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SOME ASPECTS OF THE ANTIBACTERIAL MODE OF ACTION OF 4,7-PHENANTHROLINE-5,6-QUINONE (PHANQUONE)

A THESIS

Submitted in partial fulfilment of the requirements for the Award of the Degree of

DOCTOR OF PHILOSOPHY

by

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TO

The holiest city in the world

TO

The city where wars are fought
in the name of peace

TO

JERUSALEM, my birthplace

I dedicate this work

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PART I

INTRODUCTION

SECTION ONE

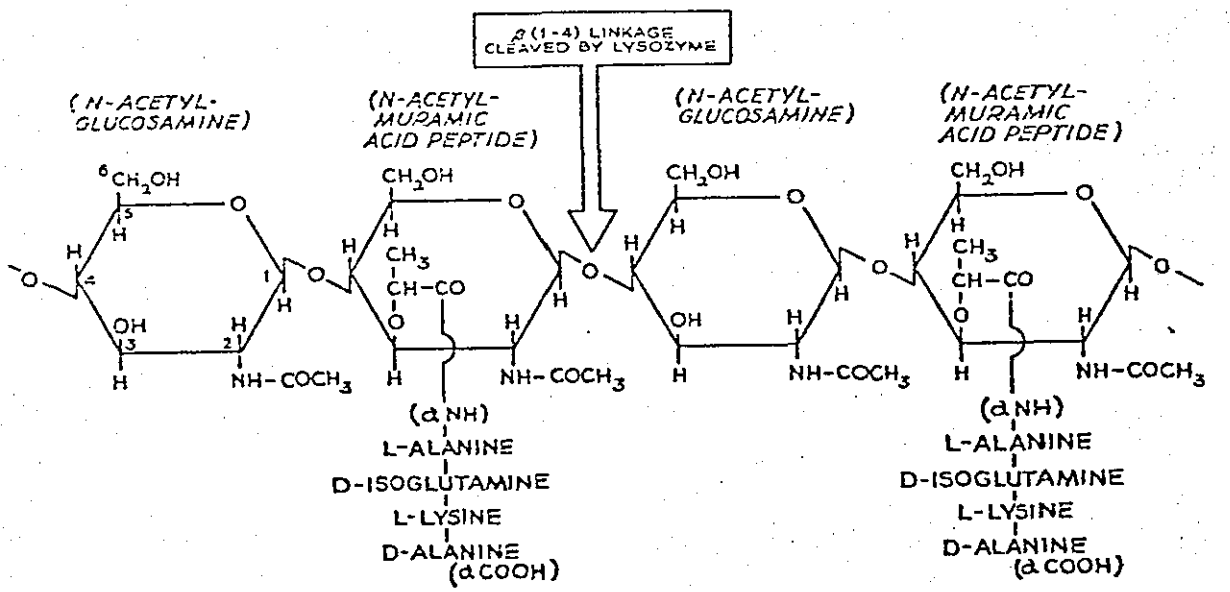
THE INTERACTION OF ANTIBACTERIAL AGENTS WITH THE BACTERIAL CELL

1. THE CELL WALL

Although there is a great diversity in the structure of the cell walls of different bacterial species, two main types of cell wall are recognised, the Gram-positive and the Gram-negative walls. The simpler Gram-positive wall consists mainly of a peptidoglycan, whose basic framework is made up of alternating subunits of N-acetylglucosamine and N-acetylmuramic acid, connected by β(1,4) linkages (Fig. 1.1(a)). The N-acetylmuramic acid is substituted at the acidic group with a short peptide chain, containing the unusual D-isomers of glutamic acid and alanine; these peptides are cross-linked together giving rise to a rigid polymer (Fig. 1.1(b)). In addition, the Gram-positive walls contain teichoic acid, and a mucopolysaccharide.

The more complex Gram-negative wall consists of three layers: a peptidoglycan inner layer, similar to that of Gram-positive walls, and outer layers of lipopolysaccharide and lipoprotein. Teichoic acids are, however, absent.

The structure of the bacterial cell walls have been extensively reviewed by Jawetz, Melnick and Adelberg (1972), Gale *et al* (1972) and Franklin and Snow (1975).



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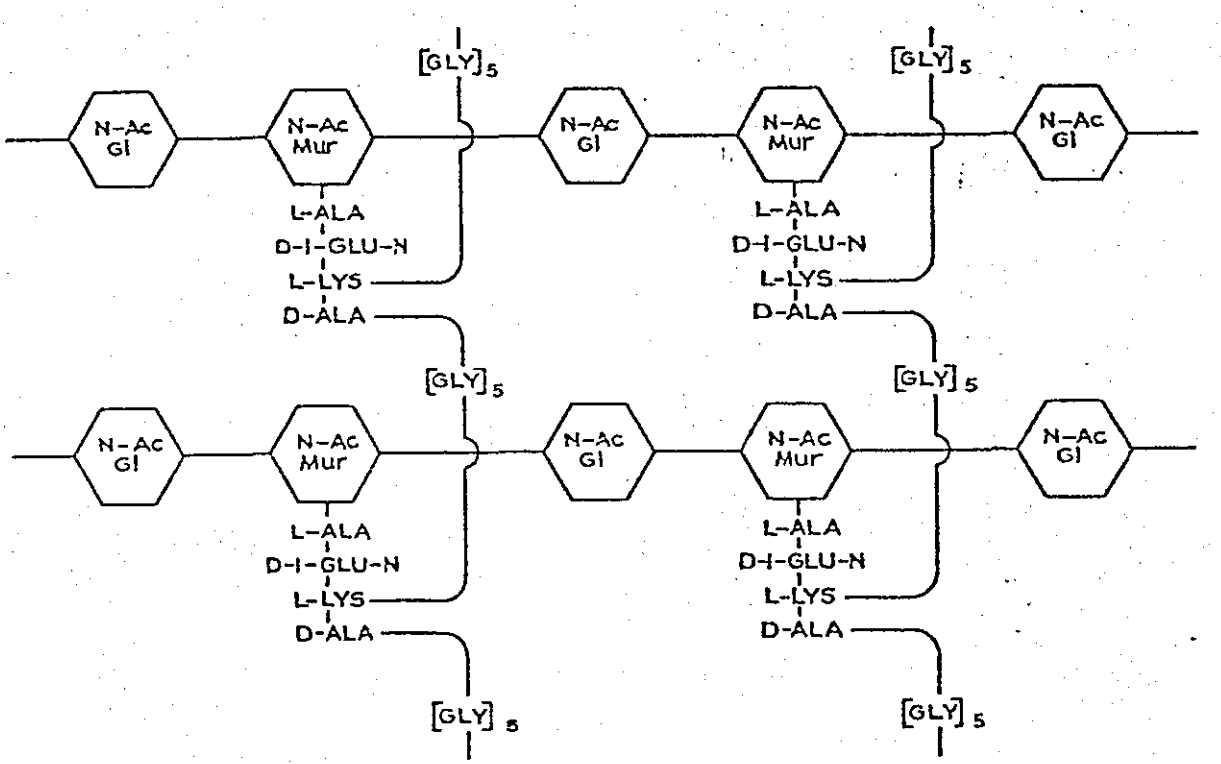

(b)

Fig (1.1)*

a) The peptidoglycan of *Staphylococcus aureus*, with its backbone consisting of alternating units of N-acetylglucosamine and N-acetylmuramic acid connected by β(1-4) linkages.

b) Schematic representation of the peptidoglycan lattice; short peptides, linked to the muramic acid, are cross-linked between parallel polysaccharide backbones.

