solubilized enzyme was incubated for 10 minutes in Tris-Mg buffer (0.1M Tris chloride 2 mM Mg++ PH 7.5) at room temperature with or without

inhibitors. The suspension was then diluted with an equal volume of Tris chloride (0.1M) - ATP, (10 mM) - Mg^{++} (8mM) and incubated at 38°C. Pi release was determined after 10 minutes by the method of Chen et al (1956).

The use of Triphenyltetrazolium chloride (T.T.C) in determining the dehydrogenase activity of E. coli.

E. coli grown on nutrient agar slant was washed off and agar fragments removed by centrifugation at 130g. The cells were then washed three times and resuspended in 0.1M phosphate buffer. One ml of this suspension was dried and weighed to determine the dry weight of the cells. For determination of the dehydrogenase activity the method of Hugo (1954) was used. Into a series of sterile universal bottles 1.0 ml of T.T.C. (0.1% w/v); 0.5 ml of cell suspension and 1 ml of substrate were added and made up to 5 ml with 0.1M phosphate buffer. These bottles were used as controls. The same solutions with 0.5 ml of various concentrations of diphenyliodonium chloride were also prepared. All bottles were kept in a water bath at 37°C. At 20 minute intervals one test and one control bottle was removed. The reaction mixture was acidified with 5 ml of glacial acetic acid. The triphenylformazan produced by the reduction of T.T.C. by dehydrogenases was extracted using 5 ml of toluene and estimated colourimetrically at 490 nm.

Oxygen consumption of E. coli as measured by Warburg apparatus.

E. coli was inoculated onto the surface of nutrient agar and incubated at 37°C for 24 hours. The culture was washed off and agar fragments removed by centrifuging at 130g. The cells were then washed three times by centrifugation at 8000g and resuspended in 15 ml 0.5 M phosphate buffer. The uptake of oxygen was determined by the direct method of Warburg in an atmosphere of air at 100 oscillation/min. Each control flask contained in 3 ml, 70 mg of cells, 0.5M phosphate buffer (pH 7.2) and 0.02M glucose. The same solutions with 0.025 ml of various concentrations of diphenyliodonium chloride were prepared (Dawes 1972).

Effect of diphenyliodonium chloride on the morphology and size of the cell.

E. coli was grown at 37°C for 24 hours in sterile nutrient broth containing 100 µg/ml diphenyliodonium chloride. The cells were harvested by centrifugation at 4000g at 4°C, washed and resuspended with 0.9% sodium chloride, to give a cell density of ca 1.5 x 10^7 cells/ml. The cell size was then determined by the method of Bulman & Stretton (1974).

Electron Microscopy

1) Scanning Electron Microscopy

Glutaraldehyde was added to an E. coli broth culture to give a final concentration of 1.5% (w/v). After two minutes contact, cells were removed by centrifugation at 3000g for 15 minutes at 4°C. The cells were then resuspended in two ml of gluteraldehyde (5% w/v) in ¼ strength Ringers solution and stored at 4°C for 16 hours.

The cells were removed by centrifugation, washed three times with distilled water and resuspended to give the required cell density. One drop of this suspension was allowed to air dry on a cover slip and then dehydrated over calcium chloride under a partial vacuum (Bulman & Stretton 1974).

2) Transmission Electron Microscopy

100 ml of sterile nutrient broth containing 100 µg/ml of diphenyliodonium chloride was prepared and inoculated with an overnight culture of E. coli. The inoculated broth was kept in a shaking water bath at 37°C for 18 hours. Cells were harvested by centrifugation and transmission electron microscopy was carried out by the method of Glauert (1958).

Titration curve for diphenyliodonium chloride

The pH meter was standardised initially using pH 7 buffer. 0.2g diphenyliodonium chloride was dissolved in 100 ml of distilled water and 25 ml of this solution i.e. 0.05g diphenyliodonium chloride was titrated with 0.1N Hcl. This titration was repeated with 25 ml distilled water. Then, using the same conditions, a further 25 ml aliquot of diphenyliodonium chloride solution was titrated against 0. 1N NaOH. Using the titration values obtained for diphenyliodonium chloride and water,a table of milli-equivalents of titrant versus pH was obtained.

SECTION 3

Results

Antibacterial activity

1) Bacteriostatic and Bactericidal action

The MIC values(table 1) of diphenyliodonium chloride shows that this compound has greater activity on gram-positive than gram-negative bacteria. The greatest activity was shown with vegetative cells of *B. subtilis* and *B. megaterium*.

Table 1

Minimum inhibitory concentrations for diphenyliodonium chloride, using one loopful of culture containing *ca.* 4×10^4 cells/ml.

Organisms	MIC (µg/ml)
S. aureus	30
S. aureus (Oxford)	30
B. megaterium	20
B. subtilis	10
E. coli (9001)	100
E. coli (Texas)	100
P. aeruginosa	70
S. faecalis	100

Using the same inoculum the approximate bactericidal concentration was generally found to be 10-20 µg/ml higher than the MIC value. The MIC value rose slightly when the cell concentration was increased from 10^2 to 10^7 cells/ml (Fig 1).

FIG 1.

The Effect of cell numbers on the MIC value for diphenyliodonium chloride using *E. coli* as the test organism.

Fig. I

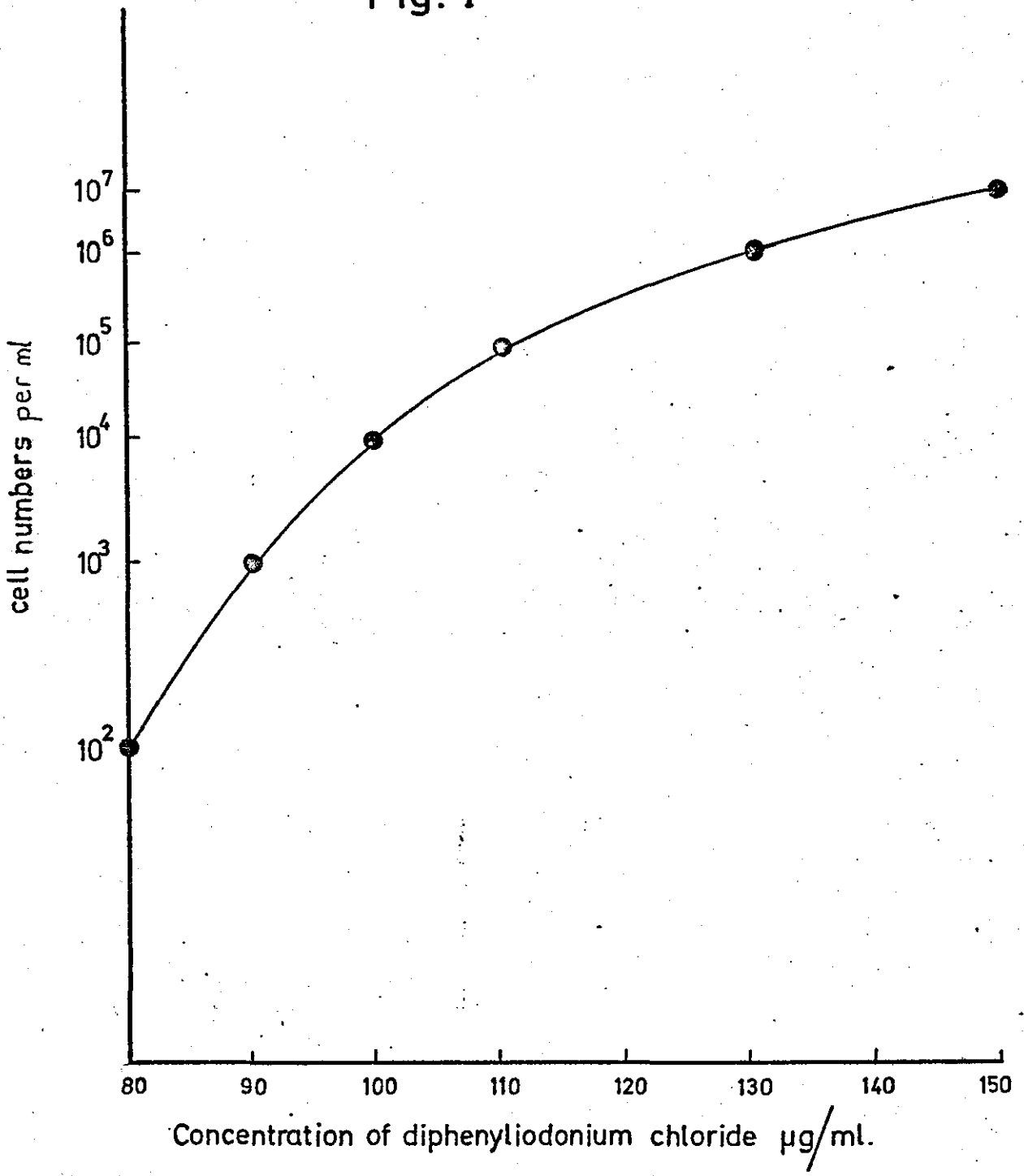

cell numbers per ml
10^7
10^6
10^5
10^4
10^3
10^2
80
90
100
110
120
130
140
150
Concentration of diphenyliodonium chloride µg/ml.

Table 2 shows the MIC values using ca. 10^7 cells/ml.

Table 2

Minimum inhibitory concentration and minimum bacterial concentration values for diphenyliodonium chloride using ca 10^7 cells/ml.

Organisms	MIC	MBC
S. aureus	70	200
S. aureus (Oxford)	70	200
B. megaterium	50	100
B. subtilis	10	10
E. coli (9001)	150	>200
E. coli (Texas)	150	>200
P. aeruginosa	140	>200
S. faecalis	150	>200

2) Effect of oxygen

The effect of oxygen on MIC values was carried out by measuring zones of inhibition of E. coli and S. aureus in both aerobic and anaerobic conditions. Table 3 shows that diphenyliodonium chloride is more active under aerobic conditions.

Table 3

Zones of inhibition for E. coli, S. aureus, C. albicans and Clostridium sporogenes under aerobic and anaerobic conditions using AA disc.

Organisms	Aerobic	Anaerobic
S. aureus	32.4 mm	25.8 mm
E. coli	29.8 mm	17.2 mm
C. albicans	43.5 mm	-
Clostridium sporogenes	-	57.2 mm

The MIC value for diphenyliodonium chloride for C. albicans was determined at both pH 5 and pH 7. At the higher pH value, the MIC is lower than that obtained at pH 5 (table 4). These determinations were carried out by the plate method.

Table 4.

Minimum inhibitory concentration of diphenyliodonium chloride for C. albicans at pH 5 and pH 7.

Organism	MIC at pH 5	MIC at pH 7
C. albicans	100 µg/ml	40 µg/ml

Table 5 shows the MIC values for E. coli under aerobic and anaerobic conditions using plate and tube methods.

Table 5

Minimum inhibitory concentration of diphenyliodonium chloride for E. coli by plate and tube methods under aerobic and anaerobic conditions.

MIC aerobic		MIC anaerobic
Plate	Tube	Plate
50 µg/ml	100 µg/ml	>100 µg/ml

Effect on growing culture

When diphenyliodonium chloride was added during the log phase, there was an inhibition of growth, which is shown in Fig 2.

The effect of diphenyliodonium chloride on a growing culture of S. aureus is shown in Fig 3.When a concentration of diphenyliodonium chloride below the MIC value were used there was an initial reduction in growth rate, but growth continued and almost reached the same level as in the control system.

The bactericidal activity of diphenyliodonium chloride was measured by carrying out viable counts using proliferating and non-proliferating cells. (Fig 4 and 5).

FIG 2.

The effect of diphenyliodonium chloride on a log phase culture of E. coli. Point A is time of addition of diphenyliodonium chloride;

○–○, control culture;

●–●, 50 µg/ml diphenyliodonium chloride;

▲–▲, 100 µg/ml diphenyliodonium chloride;

■–■, 200 µg/ml diphenyliodonium chloride.

Fig. 2

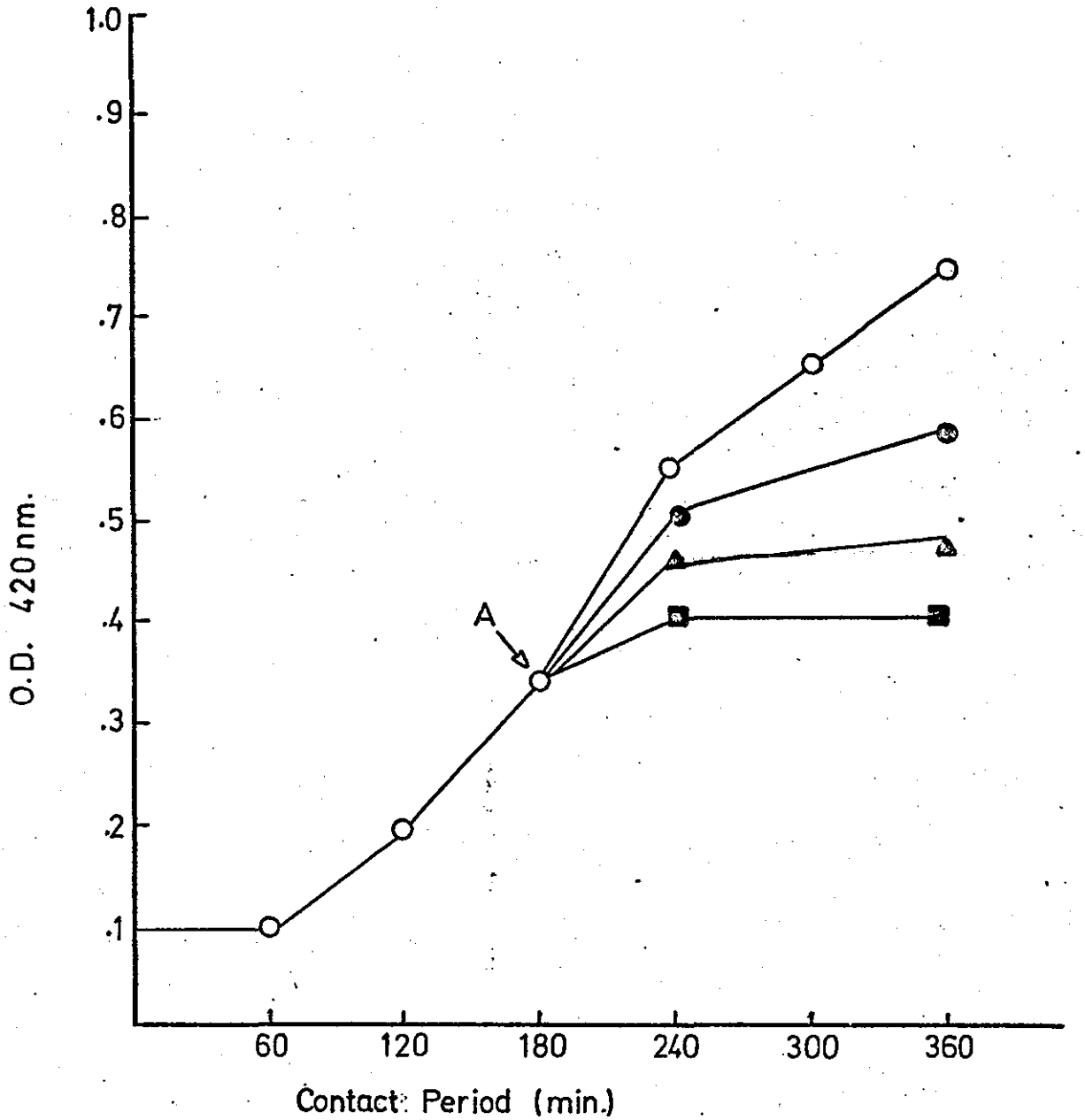
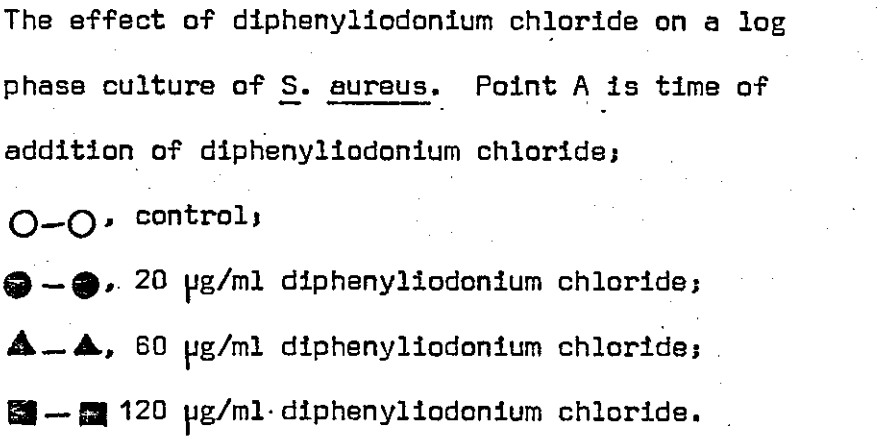

1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
O.D. 420nm.
A
60
120
180
240
300
360
Contact Period (min.)