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* After Jawetz, Melnick and Adelberg (1972).

The cell wall is a target for the action of a number of important antibiotics and antibacterial agents. Among these are penicillins and cephalosporins, which inhibit the synthesis of bacterial cell wall mainly by blocking the terminal cross-linking of linear glycopeptides into the complex peptidoglycan. Bacitracin blocks the formation of the linear peptidopolysaccharide by complexing with a pyrophosphate needed in the process. D-cycloserine competitively inhibits alanine-racemase and D-alanine-D-alanine synthetase. Phosphonomycin acts as an analogue of phosphoenol-pyruvate, and binds covalently to the pyruvate-UDP-N-acetylglucosamine transferase; thus preventing the formation of UDP-N-acetylaglucosamine enolpyruvate, while vancomycin inhibits the incorporation of amino acids into peptidoglycan.

Detailed reviews of the action of antibacterial agents and antibiotics against bacterial cell walls are given by Reynolds (1966), Russell (1969), Gale *et al* (1972), and Franklin and Snow (1975).

2. THE CELL MEMBRANE

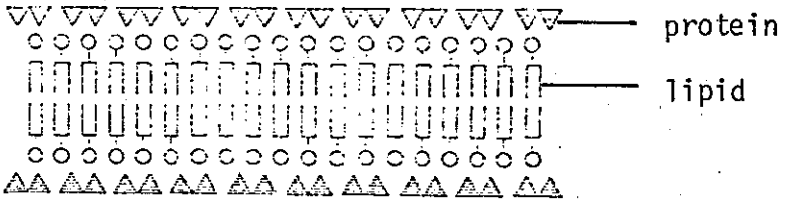
Apart from being the osmotic barrier of the cell, the bacterial cytoplasmic membrane is the site of respiration and oxidative phosphorylation, and their associated enzymes (Russell, 1969; Hatefi, 1976). These two processes have been discussed in great detail by Wendler (1976) and Haddock and Jones (1977). The membrane lipid fraction constitutes up to 30% of its dryweight, of which 80% is phospholipid, the rest being neutral lipid (Hughes, 1962). The fatty acid chains in some Gram-negative bacteria may be mainly saturated and even-numbered, while Gram-positive organisms contain

a high proportion of branched chains (Brundish, Shaw and Baddily, 1966). A carbohydrate fraction is usually present, but its proportion depends on the adhering cell wall, and therefore the membrane content fluctuates between 0.2 - 19% (Salton, 1967).

The protein fraction constitutes 40-85% of the membrane dry-weight, of which its largest proportion is the enzymes of the electron transport system (Salton, 1967). Its amino acid composition is similar to the cell protein, but different from the cell wall, in that it contains all the common L-amino acids but not the D-amino acids (Hughes, 1962). RNA is found to constitute 0.8-15% of its dry-weight (Salton, 1967).

Although the cytoplasmic membrane has a very low mechanical strength, it possesses special and complex properties of permeability, and also regulates the passage of metabolites into the cell (Hugo, 1967). A simple model of the "unit membrane" has been proposed by Danielli and Davson (1935), and consists of a three layer structure: protein-lipid-protein (Fig. 1.2(a)). The middle layer consists of two monolayers of lipid with hydrocarbon chains in end-to-end contact, and hydrophilic ends pointing outwards, in contact with the dense protein layer on either side (Hughes, 1962).

This membrane model has been subject to criticism. Vanderkooi and Green (1970) suggested that the membrane may consist of two layers of globular protein molecules in contact with each other, with the phospholipid filling the interglobular spaces (Fig. 1.2(b)). Stoeckhenius and Engelman (1969) postulated that the inner lipid layer was equivalent to a leaflet, of thickness less than that of two phospholipid molecules, with a disordered hydrocarbon centre bonded by hydrophilic groups which were not closely packed (Fig. 1.2(c)).



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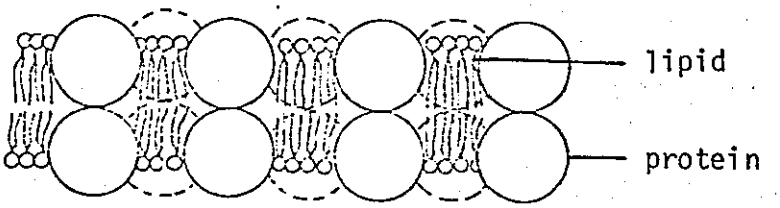

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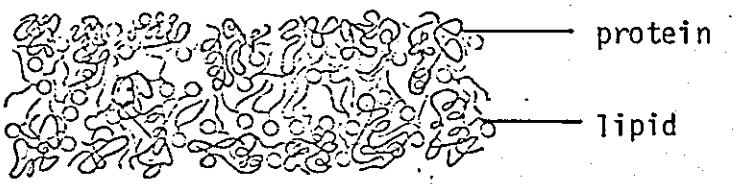

(c)

Fig. (1.2)

Models of Membrane Structures*

a) Danielli and Davson (1935) model.
b) Vanderkooi and Green (1970) model.
c) Stoeckenius and Engelman (1969) model.

* After Gale *et al* (1972).

Transport across the membrane can occur by one of three methods; passive diffusion, in which the substance passes the membrane due to a lower solute concentration or an electrical gradient within the cell; facilitated diffusion, where the substance reacts with a carrier to form a complex that can cross the membrane; and/or energy dependent transport, in which the substance cannot pass without the presence of an energy source that supplies the energy required to carry the substance against the concentration gradient (Gale *et al*, 1972).

There are three major hypotheses attempting to explain the coupling of energy production with transport across the membrane. The coupling factor theory proposes the involvement of high energy intermediates (Slater, 1966). The conformational theory proposes conformational changes in the membrane, mediated by ATP (Young, Blondin and Green, 1971). While the chemiosmotic theory suggests the setting up of a proton gradient across the membrane that provides the energy to drive not only the transport process, but oxidative phosphorylation and ATP synthesis (Mitchell, 1967; 1970; 1972). Mitchell's chemiosmotic theory is further substantiated by the finding that uncoupling agents such as 2,4-dinitrophenol (DNP) act as specific conductors of protons across bacterial, mitochondrial, and artificial membranes, thereby modifying part of the proton motive force across the membrane (Mitchell, 1961; 1968), and that these uncouplers are able to discharge transport processes (Hamilton, 1968).

Comprehensive reviews of the membrane structure and function are presented by Hughes (1962), Hugo (1967), Russell (1969), Harold (1970), Gale *et al* (1972) and Boos (1974).

Many drugs affect the membrane structure and cause small molecules to leak across. Among these are tyrothricin (Hotchkiss, 1944), cetyl trimethylammonium bromide (CTAB; Salton, 1951), phenols (Hugo, 1957), polymyxin (Newton, 1956), and chlorohexidine (Hugo, 1967).

Many other antibacterial agents affect the membrane processes rather than structure. DNP (Mitchell, 1968), 3,5,3',4'-tetrachloro-salicylanilide (TCS; Harold and Baarda, 1968), gramacidins (Cross, Taggart, Covo and Green, 1949), and others, act by uncoupling oxidative phsophorylation and inhibiting active transport; while oligomycin, dicyclohexylcarbodiimide (DCCD), and chlorohexidine inhibit the membrane-bound enzymes involved in energy transfer.

Extensive discussions of the mode of action of antibacterial agents against the cell membrane are presented by Russell (1969), Harold (1970; 1972) and Gale *et al* (1972).

3. THE NUCLEIC ACIDS

The chromosome of the bacterial cell consists of a single circular molecule of double-stranded DNA, which does not differ in structure or composition from DNA of the eukaryotic cell (Hayes, 1965). The processes of transcription of DNA into mRNA, tRNA and rRNA, and translation into proteins in the bacterial cell is similar to that of the eukaryotic cells. This is why most of the antibacterial agents acting on the DNA or the RNA of the bacteria, are quite toxic to the host cells as well.

The interaction of the different antibacterials and antibiotics with the nucleic acids and their synthesis has been reviewed in detail by Waring (1966), Reich (1966), Russell (1969), Gale *et al*

(1972) and Franklin and Snow (1975).

According to Gale *et al* (1972) the inhibitors of nucleic acid synthesis may be classified into three major categories. The first includes agents which interfere with nucleotide metabolism; azaserine inhibits nucleotide synthesis, hadacidin and 5-flurouracil inhibit nucleotide interconversion, cytosine arabinoside inhibits its utilization, while 5-bromo and 5-iododeoxyuridine are incorporated into polynucleotides, causing the distortion of their structure and function. The second category include agents which impair the DNA template function; proflavine, ethidium, and chloroquine intercalate with the DNA by becoming inserted in the grooves between base pairs, usually occupying the space of an extra base pair, thus causing frameshift mutagenesis; actinomycin D inhibits RNA polymerase, while intercalating with the DNA as well; mithramycin, chromomycin, phleomycin and others inhibit DNA and/or RNA polymerase; while mitomycin C, and nitrogen and sulphur mustards react covalently with the DNA by cross-linking it, and thus cause the breaking of its strands.

The third category includes those agents which inhibit the enzymic processes in nucleic acid synthesis; both rifampicins and streptolydigins bind directly to RNA polymerase, while nalidixic acid and β-phenethyl alcohol inhibit the DNA synthesis and replication.

Fig. (1.3) shows the sites for inhibition of nucleic acid synthesis by different antibiotics and drugs.

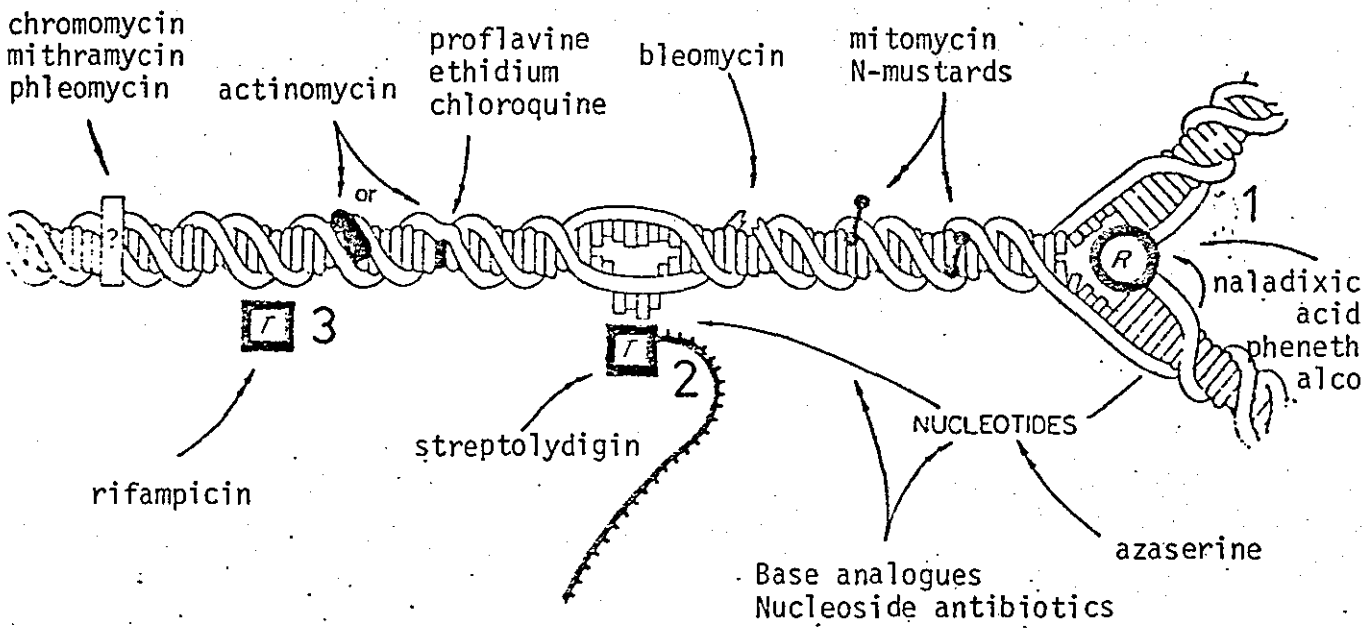

Fig. (1.3)

Sites For Inhibition Of Nucleic Acid Synthesis

By Drugs And Antibiotics*

The figure shows a DNA double helix, being replicated at (1) by replicating enzyme R, transcribed into RNA at (2) by RNA polymerase T, which is about to form an initiation complex with the DNA at 3, to start the synthesis of new RNA.

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* After Gale *et al* (1972)

4. THE RIBOSOMES

The main difference between ribosomes of prokaryotic cells and those of eukaryotic ones is that the former are smaller in size: the '80S' (S refers to the sedimentation coefficient) ribosomes of the eukaryotic cells, dissociate to give '60S' and '40S' fractions, while the '70S' prokaryotic ribosomes give '50S' and '30S' fractions. Thus, many antibacterial agents acting on the ribosomes show selective toxicity against the bacterial '70S' ribosomes; these antibacterial agents play a very important role in chemotherapy of bacterial infections.

Inhibitors of the smaller ribosomal subunit include the aminoglycosides (streptomycin, neomycin, kanamycin, gentamycin etc.), which bind to the '30S' subunit causing a misreading of the genetic message, thus inhibiting the peptide chain elongation (Orias and Gartner, 1966; Modelell and Davis, 1970). The tetrocyclines act on the 30S-moiety by binding aminoacyl-tRNA into the A site (Franklin, 1966; Cundliffe, 1972).

Inhibitors of the larger ribosomal unit include chloramphenicol, which inhibits peptide chain elongation by blocking the enzyme-substrate interaction in the '50S' moiety (Vazquez, 1966; Gale *et al*, 1972). The macrolide antibiotics (eg. erythromycin, oleandamycin and spiramycin) bind to the larger ribosomal subunit, inhibiting translocations (Cannon and Burns, 1971), as does lincomycin (Chang and Weisblum, 1967), fusidic acid (Modollel, Vasques and Monro, 1971) and thiostrepton (Weisblum and Demohn, 1970).

Fig. (1.4) shows the binding sites and the mode of action of some ribosome inhibitors.