FIG 3.



The effect of diphenyliodonium chloride on a log phase culture of S. aureus. Point A is time of addition of diphenyliodonium chloride;

○—○, control;

●—●, 20 µg/ml diphenyliodonium chloride;

▲—▲, 60 µg/ml diphenyliodonium chloride;

■—■ 120 µg/ml diphenyliodonium chloride.

Fig. 3

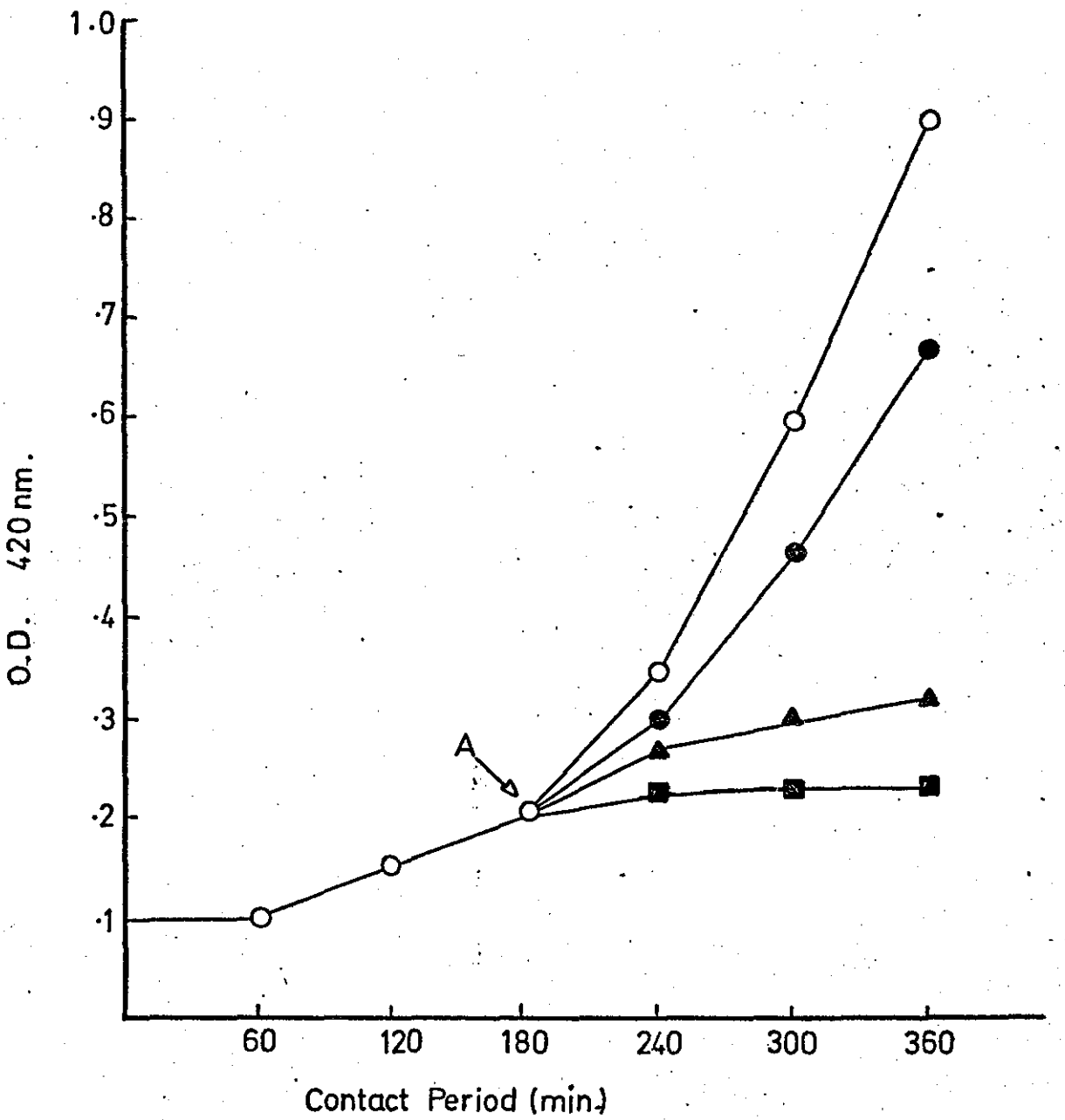

1·0
·9
·8
·7
·6
·5
·4
·3
·2
·1
O.D. 420 nm.
A
60
120
180
240
300
360
Contact Period (min.)

FIG 4.

The effect of diphenyliodonium chloride on a suspn. of ca. 10^6 cells/ml of E. coli in the presence of 0.9% NaCl at 37°C; ○—○, control; ●—●, 200 µg/ml diphenyliodonium chloride; ▲—▲ 400 µg/ml diphenyliodonium chloride; ■—■ 800 µg/ml diphenyliodonium chloride; Point A is time of addition of diphenyliodonium chloride.

Fig.4

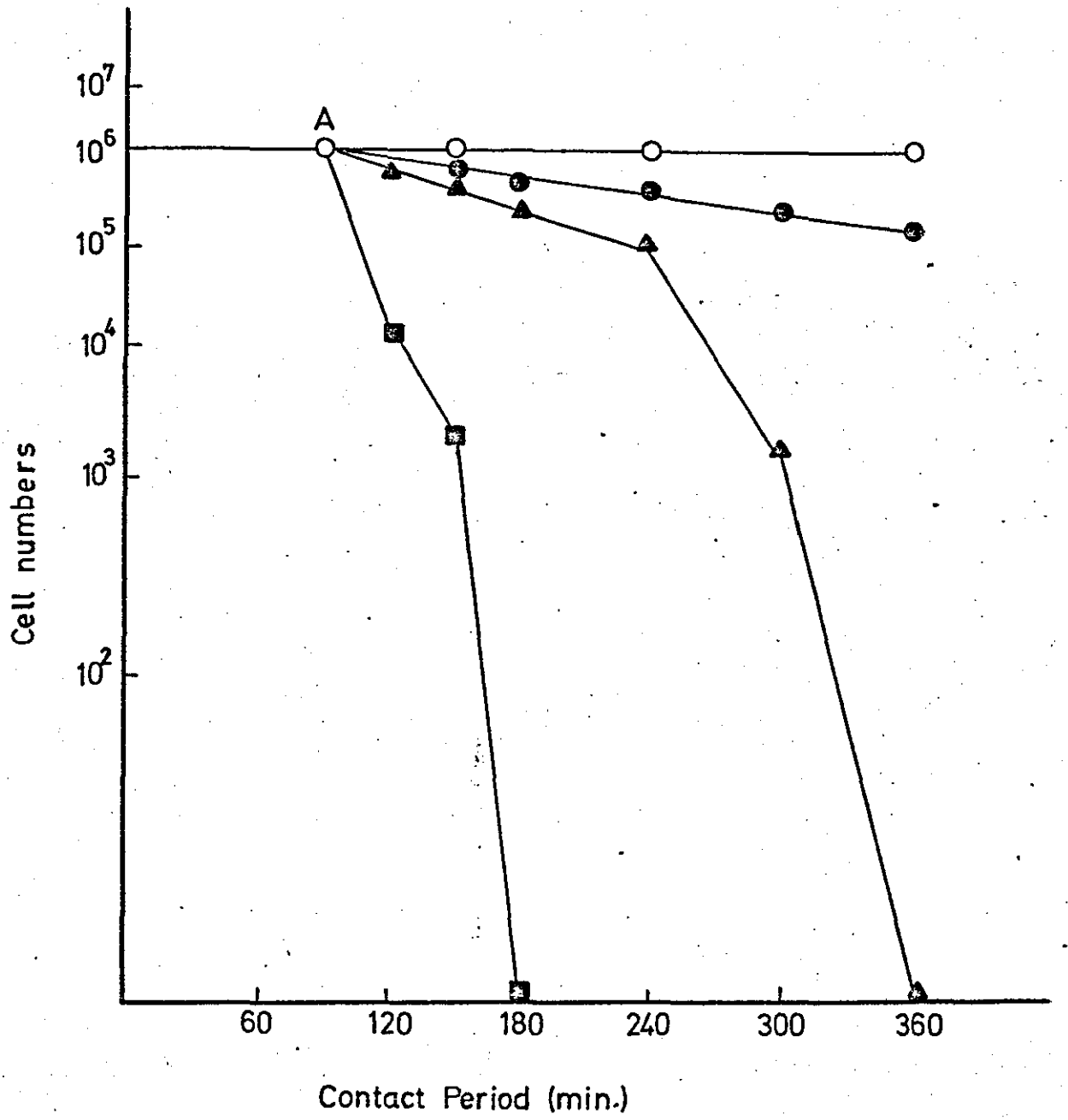

A
10^7
10^6
10^5
10^4
10^3
10^2
Cell numbers
60
120
180
240
300
360
Contact Period (min.)

FIG 5.

The effect of diphenyliodonium chloride on a broth culture of *E. coli* containing *ca.* 10^6 cells/ml at 37°C

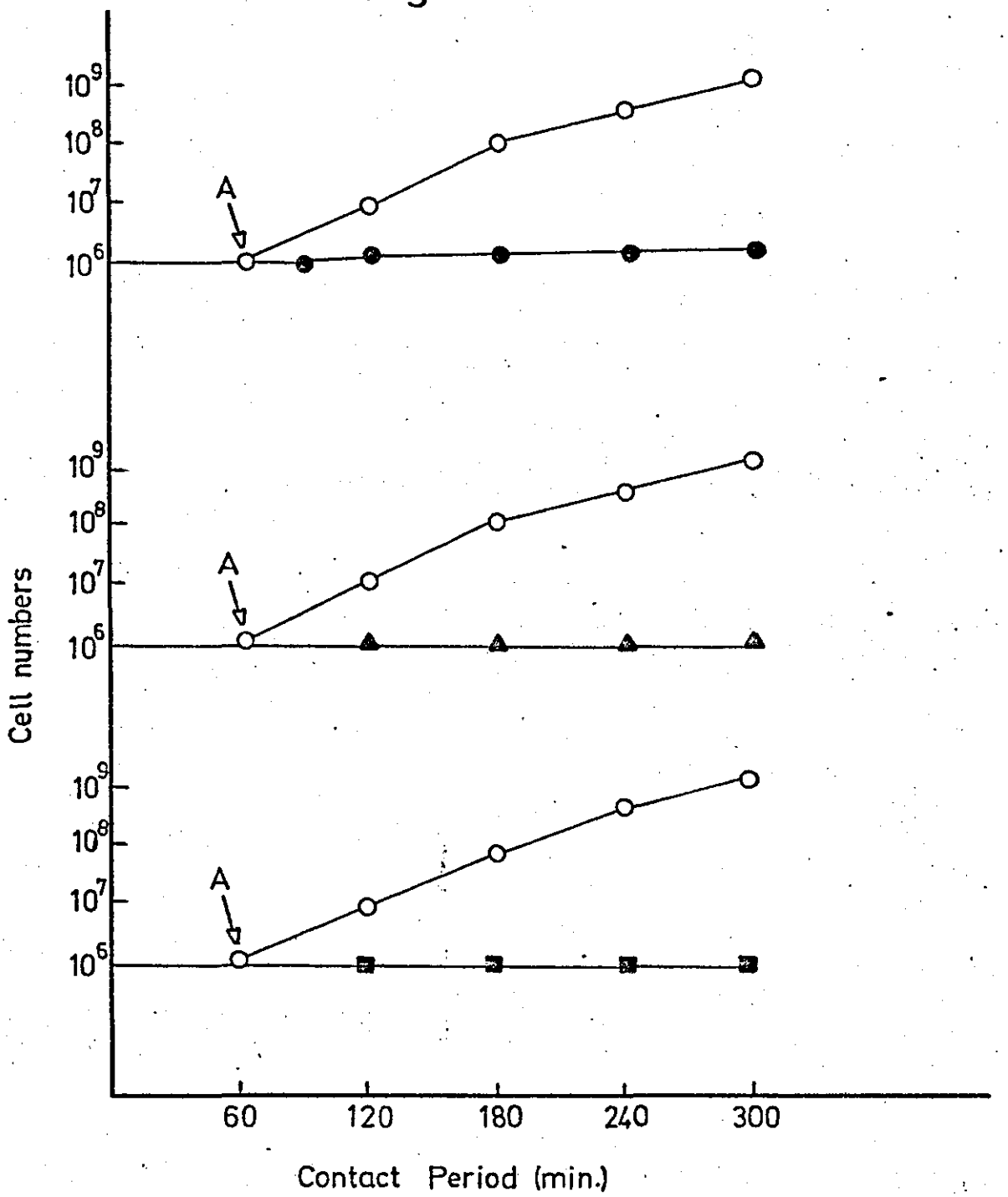
Point A is time of addition of diphenyliodonium chloride; ○—○, control;

●—●, 200 µg/ml diphenyliodonium chloride;

▲—▲, 400 µg/ml diphenyliodonium chloride;

■—■, 800 µg/ml diphenyliodonium chloride.

Fig. 5


Cell numbers
10^9
10^8
10^7
10^6
A
60
120
180
240
300
Contact Period (min.)

Effect of pH on activity of diphenyliodonium chloride.

The effect of pH on activity of diphenyliodonium chloride is shown in Figs 6 and 7, as the pH increased the antibacterial activity of diphenyliodonium chloride also increased. Strip A was impregnated with 1000 µg/ml of diphenyliodonium chloride.

Loss of intracellular constituents.

The loss of intracellular constituents was estimated by measuring RNA leakage (Fig 8). Leakage was not pronounced and reached, in all cases, an almost constant value in the presence of 100 µg/ml of diphenyliodonium chloride. This loss of cytoplasmic constituents was rapid (Fig 9) occurring within the first few minutes of contact. Thereafter, there was only a further slight loss with an increasing time of contact.

Interaction of diphenyliodonium chloride with other antibacterial agents.

There was no interaction between diphenyliodonium chloride and any of the antimetabolites used. (Table 6).

Estimation of diphenyliodonium chloride.

The use of the dipicrylamine reagent enabled diphenyliodonium chloride to be determined at low concentrations. The response curve versus O.D. at 420 nm was linear from 50 to 400 µg/ml (Fig 10).

TABLE 6

Interactions of diphenyliodonium chloride with antibiotics, preservatives and antimetabolites.