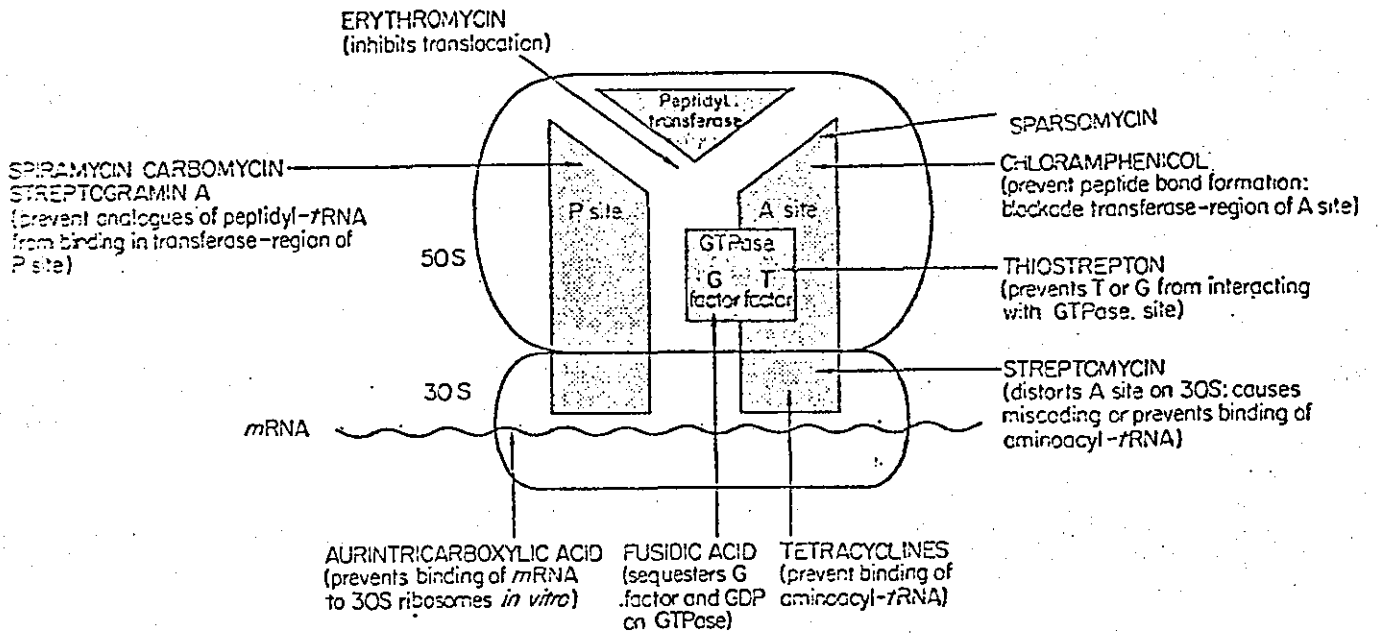

Fig. (1.4)

Modes Of Action Of Some Ribosomal Inhibitors*.

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* After Gale *et al* (1972).

Finally, some antibacterial agents act on the bacterial cell by competitively inhibiting some enzyme reactions. For example, the sulphonomides act on bacteria by competing with PAB, becoming incorporated in dihydropteroic acid and, therefore, retarding folic acid biosynthesis (Seydel, 1968; Franklin and Snow, 1975). Trimethoprim acts by binding to dihydrofolate reductase, thus interfering with folic acid biosynthesis as well (Franklin and Snow, 1975).

SECTION TWO

THE IMPORTANCE OF METAL IONS IN THE BACTERIAL CELL

Metal ions are essential components of all living cells, including bacteria. The importance of metals is realized more and more as further work is carried out on cell biochemistry.

Of the relatively light (Group I and II) metal ions that are abundant in the bacterial cell, sodium, potassium, calcium and magnesium, are the most important. Ca(II) is most abundant in bacterial membranes, and functions in controlling the permeability of semi-permeable membranes and maintaining their integrity (Albert, 1973). Mg(II) is the most abundant divalent ion in bacteria, and has several functions, for example, acting as a co-factor for many enzymes, keeping ribosome component parts together, and helping to attach the m-RNA to the ribosomes (Davis *et al*, 1970; Albert, 1973).

Many heavy transition metals such as cobalt, copper, iron, manganese, nickel and zinc, are essential to the bacterial cell in trace amounts. They can act as cofactors for enzymes and enzyme systems; for example, Co(II) is a cofactor for carboxypeptidase, Mo(II) is essential for xanthine oxidase, aldehyde oxidase, nitrate reductase and introgenase, Mn(II) is essential for the activity of oxaloacetic decarboxylase, arginase and prolidase, while Fe(II) and Fe(III) are a vital constituent of porphorin enzymes, catalase, peroxidase and various cytochromes and is absolutely required by

bacteria (Albert, 1973). In fact, of the 840 enzymes known in 1964, 27% had metals built in their structure, required metals for activity, or were further activated by metal ions (Boyer, Lardy and Myrback, 1964).

Although metals can activate enzyme reactions (O'Dell and Campbell, 1971; Worwood, 1974), they can, nevertheless, deactivate or retard some enzyme reactions (Milstein, 1961; O'Sullivan and Morrison, 1963).

1. METALLOENZYMES

A metalloenzyme is that enzyme which retains stoichiometric tightly bound, functional metal ions upon purification, in contrast to metal activated enzymes which require the addition of metal ions for activity (Boyer, Lardy and Myrback, 1964).

Table (1.1) lists some known metalloenzymes.

Table (1.1)

Some Known Metalloenzymes

Enzyme	Metal	Reference
Aldolase	Zn	Albert (1973)
Alkaline phosphatase	Zn	
Carbonic anhydrase	Zn	
Carboxypeptidase	Zn	
Carboxytransphosphorylase	Co	
Glycol dehydrase	Co	
Cytochrome oxidase	Cu, Fe	
Phenol oxidases	Cu	
Cytochrome C	Fe	
Pyruvate oxidase	Mn	
ATPase	Mg or Ca	
Alcoholdehydrogenase	Zn	Albert (1973); Boiwe and Branden (1977).
Dipeptidase	Zn	Hayman, Gatmitan and Patterson (1974).
Nucleotide pyrophosphatase	Zn	Twu, Haroz and Brethhauer (1977).
Cysteamine oxygenase	Fe	Cavallini *et al*, (1969).
Succinate dehydrogenase	Fe	Warringa, Smith, Giuditta and Singer (1958); Warringa and Giuditta (1958); Singer (1965).
Malate dehydrogenase	Zn	Vallee, Hock, Adelstein and Wacker (1956); Kun (1963).
Lactate dehydrogenase	Zn	Vallee and Wacker (1956); Boeri and Tosi (1956); Vestling, Hsieh, Teramaya and Baptist (1963); Everse and Kaplan (1973).

2. CYTOCHROMES

Cytochromes are defined as haem proteins whose principle biological function is electron and/or hydrogen transport by virtue of a reversible valency change of their haem iron (Lemberg and Barrett, 1973). Fig. (1.5) shows the haem prosthetic group structure of cytochrome a.