N = no reaction

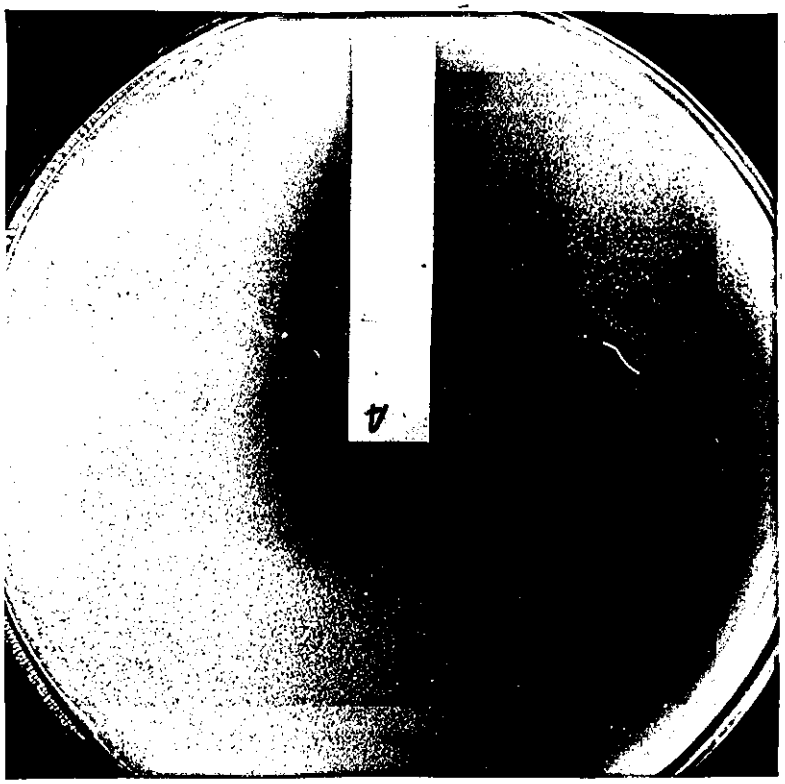
Strip A	Strip B	S. aureus	E. coli
diphenyliodonium chloride	Penicillin	N	N
	Methicillin	N	N
	Ampicillin	N	N
	Cloxacillin	N	N
	D-Cycloserine	N	N
	Vancomycine	N	N
	Fosfonomycin	N	N
	Streptomycin	N	N
	Gentamycin	N	N
	Tetracycline	N	N
	Lincomycin	N	N
	Clindomycin	N	N
	Ethiodium bromide	N	N
	Acriflavine	N	N
	Phenol	N	N
	Cetylpyridinium chloride	N	N
	Cetrimide	N	N
	Dinitrophenol	N	N
	Sodium lauryl sulphate	N	N
	Thioglycolic Acid 1%	N	N
	Thioglycerol 1%	N	N
	Mercaptoethanol 1%	N	N
	Tween 80 (25%)	N	N
	Adenine	N	N
	Thymine	N	N
	Uracil	N	N

FIG 6.

The effect of pH on the activity of diphenyliodonium chloride on S. aureus.

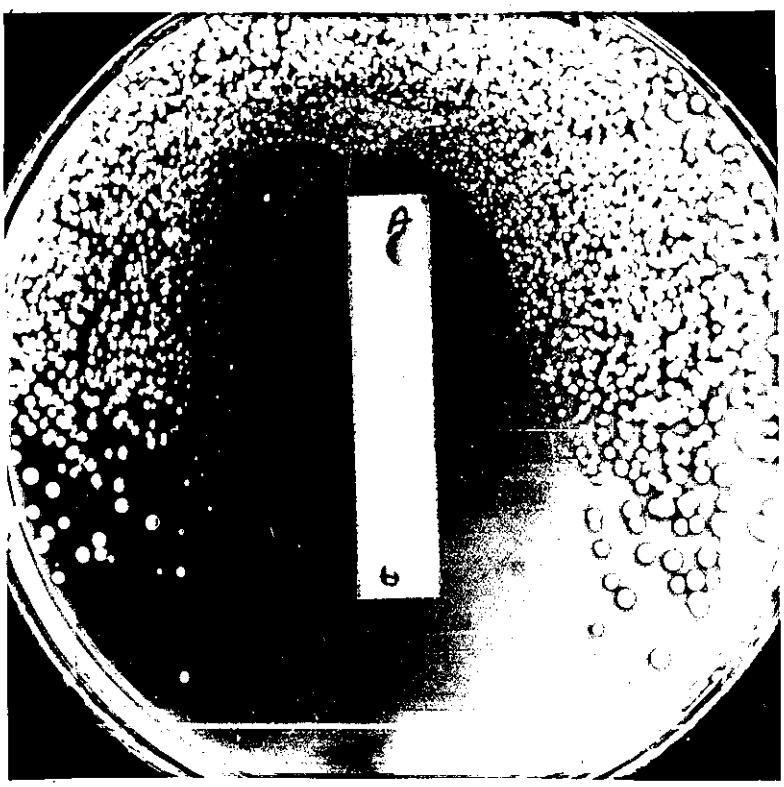
FIG 7.

The effect of increase in pH (pH 5 to 9) on the activity of diphenyliodonium chloride on B. megaterium.



pH 5

pH 9.



pH 5

pH 9

FIG 8.

The effect of diphenyliodonium chloride on the interacelluler leakage of RNA. Test organism, E. coli suspension 26.7 mg dry weight cells/ml in 0.01M phosphate buffer (pH = 7.2) maintained at 37^{o}C for 2 hours.

Fig.8

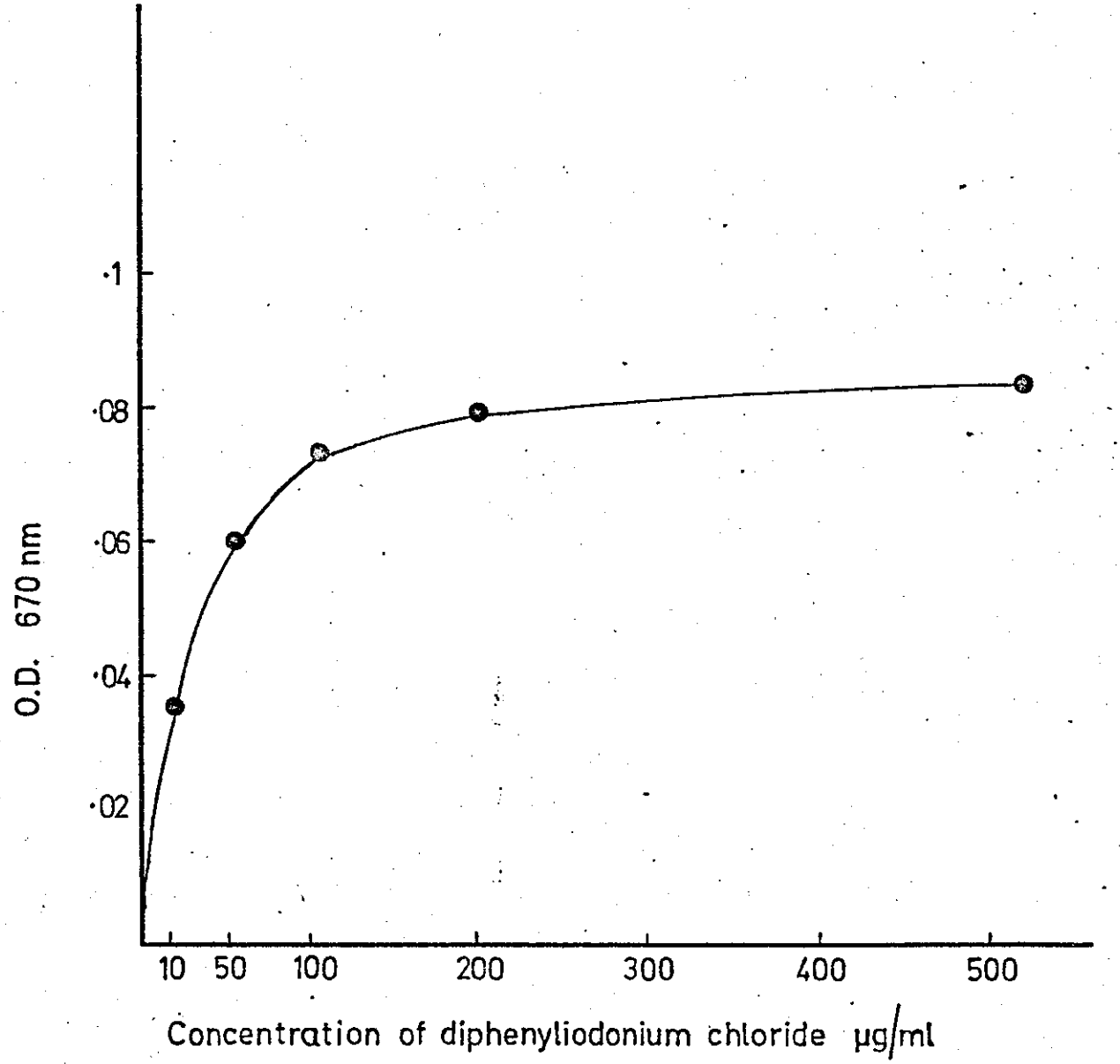

·1
·08
·06
·04
·02
O.D. 670 nm
10
50
100
200
300
400
500
Concentration of diphenyliodonium chloride µg/ml

FIG 9.

RNA leakage from a suspension of *E. coli*, 26.7 mg dry weight cells/ml in 0.01 M phosphate buffer (pH = 7.2), maintained at 37°C in the presence of 100 µg/ml diphenyliodonium chloride.

Fig·9

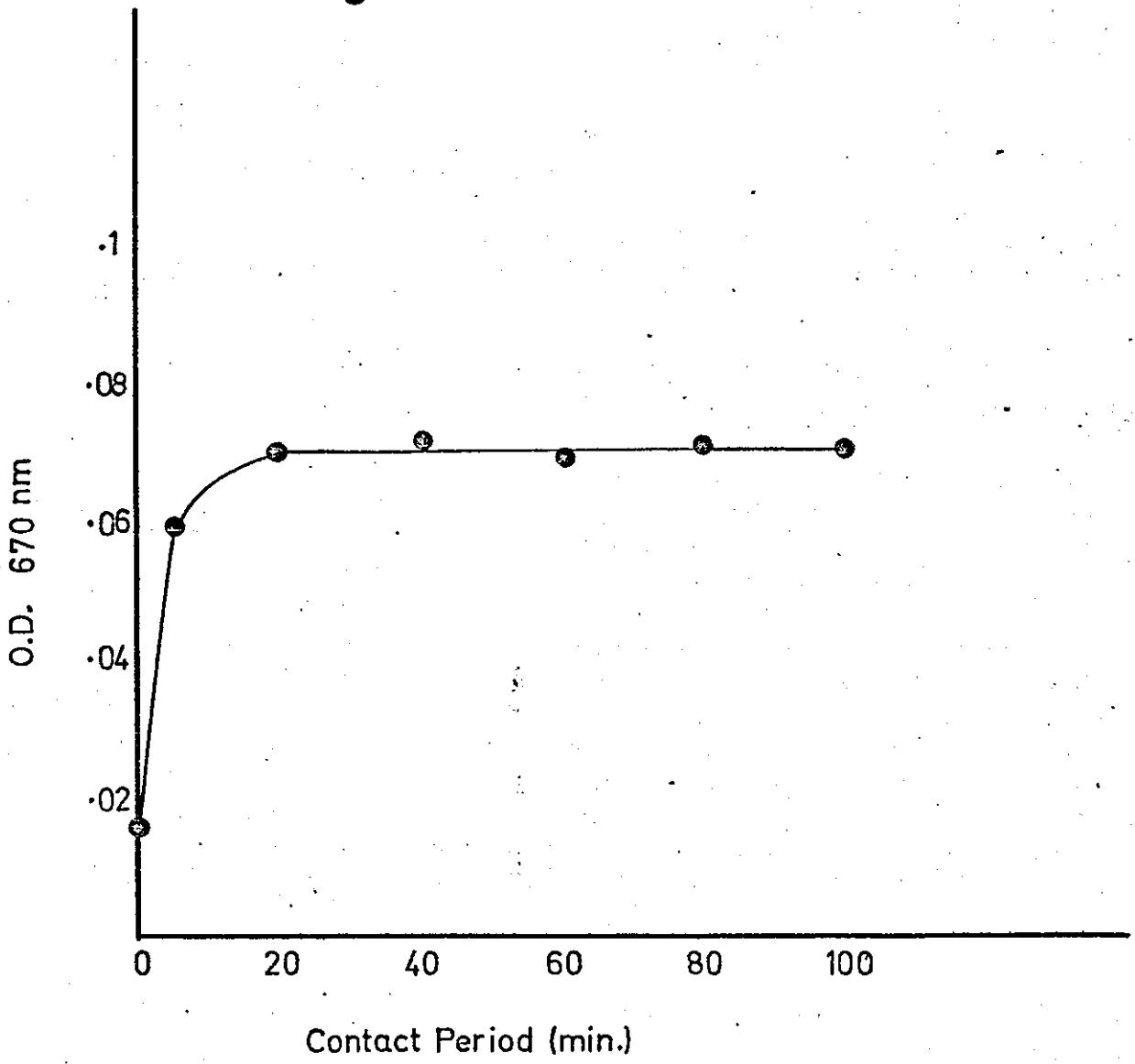
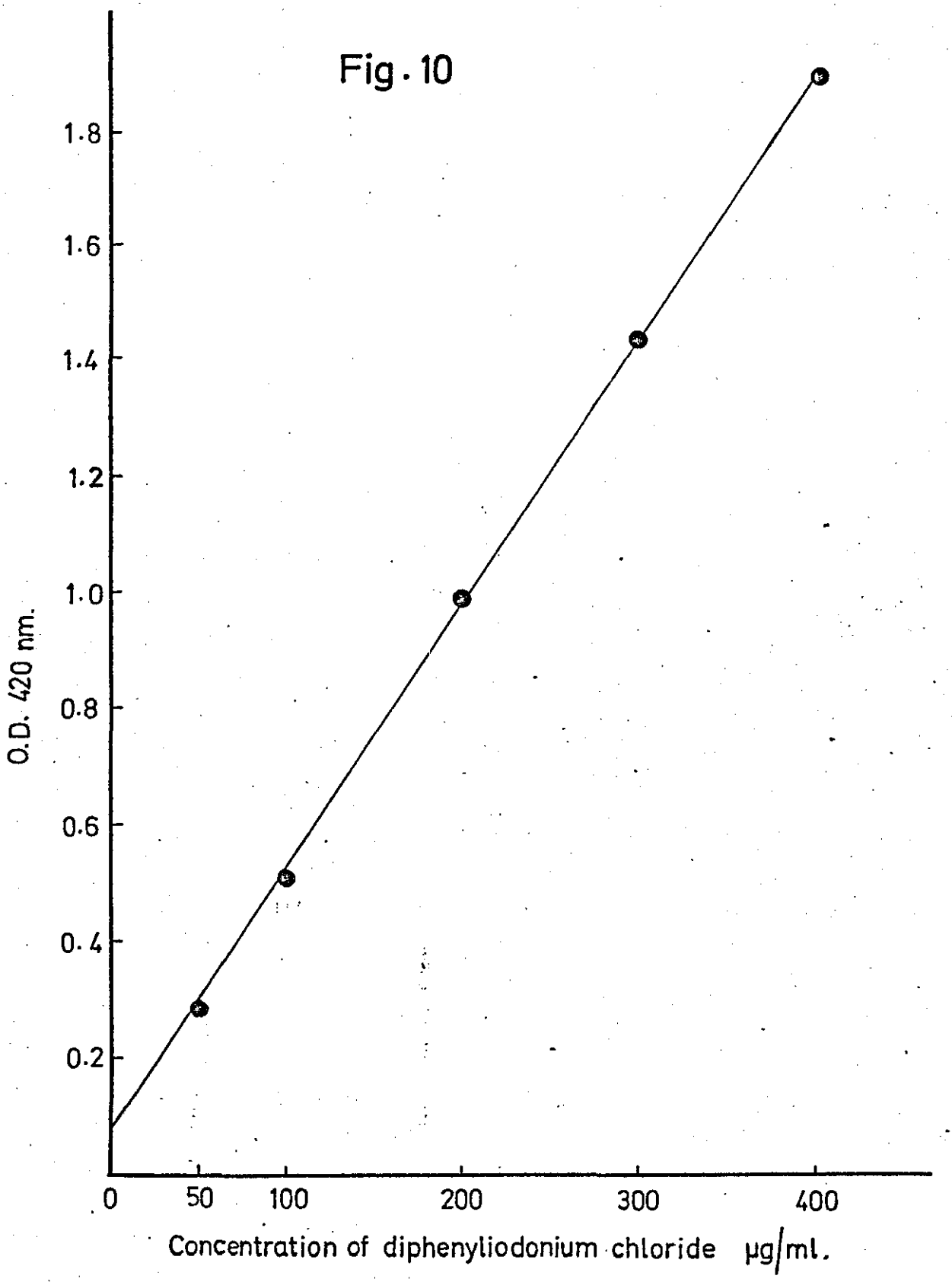


FIG 10.

Calibration curve for concentration of diphenyliodonium chloride in aqueous solution.

Fig. 10


1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
O.D. 420 nm.
0
50
100
200
300
400
Concentration of diphenyliodonium chloride µg/ml.

Uptake of diphenyliodonium chloride by bacterial cells.

The adsorption isotherm for the uptake of diphenyliodonium chloride by E. coli suspension 2.8 mg dry weight cells/ml from distilled water and phosphate buffer (0.05M, PH 7.3) after 3 hours at 37°C is shown in Fig 11.
The amount of drug adsorbed increased with an increase of pH (Fig 12). The uptake was rapid and the cells were saturated after 5 minutes contact (Fig 13).

Inhibition of the membrane-bound ATPase and of cation transport in Streptococcus faecalis.

Membrane bound ATPase activity was inhibited by diphenyliodonium chloride (-), chlorhexidine ( ) and dicyclohexylcarbodiimide ( ). Fig 14. Diphenyliodonium chloride appears to be the most active when compared to chlorhexidine and dicyclohexylcarbodiimide. Also the potency of the chlorhexidine and diphenyliodonium chloride when compared on a molar basis at 0.3 mM was similar (38% inhibition). However even at 0.06 mM diphenyliodonium chloride did not completely inhibit the enzyme activity.

Inhibition of K^+ accumulation by intact cells.

Cells harvested from medium NaTy are relatively depleted of K^+ but contain large amounts of Na^+ and H^+. In the presence of an energy source such cells can accumulate K^+, with concurrent extrusion of Na^+ and H^+, this cation exchange is accompanied by marked stimulation of glycolysis. (Figs 15 & 16).

FIG 11.

Uptake of diphenyliodonium chloride by E. coli suspension 2.8 mg dry weight cells/ml, from solution of diphenyliodonium chloride after 3 hours at 37°C, adsorption from 0.05 M phosphate buffer (pH = 7.3).

Fig. 11

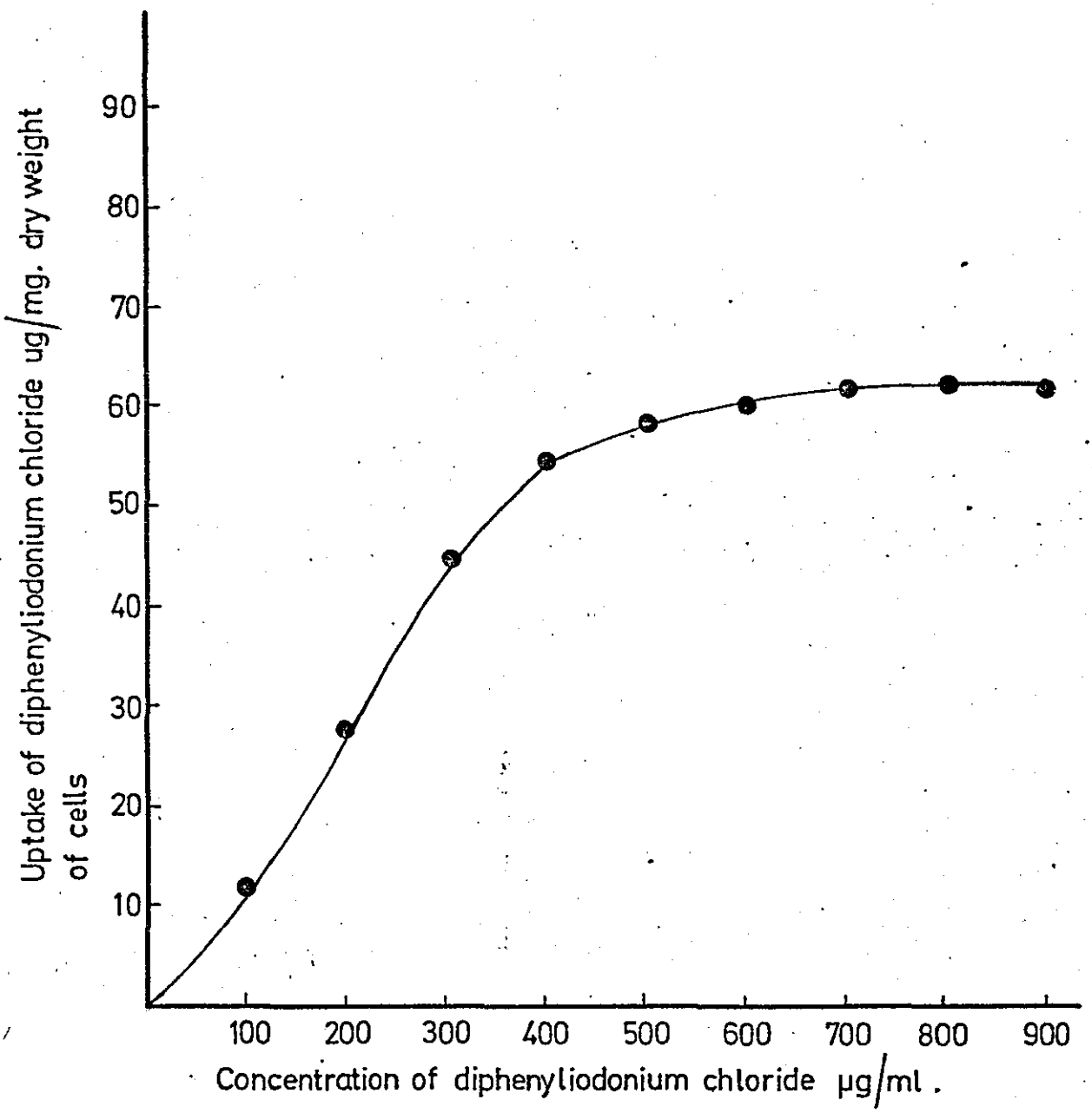

Uptake of diphenyliodonium chloride ug/mg. dry weight of cells
90
80
70
60
50
40
30
20
10
100
200
300
400
500
600
700
800
900
Concentration of diphenyliodonium chloride µg/ml.

FIG 12.

The effect of pH on adsorption of diphenyliodonium chloride by *E. coli* suspension 2.8 mg dry weight cells/ml from 0.05M phosphate buffer solution containing 200 µg/ml diphenyliodonium chloride after 20 minutes contact at 37°C.

Fig. 12

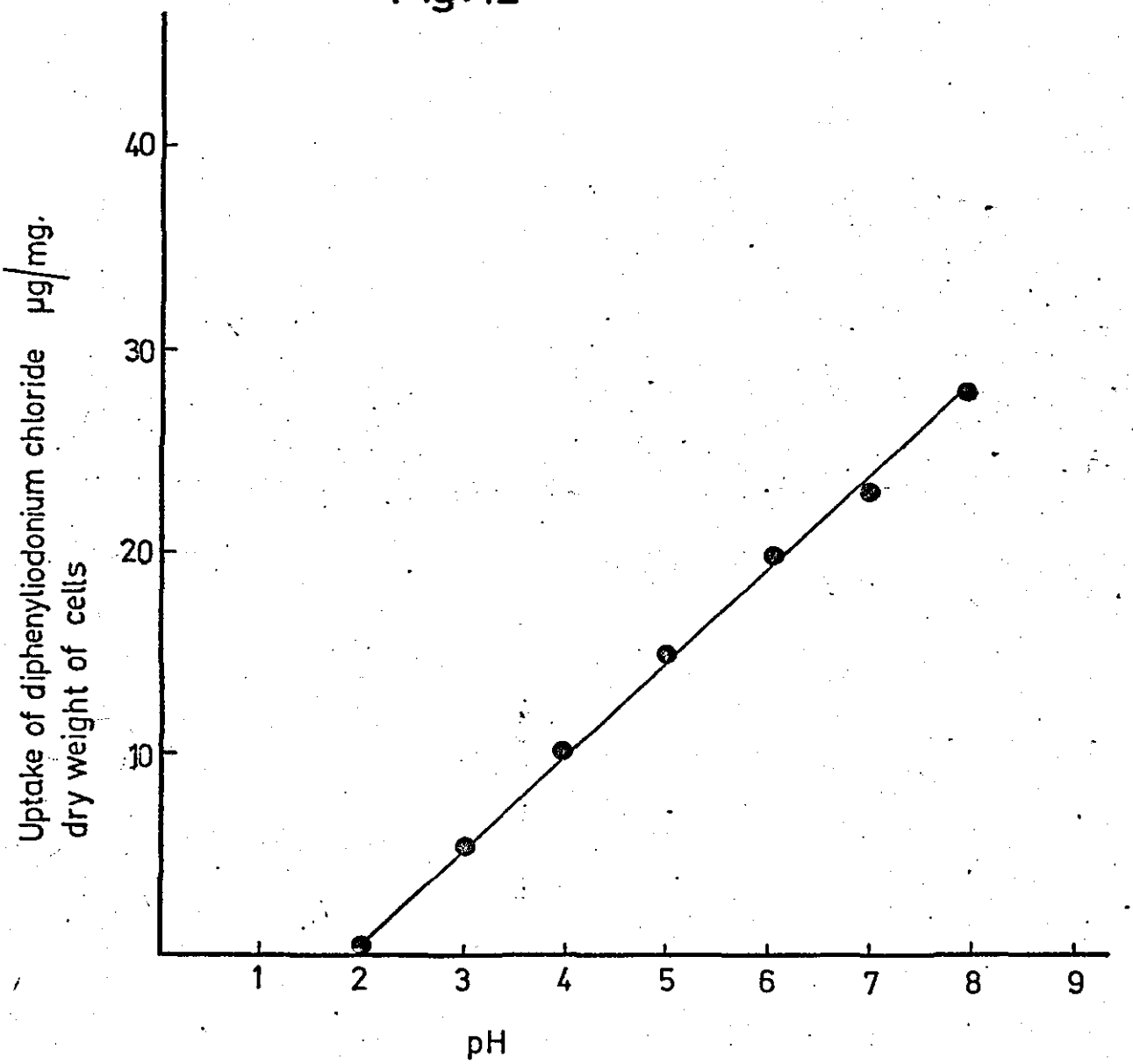

Uptake of diphenyliodonium chloride µg/mg. dry weight of cells
40
30
20
10
1
2
3
4
5
6
7
8
9
pH

FIG 13.

The effect of time on uptake of diphenyliodonium chloride by *E. coli* suspension 2.8 mg dry weight cells/ml from solution of phosphate buffer (0.05M, pH 7.3) containing 300 µg/ml diphenyliodonium chloride.

Fig.13

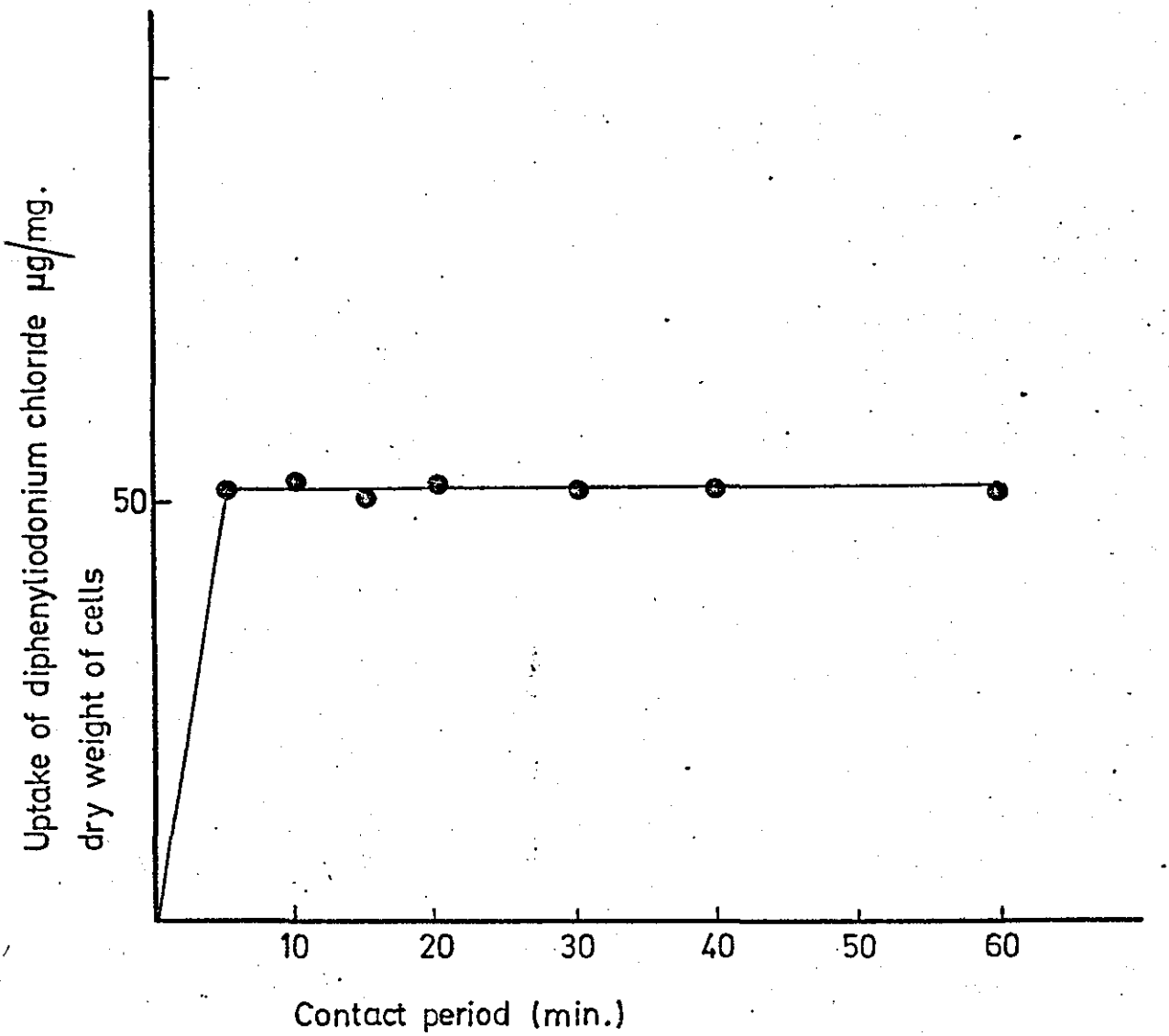

Uptake of diphenyliodonium chloride µg/mg.
dry weight of cells
50
10
20
30
40
50
60
Contact period (min.)

FIG 14.

Inhibition of the membrane-bound ATPase. The enzyme was prepared in Tris-Mg buffer (0.1M Tris chloride, 2m MMg^{++} pH 7.5), [illegible] incubated for 10 minutes at room temperature with or without inhibitors [illegible] and then diluted with an equal volume of Tris - chloride (0.1 M) - ATP (10 mM) - Mg^{++} (8mM) and incubated at 38^{o}C. Pi released was determined after 10 minutes.

●—● diphenyliodonium chloride;

▲—▲ dicyclohexylcarbodiimide;

■—■ chlorhexidine.