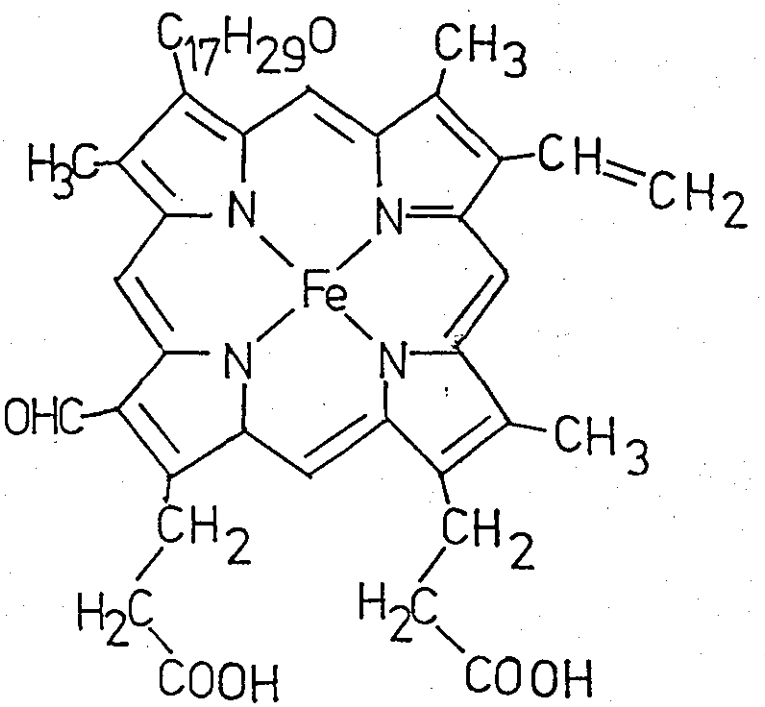


Fig. (1.5)

Structure of Cytohaem a.

The pattern of arrangement of the cytochrome chain seen in the mitochondria (Fig. 1.6), was found to be very similar, though not identical, to the bacterial chain found on the membrane (Rothfield, Weiser and Endo, 1969).

These cytochromes (of the inner mitochandrial membrane) were separated - physically and conceptually - into four functional systems as shown in Fig. (1.7).

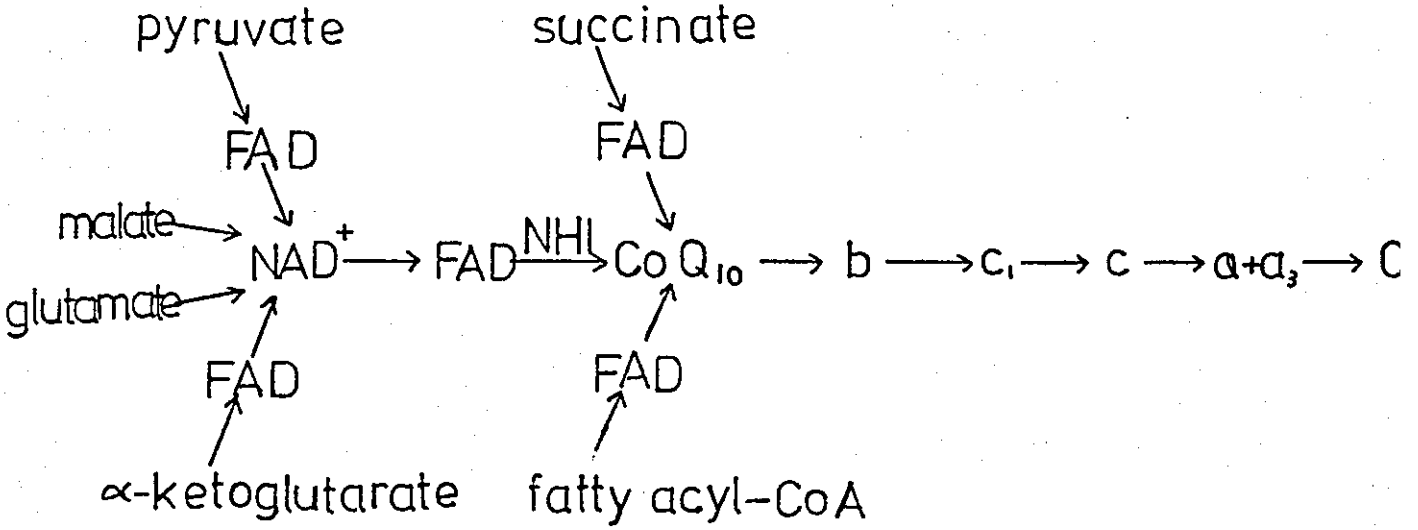


Fig. (1.6)

Schematic representation Of The Respiratory Chain

NAD = nicotinamide adenine dinucleotide;
FAD = flavin adenine dinucleotide;
NHI = non-haem iron.

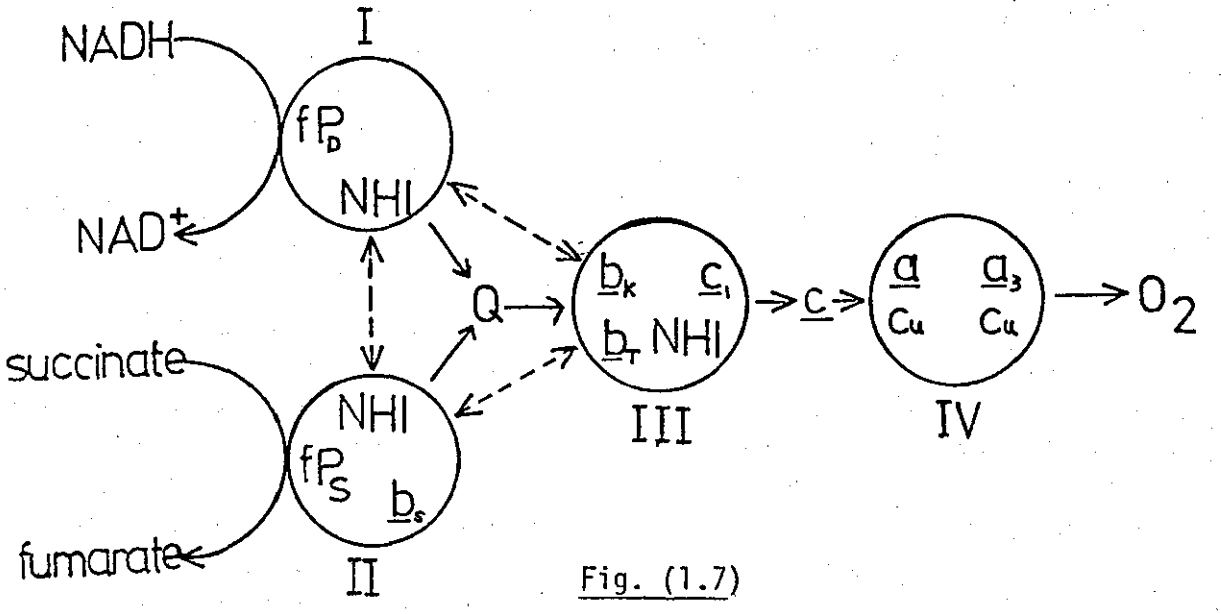


Fig. (1.7)

The Four Complexes Of The Cytochrome Chain*

Solid arrows represent major pathways; broken arrows represent minor pathways; fP = flavoprotein.

* After Hatefi, Haavik, Fowler and Griffiths (1962); Tzagoloff, Maclennan, McConnell and Green, (1967).

A comparison of the functional organization of the mitochondrial and the bacterial electron transport chain is shown in Fig. (1.8).

3. NON-HAEM IRON PROTEINS

Until recently the haem iron proteins were considered to be all-important in the electron transport. Now, however, non-haem iron (NHI) proteins are known to have a very wide range of functions, such as steroid hydroxylation, CO_2 and N_2 fixation, oxidative phosphorylation and photosynthesis.

Evidence for the participation of non-haem iron proteins as functional components in the electron transport chain has been firmly established (Phelps, Harmon and Crane, 1974; Sun, Phelps and Crane, 1975). NHI proteins have been isolated from anaerobic bacteria, aerobic bacteria, algae, plants and all animals so far investigated (Hall, Cammack and Rao, 1974).

NHI proteins may be classified into two main categories. The first, being the Iron-Sulphur (Fe-S) protein, includes ferrodoxins, which have equimolar amounts of iron and sulphide and negative redox potentials (eg. *Clostridium* ferrodoxins); high potential iron protein (HIPIP), which have equimolar amounts of iron and sulphide, but a positive redox potential (eg. *Chromatium* HIPIP); Fe-S flavoproteins, which contain flavins in addition to equal amounts of Fe and S (eg. succinate dehydrogenase); and rubredoxins, which contain only one Fe atom bound to cysteinyl sulphurs (eg. rubredoxins from *Clostridium*). The second NHI protein category includes those proteins that contain iron but is not bound to sulphur (eg. phenylalanine hydroxylase and sidrochromes).

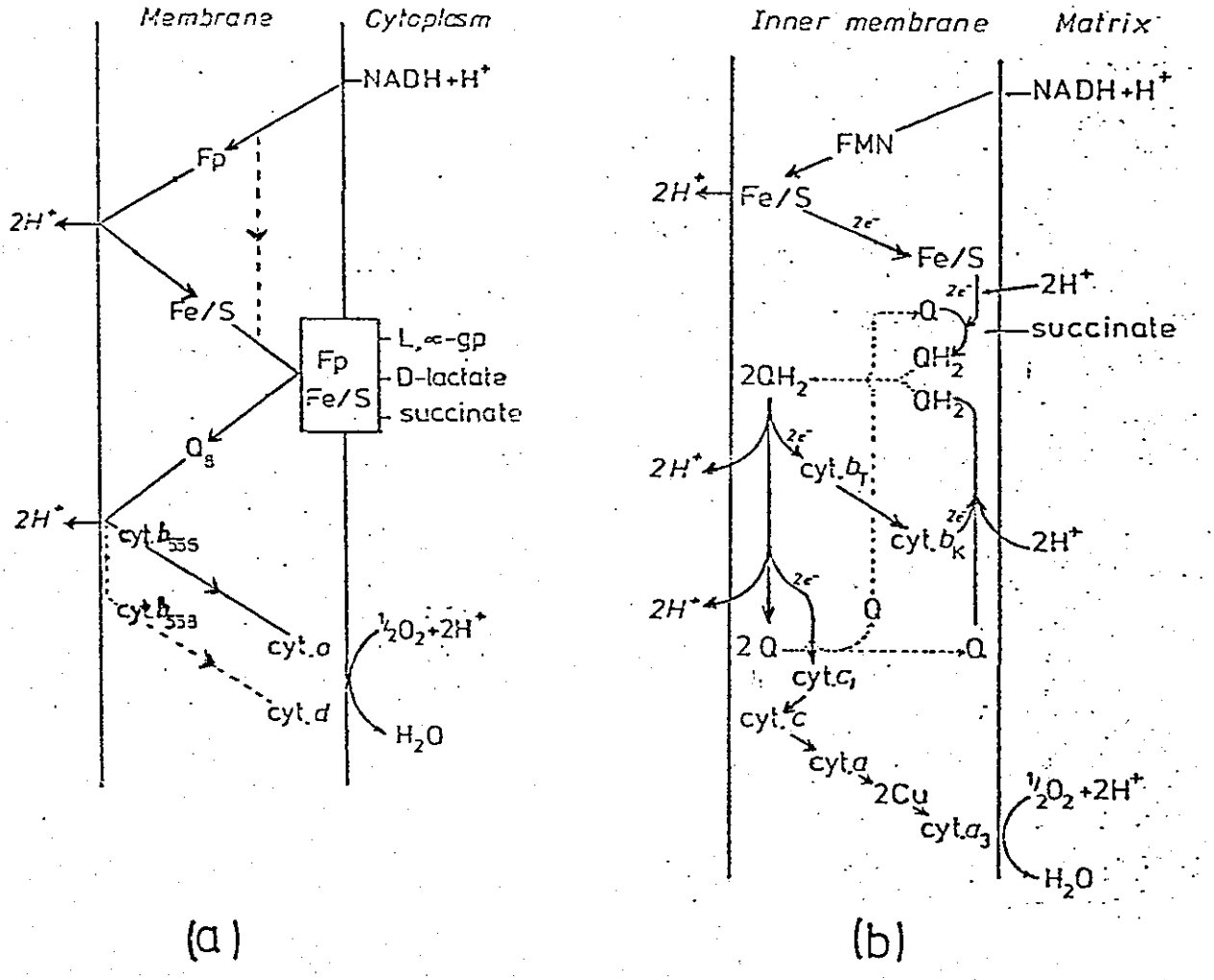


Fig. (1.8)

Proposed Functional Organization Of The Electron Transport Chains*

a) bacterial aerobic (*Escherichia coli*) chain;
b) mitochandrial chain;

FMN = Flavoprotein; Fe/s = iron/sulphur protein;
Q = Ubiquinone; QH_2 = ubiquinol;
Cyt = Cytochrome; Cu = copper-containing redox proteins;
L,∝-gp = L-∝-glycerophosphate

Dashed lines indicate alternative pathways.

* After Haddock and Jones (1977).

A comprehensive review of NHI protein is presented by Hall, Cammack and Rao (1974).

S E C T I O N T H R E E

METAL CHELATORS AND ANTIMICROBIAL ACTIVITY

1. GENERAL APPROACH

1.1 The Process of Chelation

When a metal is held between any two of the elements nitrogen, oxygen or sulphur (that are constituents of the same molecule), a chelate ring is formed (Fig. 1.9), and the metal is more tightly bound than if it was not part of a ring (Albert, 1961).

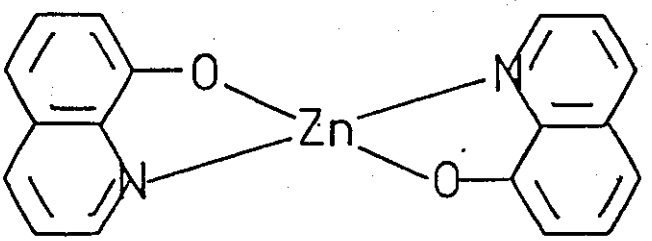


Fig. (1.9)