Fig. 14

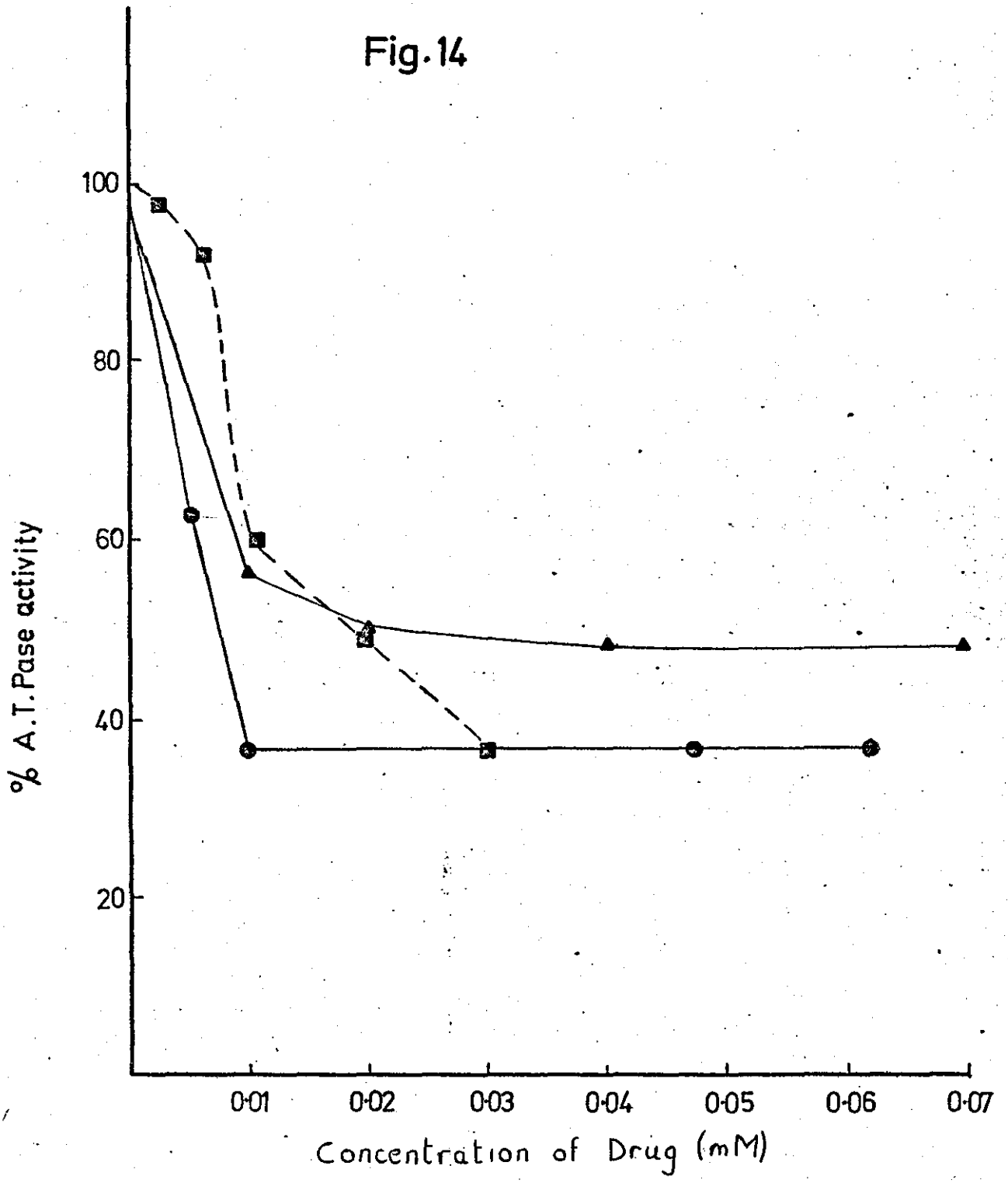

100
80
60
40
20
% A.T.Pase activity
0·01
0·02
0·03
0·04
0·05
0·06
0·07
Concentration of Drug (mM)

FIG 15.

Inhibition of net K^+ uptake by diphenyliodonium chloride and dicyclohexylcarbodiimide. Cells grown on medium NaTy were resuspended in water at 1.3 mg cells/ml. Aliquots of 20 ml each were prepared. O—O, control; X—X, 50 µg/ml diphenyliodonium chloride (1×10^{-4}M); ●—●, 100 µg/ml diphenyliodonium chloride (3×10^{-4}M); ▲—▲, dicyclohexylcarbodiimide (1×10^{-4}M); after 5 minutes glucose (4 mg/ml) was added to each and glycolysis was allowed to proceed, the pH was kept constant on the pH-stat at 7.5 for 5 minutes. At this point (designated 0 min) KCl was added to 1 mM.

Test organism - S. faecalis

Fig. 15

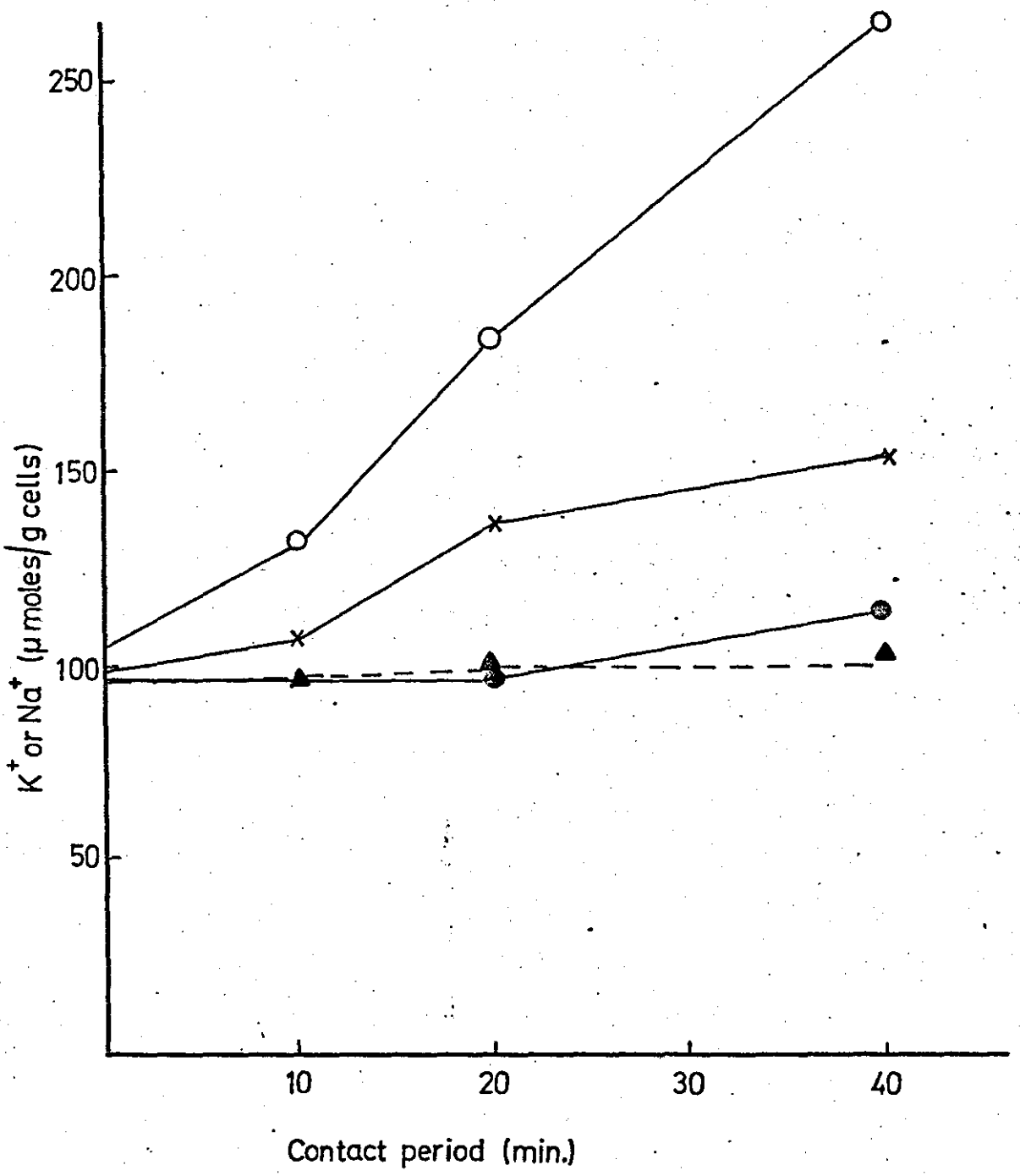
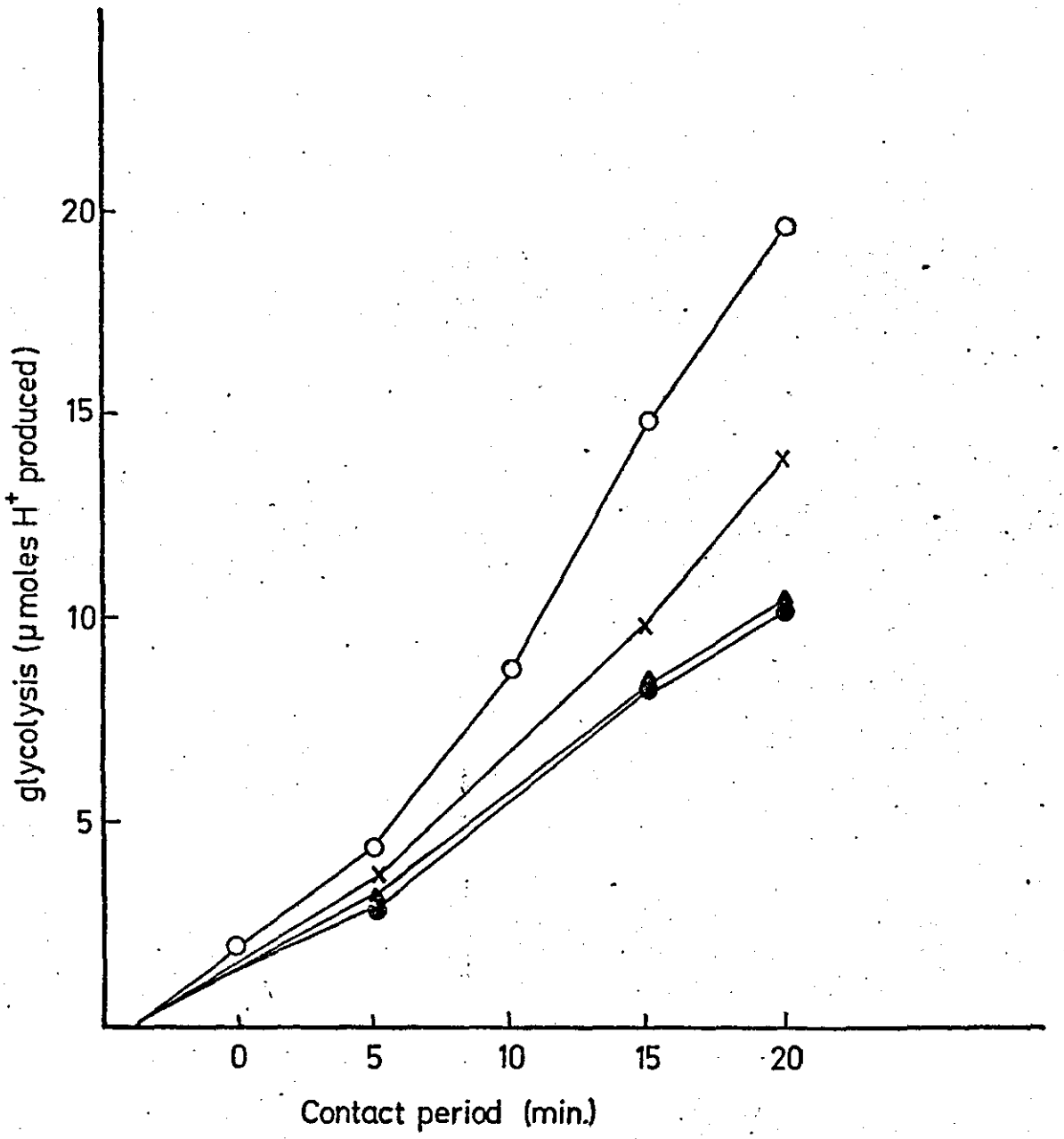

250
200
150
100
50
K^+ or Na^+ (μ moles/g cells)
10
20
30
40
Contact period (min.)

FIG 16.

The effect of diphenyliodonium chloride on the stimulation of glycolysis associated with net K^+ uptake. Cells grown on medium NaTy were resuspended in water 1.3 mg cells/ml. Aliquots of 20 ml each were prepared. O—O, control; ●—●, 100 µg/ml diphenyliodonium chloride (3×10^{-4}M); X—X, 50 µg/ml diphenyliodonium chloride (1×10^{-4}M); ▲—▲, dicyclohexylcarbodiimide (1×10^{-4}M), after 5 minutes glucose (4 mg/ml) was added to each and glycolysis was allowed to proceed, the pH was kept constant on the pH-stat at pH = 7.5 for 20 minutes.

Fig. 16


20
15
10
5
glycolysis (μmoles H+ produced)
0
5
10
15
20
Contact period (min.)

Diphenyliodonium chloride inhibited both the exchange of cations and the concurrent stimulation of glycolysis. Diphenyliodonium chloride was less effective an inhibitor than dicyclohexyl-carbodiimide (mole for mole) and inhibited K^+ accumulation completely at the MIC value and at less than the MIC value by ca . 60%.

Effect of diphenyliodonium chloride on oxygen consumption.

The effect of diphenyliodonium chloride on the aerobic metabolism of glucose is shown in Fig 17. At concentration of diphenyliodonium chloride near the MIC value, respiration continues for about 120 minutes and then ceases. At concentration of compound less than this respiration continues but is still markedly depressed in comparison to the control.

The use of Triphenyltetrazolium bromide (T.T.C) in determining the dehydrogenase activity of E. coli.

The quantitative determination of succinate, lactate and malate dehydrogenase activity in E. coli is shown in Figs 18 19 & 20. A study has been made of the use of T.T.C as an indication of bacterial dehydrogenase activity, the method has been placed on a quantitative basis by determining, colorimetrically the formazan produced on reduction. All the dehydrogenase systems examined were inhibited by diphenyliodonium chloride. However, the lactate and malate systems were particularly sensitive, the malate system being completely inhibited by 100 µg/ml. The succinate system was less sensitive and continued for 30 minutes in the presence of 50 µg/ml and slowly for 20 minutes in 100 µg/ml.

FIG 17.

Inhibition by diphenyliodonium chloride of the aerobic glucose metabolism by *E. coli*, 24 mg/ml in 0.5 M phosphate buffer containing 0.02M glucose. ○–○, control; ◐–◑, 50 µg/ml diphenyliodonium chloride; ▲–▲, 100 µg/ml diphenyliodonium chloride at 37°C.

Fig.17

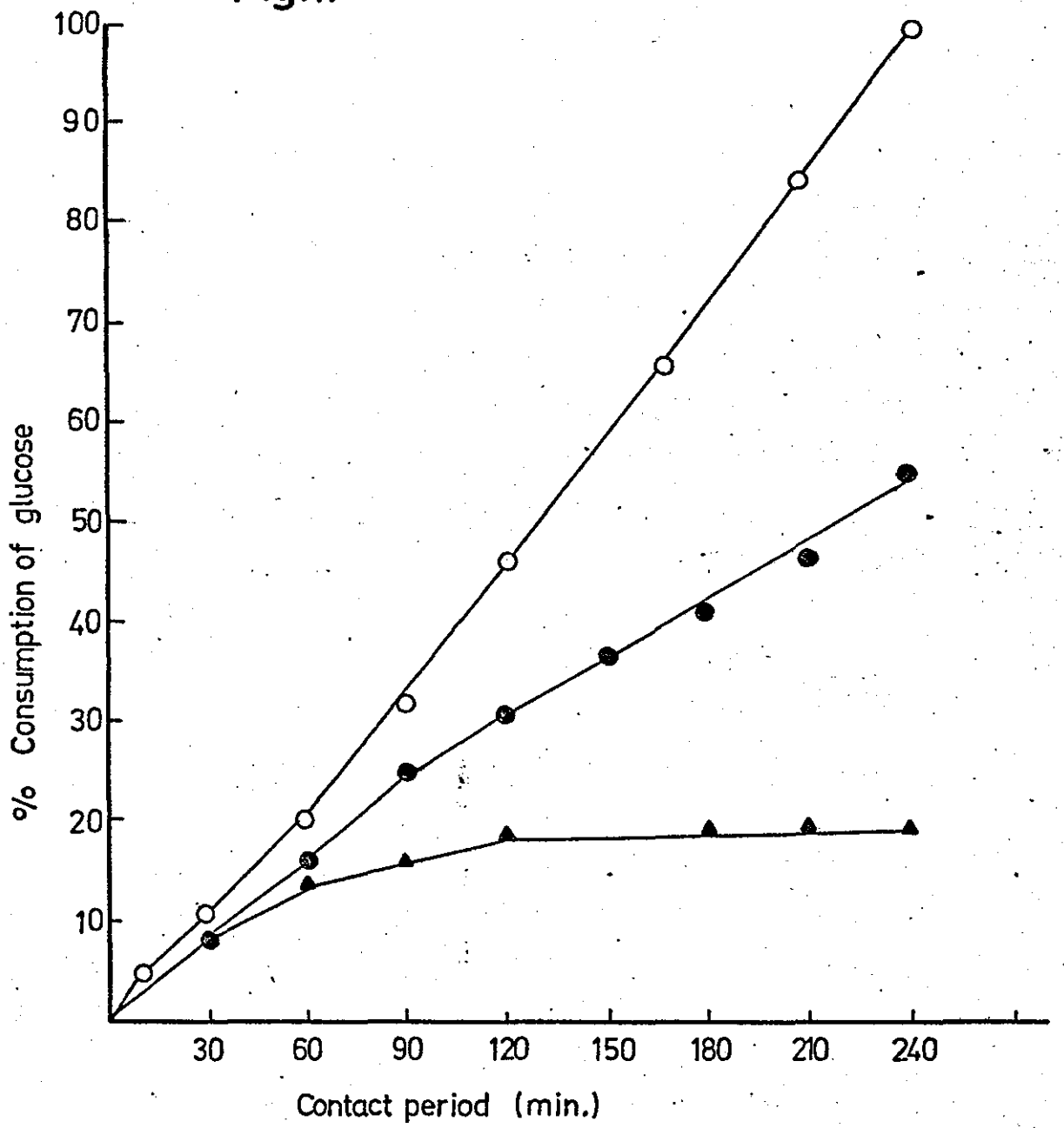

100
90
80
70
60
50
40
30
20
10
% Consumption of glucose
30
60
90
120
150
180
210
240
Contact period (min.)

FIG 18.

The production of formazan by *E. coli* 2.4 mg/ml in 0.1M phosphate buffer, (pH = 7) in the presence of succinate under aerobic conditions at 37°C; O–O, control; ●–●, 50 µg/ml diphenyliodonium chloride; ▲–▲, 100 µg/ml diphenyliodonium chloride.

Fig.18

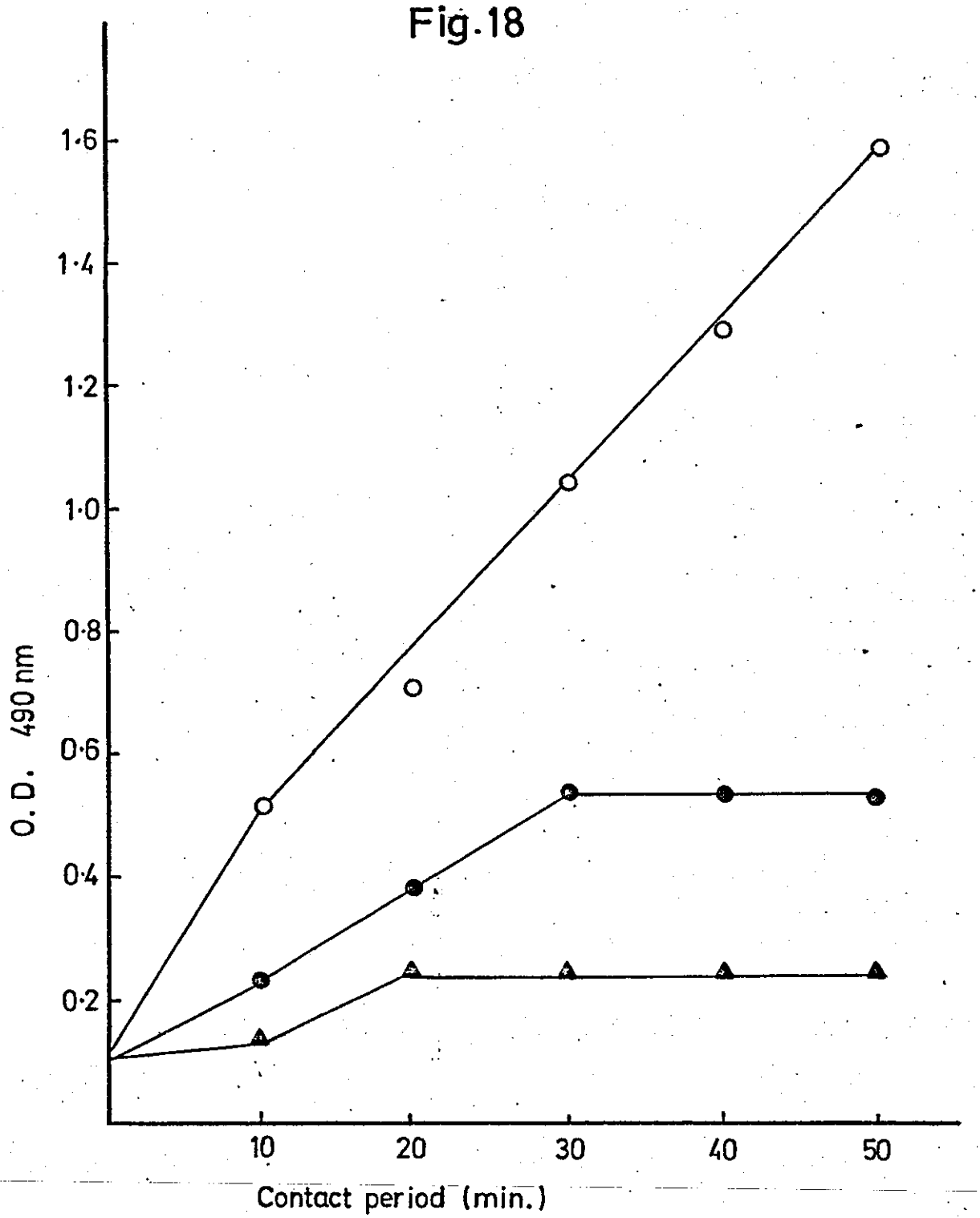


FIG 19

The production of formazan by *E. coli* 2.4 mg/ml in 0.1M phosphate buffer (pH =7) in the presence of lactate under aerobic conditions at 37°C; ○-○, control; ●-●, 100 µg/ml diphenyliodonium chloride.

Fig.19

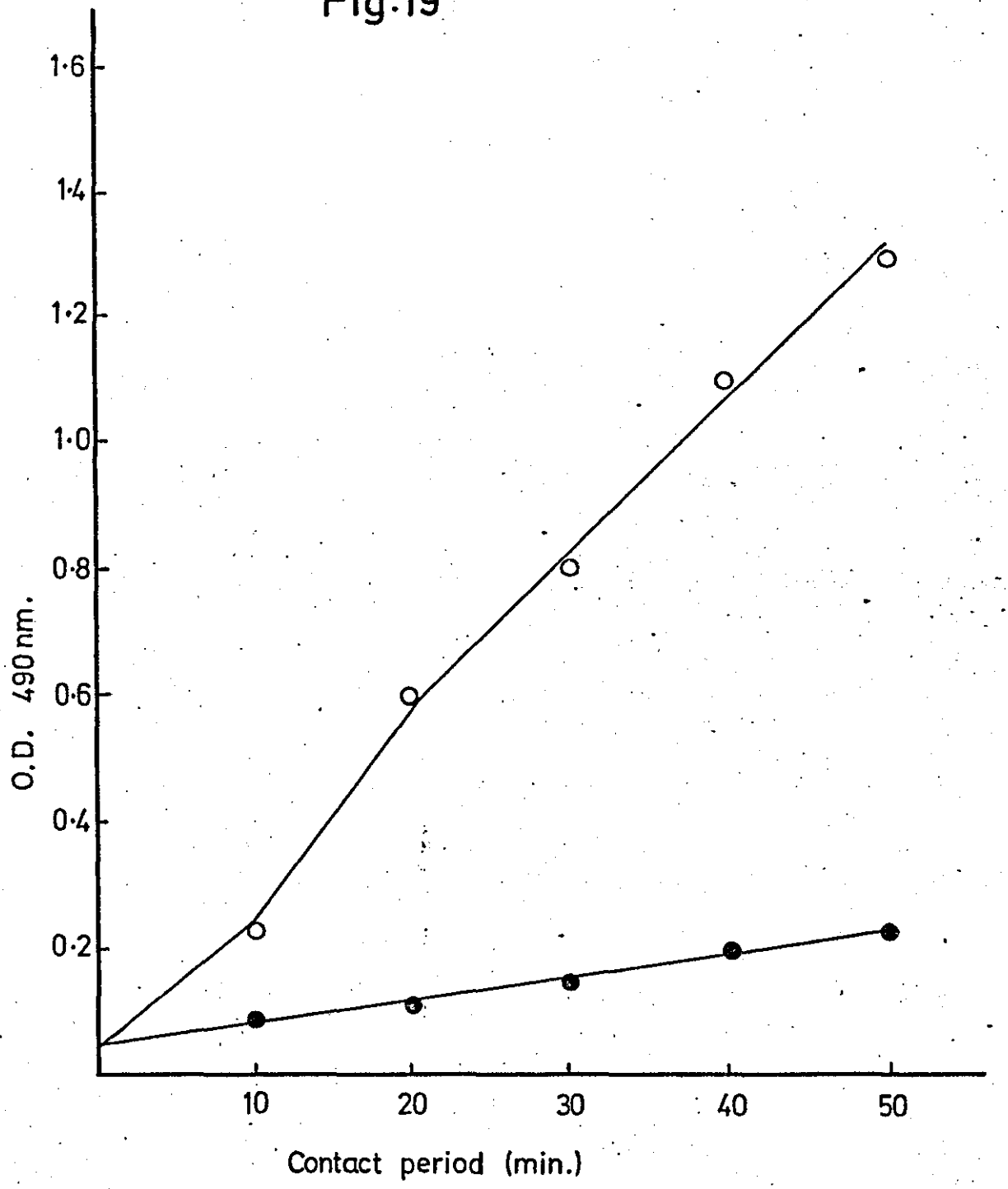
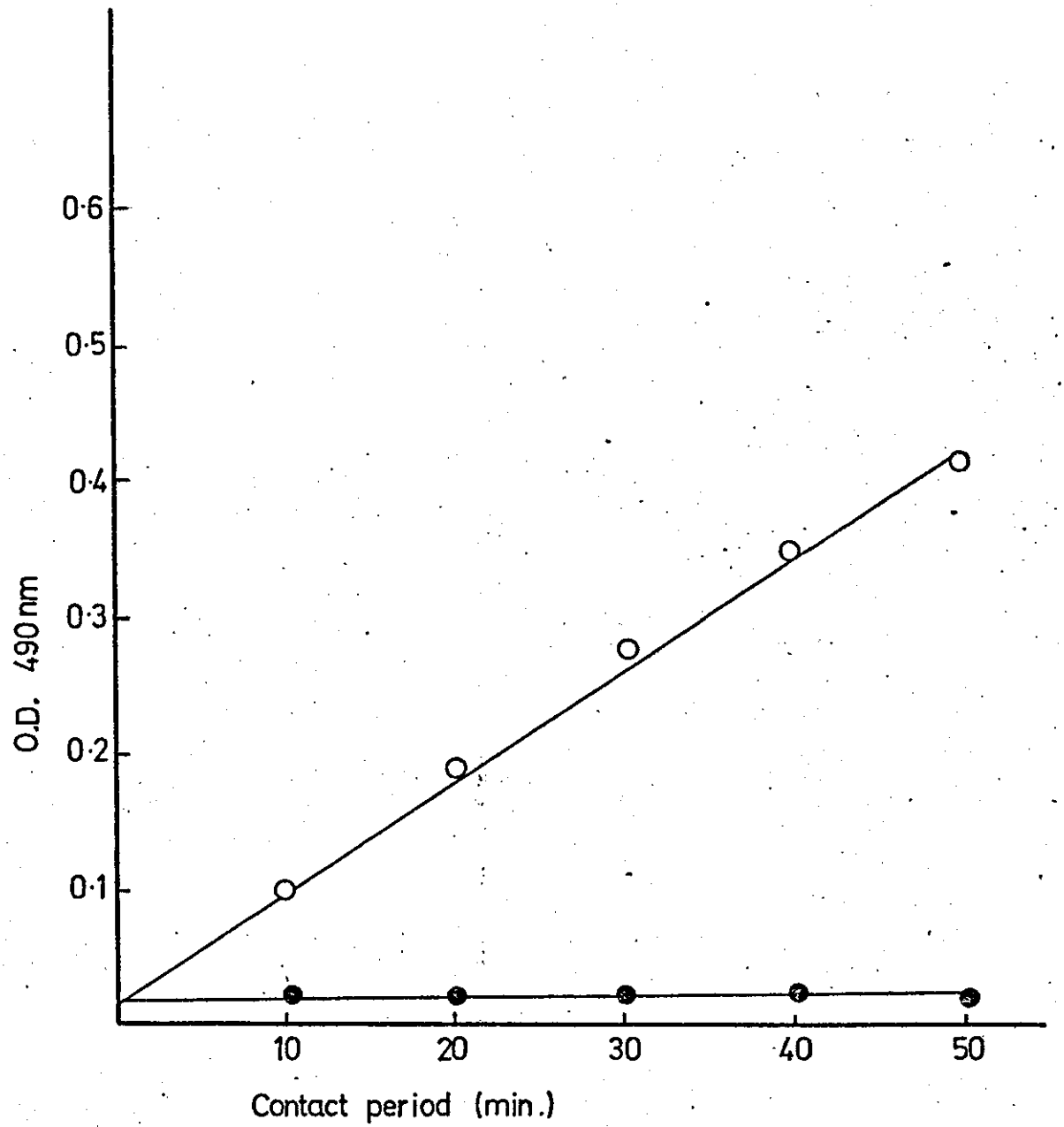


FIG 20.

The production of formazan by E. coli 2.4 mg/ml in 0.1M phosphate buffer (pH = 7) in the presence of malate under aerobic conditions at 37°C; O–O, control; ●–●, 100 µg/ml diphenyliodonium chloride.

Fig. 20



Effect of diphenyliodonium chloride on the morphology and size of the cell.

The effect of diphenyliodonium chloride on the cell size of E. coli is shown in Fig 21. There is no significant change in the size distribution of cells after treatment with 100 µg/ml diphenyliodonium chloride.

Electron Microscopy

1) Scanning Electron Microscopy.

The scanning electron microscopy study showed no alteration in the cell surface and no change in cell size of E. coli after treatment with 100 µg/ml of diphenyliodonium chloride (Figs 22 & 23).

2) Transmission Electron Microscopy.

Transmission electron microscopy of thin sections of E. coli after treatment with 100 µg/ml diphenyliodonium chloride showed that there was little alteration in cell structure, although the dark points may be sites of accumulation of diphenyliodonium chloride (Figs 24 & 25).

Titration curve for diphenyliodonium chloride

The titration curve for diphenyliodonium chloride was obtained (Fig 26).

FIG 21.