The Chelation Of The Zinc Atom By Two 8-Hydroxyquinoline Molecules

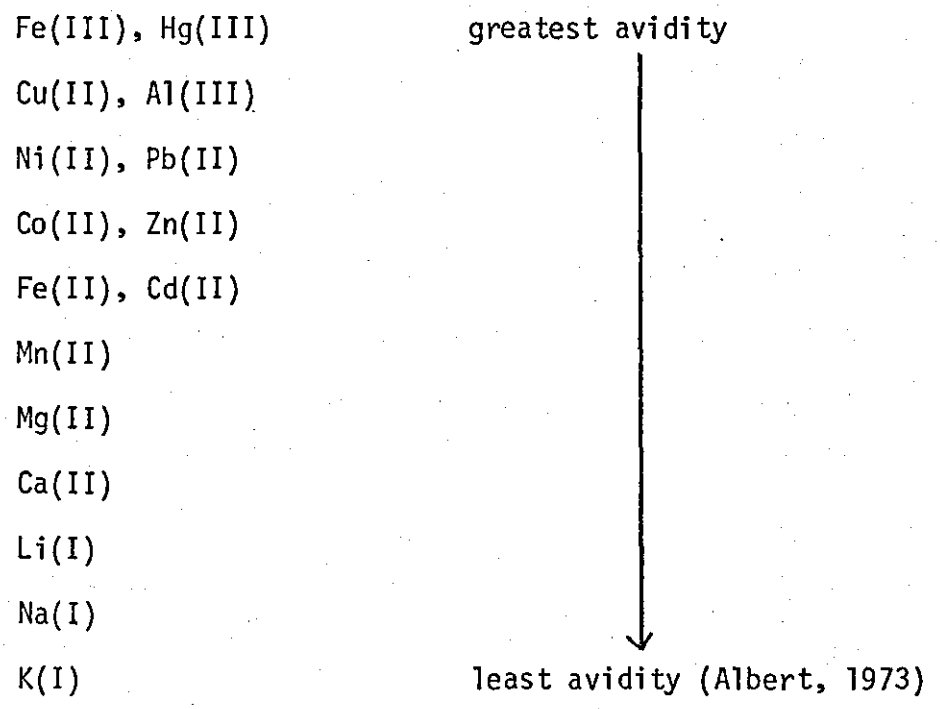
Thus, the process of chelation is defined as the process of forming a metal complex, in which one or more chelate rings are present (Mellor, 1964). In general, however, chelation through oxygen or nitrogen takes place only when 5- or 6-membered rings can be formed.

The 5-membered rings are more stable (Albert, 1961).

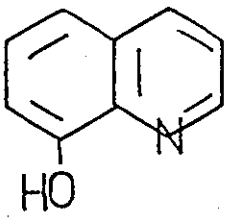
A chelating agent can be bidentate (that is having only two "claws" to hold the metal atom with, eg. 8-hydroxyquinoline and 1,10-phenanthroline tridentate, eg. triethylenetriamine; quadridentate, eg. nitrilotriacetic acid; quinquidentate, eg. tetraethylenepentamine; or even sexadentate, eg. ethylenediaminetetracetic acid (Fig. 1.10; Douglas and McDaniel, 1965).

1.2 Stability of Metal-Ligands Complexes

Chelating agents (ligands) show the following order of preference for metals:



This order is by no means rigid, for many ligands raise (or lower) one or more of these metals up (or down) the avidity scale due to individual and specific considerations (Albert, 1958).



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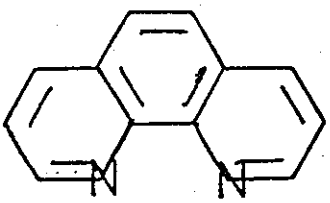

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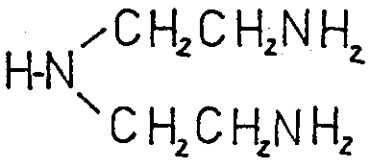
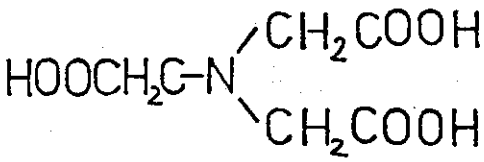


(c)



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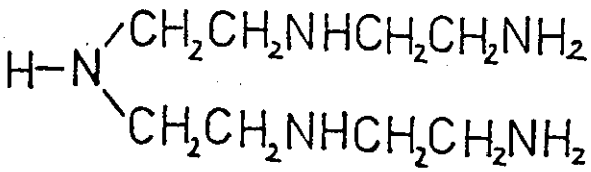

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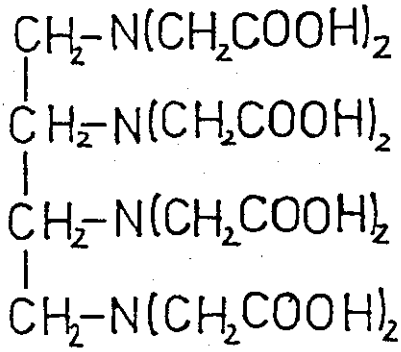


(f)

Fig. (1.10)

The Structures Of Some Chelating Agents

a) 8-hydroxyquinoline;
b) 1,10-phenanthroline;
c) diethylenetriamine;
d) nitrilotriacetic acid;
e) tetraethylenepentamine;
f) ethelyenediaminetetracetic acid.

Moreover, the chelated metal may considerably change the reactivity of the organic ligand by either changing its electronic distribution, masking its chemically active centre, forcing it into a particular stereochemical form, rendering it a conducting pathway for electron-addition or -removal and/or increasing its lipid solubility, hence helping it to penetrate into a living cell (Albert, 1973).

2. THE ANTIMICROBIAL MODES OF ACTION OF CHELATING AGENTS

Albert (1961) suggested that a metal chelator can exert its antimicrobial effect by one of two modes of action: either by immobilizing a metal, or in conjunction with a metal. In the former case, the chelating agent might be either chelating an important trace element, thus causing a deficiency in this element (Zentymer, 1944), or chelating a metal atom that is part of a metal-containing system (for example, a metalloenzyme or a non-haem iron protein), thus rendering this system inactive (Albert, Rubbo, Goldacre and Balfour, 1947; Rubbo, Albert and Gibson, 1950). In the latter case, however, the chelating agent might not necessarily damage the microbial cell into which it enters, but in the presence of a metal ion, the complex formed destroys the microorganism very quickly (Albert, 1961), as is the case with 8-hydroxyquinoline (oxine).

2.1 The Mode of Action of Ethylenediaminetetracetic Acid (EDTA)

Among many chelating agents investigated, EDTA and other related compounds (such as cyclohexan-1,2-diametetracetic acid (CDTA), N-hydroxyethylethylenediaminetriacetic acid (HDTA) and nitrilotriacetic

acid (NTA)) were found to be bactericidal to *Pseudomonas aeruginosa* and other Gram-negative species (Gray and Wilkinson, 1965; Asbell and Eagon, 1966; Roberts, Gray and Wilkinson, 1970; Haque and Russell, 1974 (a); 1976). Pretreatment of Gram-negative bacterial cells with EDTA was found to increase its subsequent sensitivity to several antimicrobial agents (Leive, 1965; 1968; 1974; Haque and Russell, 1974 (b); 1976).

The mode of action of EDTA and related compounds, has been investigated by many workers. Gray and Wilkinson (1965) and Asbell and Eagon (1966) suggested that EDTA probably extracted a lipopolysaccharide from the cell. Leive (1974) proposed that its mode of action depended on its binding to Ca(II) and Mg(II) in the cell envelope; and that the loss of these cations was followed by another change, such as loss of or alteration of material in the envelope.

Recently, Haque and Russell (1976) observed that EDTA and related compounds removed greater amounts of Mg(II) than of Ca(II), and that the most sensitive *Ps. aeruginosa* strains to EDTA were those containing the greatest amounts of Mg(II).

2.2 The Mode of Action of Oxine

Oxine is an antimicrobial with a wide spectrum of activity. It is especially active against the Gram-positive bacteria (Albert, Rubbo, Goldacre and Balfour, 1947; Gale 1949; Rubbo, Albert and Gibson, 1950; Albert, Gibson and Rubbo, 1953), and fungi (Zentmyer, 1944; Anderson and Swaby, 1951).