Size distribution of *E. coli* in the range 1.75 to 30 μm when grown in nutrient broth. A, control and B with 100 μg/ml diphenyliodonium chloride.

Fig. 21

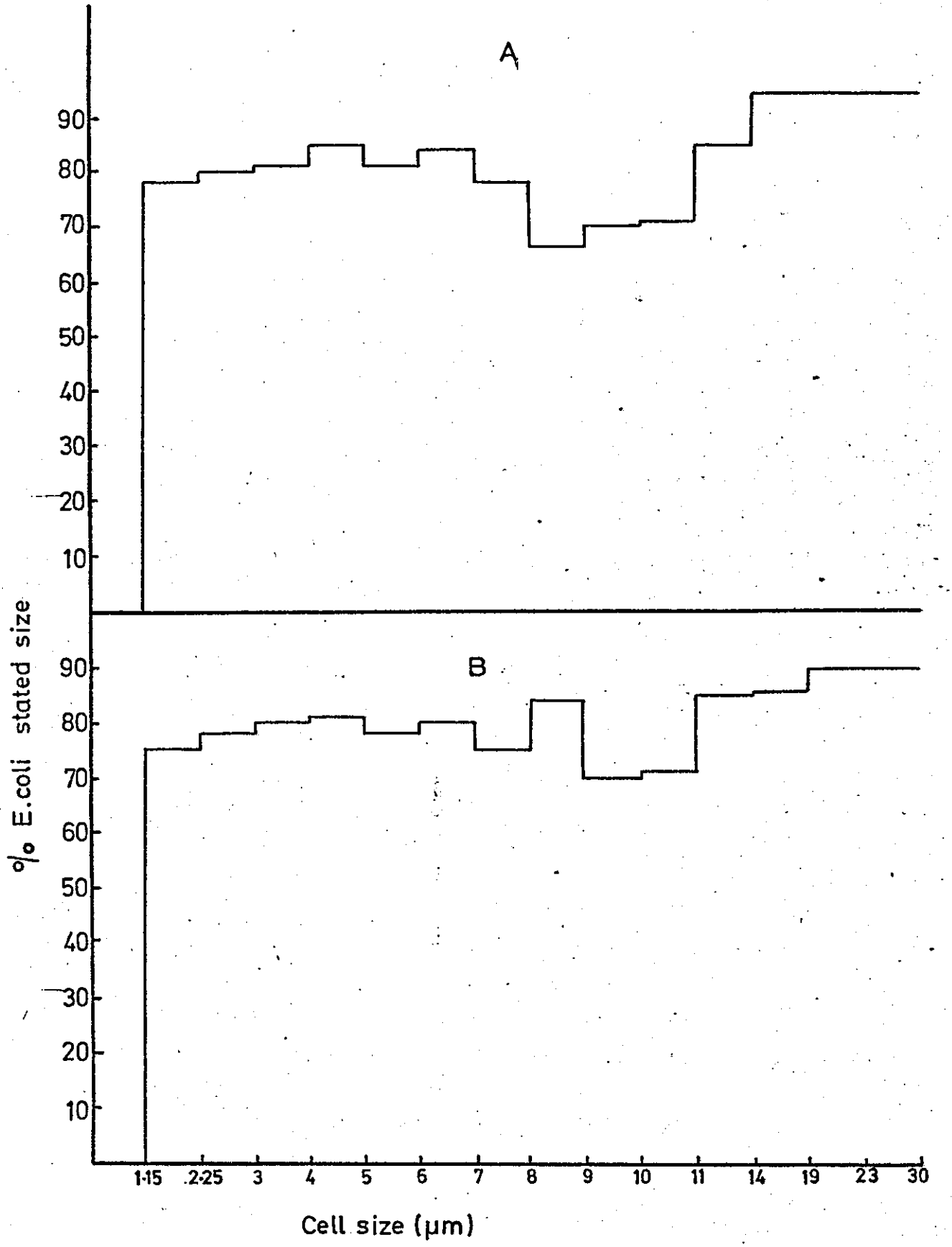

A
B
90
80
70
60
50
40
30
20
10
% E.coli stated size
1·15
·2·25
3
4
5
6
7
8
9
10
11
14
19
23
30
Cell size (μm)

FIG 22

Scanning electron microscopy of *E. coli* grown in nutrient broth; control x 9,500

FIG 23

Scanning electron microscopy of *E. coli* grown in nutrient broth containing 100 µg/ml diphenyliodonium chloride x 9,500

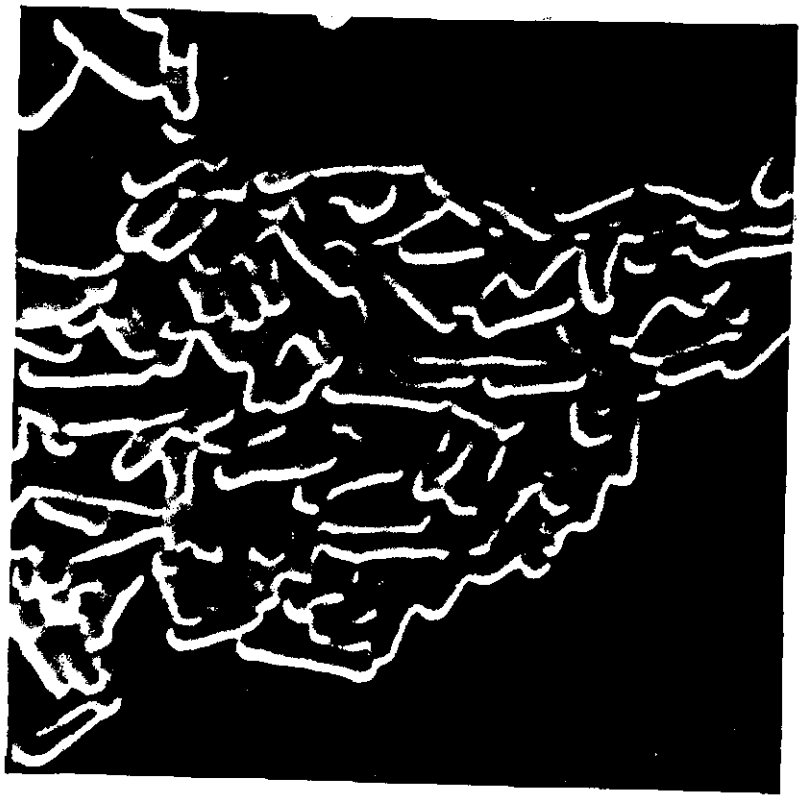
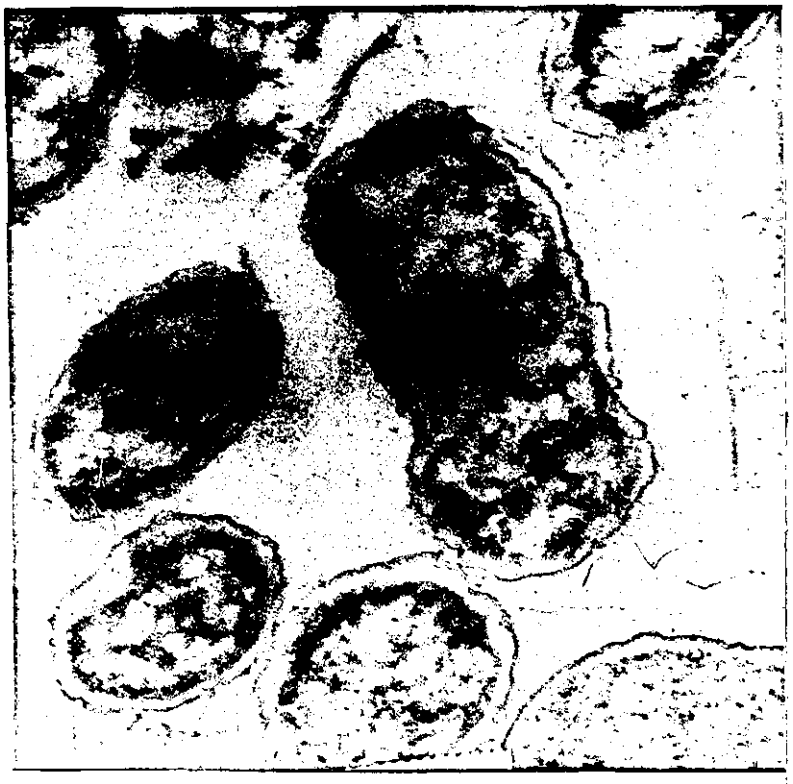


FIG 24

Electron micrograph of a cross section of *E. coli* x 40,000

FIG 25

Electron micrograph of a cross section of *E. coli* after treatment with 100 µg/ml diphenyliodonium chloride x 40,000.



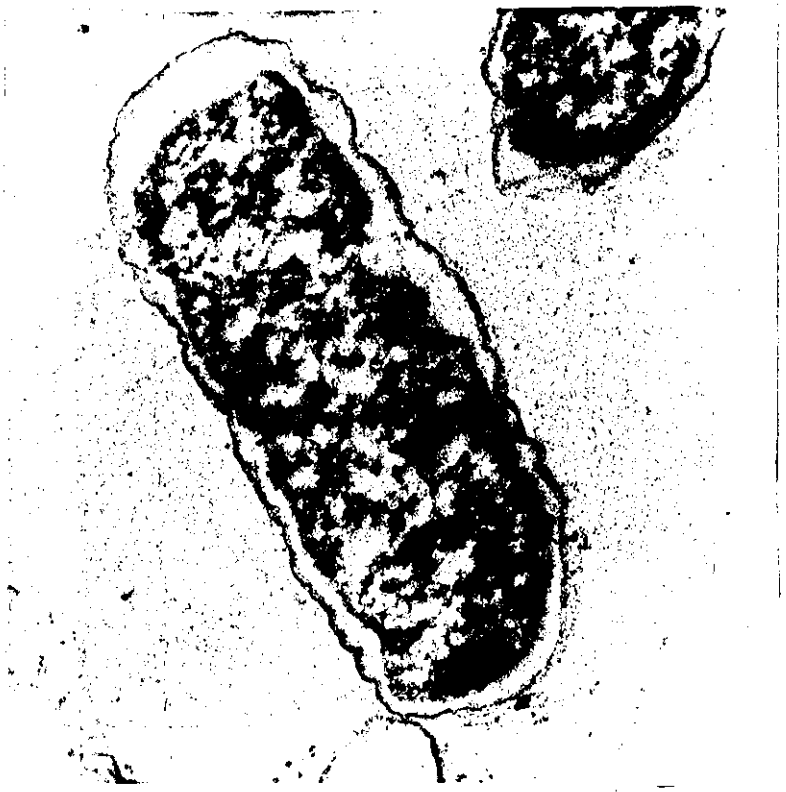
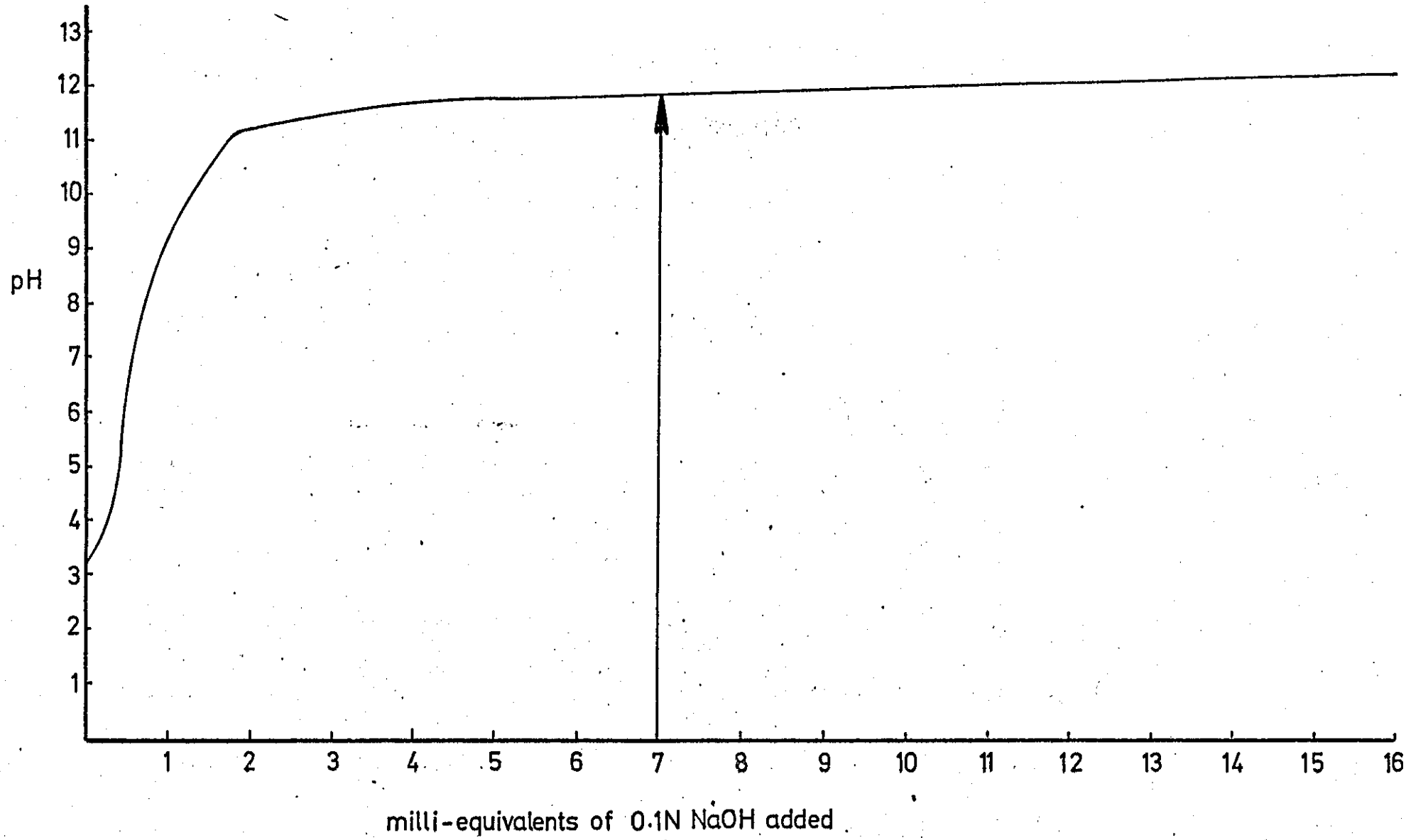


FIG 26.

Titration curve of pH against milli-equivalents of 0.1N sodium hydroxide.

Fig. 26


pH
13
12
11
10
9
8
7
6
5
4
3
2
1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
milli-equivalents of 0.1N NaOH added

SECTION 4

DISCUSSION

The determination of antibacterial activity as shown by the MIC values, indicates that diphenyliodonium chloride is considerably more active against gram-positive than gram-negative bacteria. Many antibacterial agents exhibit such selective toxicity including compounds active against cell wall or membranes. However, compounds which have greater activity against gram-positives than gram-negatives usually do not have significant action on the bacterial cell wall unless they have a specific action on cell wall biosynthesis. There is little evidence of this in the case of diphenyliodonium chloride. Firstly because no lysis occurs in growing cultures and secondly because there is no alteration in the cell surface as shown by S.E.M. This would suggest that there is no damage to the cell wall. Also there was no change in the mean cell size nor were any particularly large or small cells produced.

A study of the activity of diphenyliodonium chloride showed that the compound is bacteriostatic or bactericidal according to the concentration used. The bactericidal concentration was generally only slightly higher than the MIC value, when using an inoculum *ca.* 4×10^4 cells/ml e.g. 120 µg/ml for *E. coli*. When using *ca.* 10^7 cells/ml the bactericidal level was more than twice the bacteriostatic concentrations e.g. $>$ 200 µg/ml for *E. coli*.

The bactericidal effect was shown by the effect of diphenyliodonium chloride on proliferating and non-proliferating cells. For a suspension of 2×10^6 cells/ml a concentration of 140 µg/ml is required to inhibit the growth of E. coli. Even at concentration of 200 µg/ml (twice the MIC value) there was little bactericidal action and at double this concentration it took over 6 hours to completely kill the inoculum. At higher concentrations an increased bactericidal action was observed e.g. 400 µg/ml gave a 99.99% kill in 4 hours and 800 µg/ml in 90 minutes.

The effect of diphenyliodonium chloride on growing cultures of E. coli indicated that the action was not immediate even when added at the mid log phase and it took almost an hour to show inhibition of the test organism. At 200 µg/ml (twice the MIC value) of diphenyliodonium chloride complete inhibition of growth occurred. But this could not be studied further, since none of the common antagonists of disinfectants reversed the effect of diphenyliodonium chloride. Thus it was not possible to examine the effect on growing cultures and hence determine when a bactericidal effect was reached. One explanation of these observations could be the inhibition of protein or nucleic acid synthesis. However, this does not necessarily imply that an antibacterial agent directly effects these processes, since such syntheses could also be inhibited indirectly if the compound acts on one or more stages of metabolism.
At lower concentrations a reduction in growth occurred but eventually reached the same level as the control.

When diphenyliodonium chloride was added at the MIC value (100 µg/ml) to a mid log phase culture of E. coli, complete inhibition of growth did not occur. This is probably attributable to quenching of activity by the cells present. Such quenching effect was similarly noted above when the MIC were determined using a range of inoculum levels.

Unlike E. coli the growth of S. aureus was inhibited immediately after the addition of 120 µg/ml of diphenyliodonium chloride, confirming the greater sensitivity of S. aureus compared with E. coli.
At lower concentrations of the compound growth continued but was slightly reduced and at very low concentration e.g. 20 µg/ml a slight reduction in cell growth was noted but the final cell density was very similar to the control. Thus, this would tend to confirm the bacteriostatic nature of diphenyliodonium chloride.

Diphenyliodonium chloride appears to be more active against E. coli and S. aureus under aerobic conditions than under anaerobic conditions, both by zone diffusion and MIC studies. Under aerobic conditions the zones of inhibition are larger than under anaerobic conditions. Also using the plate method, the MIC value under anaerobic conditions was ca. twice that obtained under aerobic conditions. The different MIC values which were obtained by plate and tube methods could be partly explained by the availability of oxygen in the two systems, for the tube method there could be some limitation of oxygen.

The assessment of the interaction between diphenyliodonium chloride and the most common antibiotics and antimetabolites showed that there was no interaction between them. This kind of behaviour which Maccacaro (1961) calls "indifference" does not always have a clear significance, and may not necessarily infer that interaction does not occur.

Before an antibacterial agent can exert its action, it must gain access to the sensitive site, and the cell wall, may present a barrier to the passage of diphenyliodonium chloride. The cell wall can act as a non-absorbing barrier and so prevent access to the cell membrane or other site within the cell. The cell wallmay also have a high affinity for the drug, thus acting as an absorbing barrier and so preventing adsorption of the drug by the cell interior.
One possible site of absorption are the lipids of the cell envelope in E. coli which contains up to 20% of lipid material compared with 1-2% in S. aureus.

Since it has been found that lipid solubility for diphenyliodonium chloride is very low (the partition coefficient water: n hexane = 0.06) it is unlikely that this compound will dissolve and become trapped in the lipid of the cell envelope. It could, therefore, react with the non-lipid material present in the cell wall by an ion-exchange process. Thus passing from the external environment to the interior of the cell, where it may react with enzymes involved in electron transport processes in the

cell membrane and ultimately interacting with cytoplasmic constituents. However, such a mechanism would fail to differentiate between the relative sensitivity of E. coli and S. aureus unless there is more ionogenic material in the cell wall of E. coli.

The effect of pH on the uptake of diphenyliodonium chloride showed that at higher pH values a greater amount of the drug was adsorbed. This would suggest that the main effect of changes in pH is to influence the ionization of the adsorbing sites in the cell, because diphenyliodonium chloride is a salt and fully ionized at all pH values and so ionization of the acidic cell components is the most important factor. This increased adsorption is accompanied by an increase in antimicrobial activity as the pH value of the medium rises. This is similar to the observed rise in the activity of the cationic detergent, cetrimide at alkaline pH values which also may be due to an increased adsorption (Sykes, 1965). Similar results were obtained with chlorhexidine (Longworth, 1971) where an increase in ionized groups at the cell surface occurs with an increase in the uptake.
Where the adsorption of antibacterial agents (which exhibit selectivity), has been determined at drug levels which are inhibitory, the levels of uptake were not dissimilar. For example dequalinium (Hugo & Frier, 1969) or fentichlor (Hugo & Bloomfield, 1971 a). Under these circumstances there appears to be a difference in affinity for the compounds by S. aureus and E. coli and certainly no real difference in their mode of action.

The uptake of antibacterial agents by intact cells under various conditions permit some conclusions to be made about the adsorption process and the cell receptor sites involved.
When the adsorption of diphenyliodonium chloride by E. coli is examined it is seen that a Langmuirian isotherm or L-form (Giles et al 1960) is obtained. This indicates that drug molecules are at first readily accepted by the adsorbing material, but as the available sites become progressively filled, further adsorption is hindered.

This situation is found with many antibacterial agents including fentichlor (Hugo & Bloomfield, 1971a), and dequalinium (Hugo & Frier, 1969).
The fact that a Langmuirian isotherm is obtained, would indicate monomolecular layer adsorption. However, it is possible to calculate the approximate concentration of diphenyliodonium chloride at which a monolayer of drug molecules is formed on the surface of a bacterial cell. Thus if it is assumed that E. coli is a cylinder 2.0 μm x 0.8 μm surmounted at either end by hemispheres of radius 0.25 μm, then the surface area is $5.812 \mu m^2 = 5.812 \times 10^8 \text{ Å}^2$. If it is assumed that the diphenyliodonium chloride molecule is orientated with one of the phenyl groups at right angles to the cell surface and the rest of the molecule perpendicular to the surface (a condition for optimal packing, but unlikely to occur in fact) then the molecule will occupy an area of 26 Å^2, thus the maximum number of molecules accommodated as a monolayer is $5.812 \times 10^8 / 26 = 0.224 \times 10^8$ molecules, or, from Avogadro's number, 1.867×10^{-8} µg diphenyliodonium chloride (base) per cell . 1 mg dry weight cells of E. coli = $4 \times 10^9 = 74.68$ µg base = 85.5 µg of the diphenyliodonium chloride/mg dry weight of cells.

The actual figure is probably much less than this. Thus, it is possible that adsorption over and above this level might be due to the building up of multilayers (at the saturation level ca.5 monolayers) of the drug which may be a feature of the leakage inhibition phenomenon, or, alternatively to penetration of drug into the interior (cytoplasm) of the cell. Similarly, Cox (1966) showed that with 1-decamethylene-4-aminoguinaldinium,sufficient drug was absorbed at the saturation level to give at least 30 monolayers around the bacterial cell. Hugo & Longworth (1964) also found that multilayers of chlorhexidine may occur around the bacterial cell. Whilst uptake studies can be helpful, they are probably too simplistic because it is not always reasonable to extrapolate data from simple physical systems to complex biological systems where a dynamic situation occurs.

It is most likely that adsorption takes place at sites separate from the cell surface, that is within the cell wall and ultimately in the cytoplasm. T.E.M. examination of sections has shown that the amount of drug present in the cell wall is not high, nor is it localized in specific regions , since the electron dense iodine would be expected to be detected by this method.

It has been shown that the MIC increases with numbers of cells inoculated. This could probably be explained by the drug adsorbing to the cells and thus being inactivated, thus the greater the number of cells present, the greater the inactivation.

Diphenyliodonium chloride may affect the integrity of the cell membrane, because there is some loss of intracellular material when the drug is added to a suspension of E. coli. However, this is not likely to be the main mechanism of its lethal action because the loss of interacellular material is very low when compared to the loss in the presence of chlorhexidine (Hugo & Longworth, 1964),polymyxin (Newton, 1953) or colistin (Stretton 1965) which exert their action by membrane damage. The effect of increased concentration of diphenyliodonium chloride on the loss of cell constituents is monophasic, which is similar to the effect of hexylresorcinol (Becket et al, 1959) where the progressive addition of hexyl-resorcinol caused more extensive damage and the release of more cellular constituents until the drug molecules exerted their maximum effect upon the cytoplasmic membrane. The membrane active compounds CTAB and polymyxin (Newton, 1953) and chlorhexidine (Hugo & Longworth, 1964), have a diphasic effect. In the cases of fentichlor (Hugo & Bloomfield, 1971 b), polymyxin (Few & Schulman, 1953) and CTAB (Salton, 1951), there is a linear relationship between the proportion of cells killed and the amount of 260 nm absorbing material released. The loss of intracellular material could be due to the activation of autolytic enzymes, but this is unlikely as the loss increases only slowly with time and would not represent a significant fraction of the material lost. However, at concentrations less than 100 µg/ml of diphenyliodonium chloride,RNA loss was produced in E. coli and at 100 µg/ml (the MIC value) there was maximum loss of RNA. If the concentration of diphenyliodonium chloride was increased to a bactericidal level, then, leakage

is not longer concentration dependent.

The small amount of damage to the cell was confirmed using both scanning and transmission electron microscopy. Even at 100 µg/ml of diphenyliodonium chloride there was no detectable loss of major cell constituents when ultra thin sections were examined by transmission electron microscopy.

The effects on metabolic activity were shown by the inhibition of dehydrogenase systems, at approximately the MIC values, and on the sensitivity of aerobic glucose metabolism.

Diphenyliodonium chloride inhibits glucose metabolism of E. coli under aerobic conditions. The rate of oxygen consumption was less than the control in the presence of 100 µg/ml and was completely inhibited after 2 hours. This suggests that glucose metabolism is very sensitive to diphenyliodonium chloride. At 50 µg/ml of the drug respiration continues but is still markedly depressed.

Dehydrogenase systems were also inhibited by diphenyliodonium chloride. The malate and lactate systems are particularly sensitive to diphenyliodonium chloride. The malate system, being completely inhibited by 100 µg/ml of the drug with the succinate dehydrogenase being less sensitive.

Since it was found that there is no significant difference between the concentration of diphenyliodonium chloride required to inhibit the metabolism of glucose under aerobic and anaerobic conditions, it is unlikely that specific inhibition of cytochrome systems is involved. Woodroffe & Wilkinson (1966), using tetrachlorasalicylanilide, found a 20-fold difference existed between the concentration required to inhibit glucose fermentation and that necessary to inhibit glucose oxidation.

There was no stimulation of dehydrogenase activity at low concentrations of diphenyliodonium chloride. This is unlike the stimulation obtained where compounds are used which are known to damage the integrity of the cytoplasmic membrane e.g. chlorhexidine, (Hugo & Longworth, 1964) where an increase in membrane permeability may be responsible for the observed phenomenon. and with bronopol (Stretton & Manson 1973) which acts by oxidation of the thiol groups.

The dehydrogenase enzyme systems are located in the cell membrane as demonstrated by Sedar & Burde (1965) and Hirano et al (1969) and alteration of these enzymes may damage membrane structure and permit leakage. This type of effect is shown by the closely related diphenyleneiodonium chloride which is also an antibacterial agent approximately as active as diphenyliodonium chloride (Stretton, unpublished). This compound inhibits phosphorylations and the chloride-hydroxide balance in isolated chloroplasts (Watling & Selwyn, 1972).

It has been shown that S. faecalis may exhibit a substantial anaerobic endogenous metabolism after growth on media containing an excess of energy source. Membrane ghosts from S. faecalis contain a firmly bound-adenosine triphosphate when they are prepared and washed with buffer pH = 7.5 containing Mg^{++} ions. Several workers (Holland & Sherratt, 1972 and Gatley & Sheratt, 1976) have investigated the effect of diphenyleneiodonium chloride on mitochondria. This compound inhibits ADP- or uncoupler-stimulated oxidation of succinate or of glutamate in Cl^--containing media by a mechanism dependent on the catalysis of a linked Cl^--OH^- exchange across the inner mitochondrial membrane. In this respect diphenyleneiodonium chloride resembles the trialkyltin compounds which also mediate this exchange (Selwyn et al, 1970). Diphenyleneiodonium chloride also inhibits soluble rat liver NADH dehydrogenase and NADH oxidation by rat liver submitochondrial particles directly. NADH dehydrogenase preparations apparently contain only high affinity binding sites whilst only low affinity sites occur in erythrocytes.

Mitochondrial ATPase is thought to catalyse the terminal step in oxidative phosphorylation. (Pullman & Schatz, 1967). It is unlikely that the ATPase associated with protoplast membranes of S. faecalis serves an analogous function as this organism lacks cytochromes and generates ATP entirely via glycolysis (Deibel, 1964).

Diphenyliodonium chloride is a potent inhibitor of membrane associated phenomena, because it inhibits membrane-bound-ATPase and K^+ accumulation by intact cells and reduces the rate of glycolysis. Thus a number of energy dependent processes in S. faecalis are inhibited by diphenyliodonium chloride .

The ATPase of mitochondrial membrane appears functionally very different from the ATPase associated with the plasma membrane of fermentative cells such as erythrocytes or S. faecalis. The former is thought to catalyse the terminal step in oxidative phosphorylation, the latter apparently mediates the utilization of glycolytically generated ATP for membrane transport. Yet, it has been known for some time that oligomycin, the classical inhibitor of ATPase and oxidative phosphorylation in mitochondria, inhibits also ATPase and cation transport in erythrocytes (Whittam, et al, 1964).

The observation with Dio9 and chlorhexidine (Harold et al 1969 a) as well as earlier work with DCCD (Harold et al, 1969 b) adds to the growing evidence that a fundamental unity underlies processes apparently as diverse as oxidative phosphorylation in mitochondria and cation transport by S. faecalis.

Similarities at the molecular level between the ATPase of mitochondria and of S. faecalis (Abrams, 1965 and Abrams & Baron, 1967) point in the same direction.

Diphenyliodonium chloride, Dio9, chlorhexidine and DCCD all stop the growth of S. faecalis at concentration comparable to those which inhibit the energy dependent transport of K^+. The concentration of diphenliodonium chloride which inhibits ATPase activity is much less than the MIC value. The cessation of growth may thus be a consequence of the inhibition of energy linked transport processes.

Dicyclohexylcarliodiimide (DCCD) does not interfere with the generation of ATP (Harold, et al, 1969 a), but it inhibits the membrane-bound ATPase of the intact cell. The other compounds which were used as controls, Dio9 and chlorhexidine, probably act at the same site. The site of action of DCCD is not clearly defined and it inhibits membrane-bound ATPase but not the solubilised enzyme (Harold et al, 1969 b). Thus it appears that DCCD reacts with an unidentified component of the membrane and inhibits the ATPase indirectly, perhaps by a transmitted effect on the conformation of the enzyme (Harold et al, 1969 b and Bulos & Racker, 1968). In contrast chlorhexidine and Dio9 inhibit both the membrane-bound and solubilised ATPase of S. faecalis.

Diphenyliodonium chloride, Dio9, chlorhexidine and DCCD, all stop the growth of S. faecalis at concentrations comparable to those inhibiting the energy dependent transport of K^+. Also diphenyliodonium chloride inhibits ATPase activity at a concentration below the MIC value. This suggests that one aspect of the lethal action of diphenyliodonium chloride is to inhibit energy linked transport processes.

The evidence suggests that diphenyliodonium chloride exerts its antibacterial action primarily on systems associated with the cytoplasmic membrane. The most sensitive sites would appear to be the membrane-bound ATPase and K^+ transport. In addition membrane associated dehydrogenase systems are also very sensitive to the compound and because of its membrane involvement there is a loss of cytoplasmic constituents.

However, there are other effects produced by diphenyliodonium chloride which suggests that at higher concentrations it interferes with general cell metabolism and that these contribute to its bacteriostatic effects.

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