This antimicrobial activity was attributed to the ability of oxine to chelate (Zentmyer, 1944; Albert, Rubbo, Goldacre and Balfour, 1947). Zentmyer based his argument on the fact that inhibition caused

by oxine was removed when zinc ions were added in excess. He attributed this antimicrobial activity to the chelation of some important trace element needed by the cell causing a trace element deficiency.

Albert and co-workers (Albert, Rubbo, Goldacre and Balfour, 1947; Albert, Gibson and Rubbo, 1953) showed that while oxine had little activity against Gram-positive bacteria in media depleted of heavy elements, be it broth or distilled water, its action was enhanced by the presence of Cu(II) and Fe(II), and to a lesser extent, Mg(II), Mn(II), Co(II), Zn(II), Ca(II) and Ni(II). Thus, it was suggested that the presence of a metal ion (especially Fe(II) or Cu(II)) was essential for the activity of oxine against Gram-positive organisms.

The concentration of these metal ions, relative to that of oxine, was found to be critical; that is, oxine in excess, relative to the metal ion, reduced the bactericidal rate in broth (causing what was referred to as concentration quenching), and so did the metal ion in excess (Albert, Gibson and Rubbo, 1953). This lead many workers (Albert, Hampton, Selbie and Simon, 1954; Block, 1956; Albert, 1973) to postulate that its mode of action was inside the cell, and that the 1:1 or 2:1 oxine-metal ion complexes (Fig. 1.11) were the actual toxic substance, probably due to their unsaturation. These complexes could not penetrate into the cell probably because they were ionized and hence not lipophilic. However, oxine entered the cell as such (Beckett, Vahora, and Robinson, 1958), as well as the 3:1 complex, which was lipophilic, but non-toxic, because of saturation; and therefore, in the absence of excess oxine inside the cell, the 3:1 complex dissociated to give the 2:1 or the 1:1

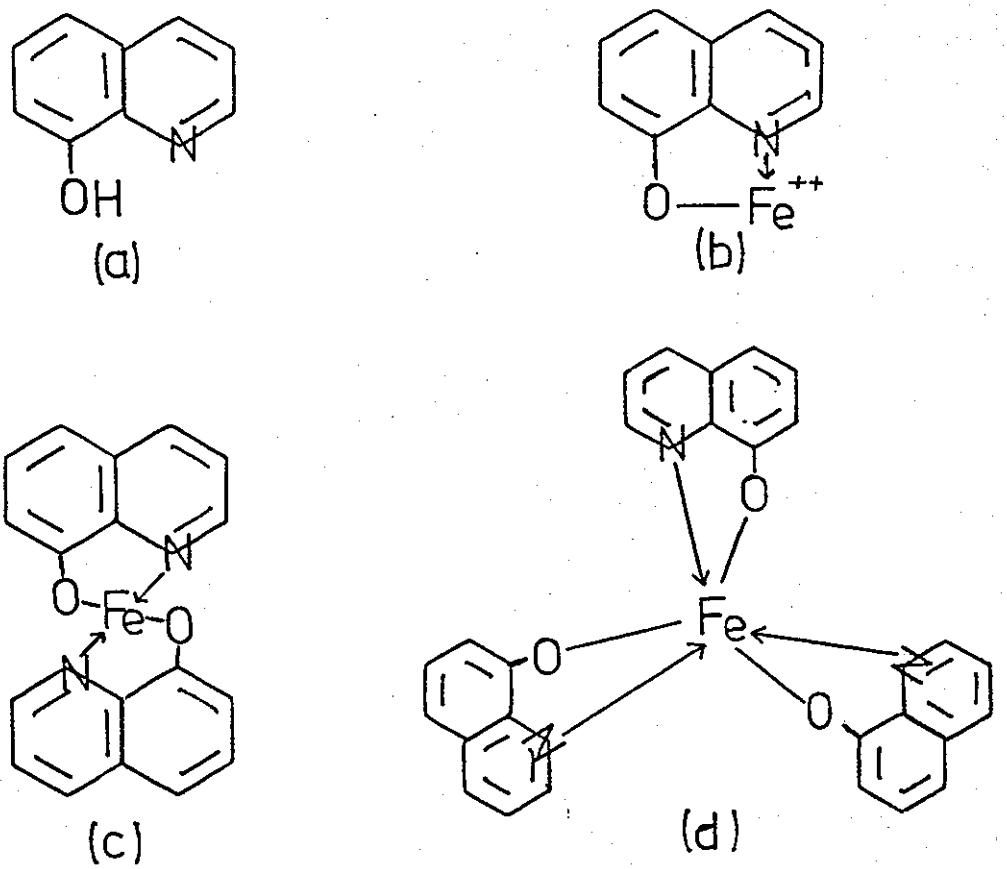


Fig. (1.11)

Structure Of Oxine And Its Iron Complexes

a) oxine; b) 1:1 complex; c) 2:1 complex; d) 3:1 complex.

complexes and death resulted. On the other hand and in the presence of excess oxine, rapid killing did not occur because the 3:1 complex persisted in the cell, and was harmless. In the presence of excess metal ions, rapid death did not occur, because the 3:1 complex could not be formed according to the law of mass action.

Only one transition metal ion, Co(II), antagonised the action of oxine against the Gram-positive bacteria (Rubbo, Albert and Gibson, 1950), yeasts (Nordbring-Hertz, 1955), and trypanosomes (Williamson, 1959). An explanation to this antagonistic effect of Co(II) was attempted by Albert (1973), who took into account the fact that several vital cell constituents, particularly mercapto-compounds

such as lipoic acid and ascorbic acid, were easily oxidized by atmospheric oxygen if traces of iron or copper were present. These oxidations lead to the formation of hydrogen peroxide, which in turn oxidised more substrate, and the combination of metal and hydrogen peroxide produced a fulminating chain reaction. Thus, a small amount of metal could catalyze a widespread destruction. Accordingly, Albert suggested that in some reactions of this kind, traces of cobalt had been found to act as an efficient chain breaker, greatly moderating the destruction of the bacterial cell.

The above described mode of action of oxine was applicable in the case of Gram-positive, but not Gram-negative bacteria. In the case of Gram-negative organisms, against which oxine - in general - had a weak antibacterial action, the picture was different, for no such metallotoxic effect could be demonstrated. The action of oxine on *Escherichia coli* was even antagonized by the presence of Zn(II), Fe(II), Cu(II), Ni(II) and (Mn(II), and death seemed to be entirely due to metal deprivation (Rubbo, Albert and Gibson, 1950).

2.3 Studies on the Mode of Action of 1,10-Phenanthroline

1,10-phenathroline (and related compounds) was found to be not only active against bacteria (McNaught and Owen, 1949; Turrian, 1951; Feeney, Petersen and Sahinkaya, 1957; Dwyer *et al*, 1969; Tochikuto, 1974; Tuszkiewicz, Pleszczynska, Mlochowski and Skrowaczewska, 1975; Berger, Johnson and Skinner, 1975), but also exhibited antifungal (Zsolnai, 1961; Cade, Cohen and Shulman, 1970), anthelminthic (Baldwin, 1948) and even antiviral activity (White, Harris and Shulman, 1963; Shulman and Dwyer, 1964; Shulman and White, 1973).

There is much confusion whether the mode of action of 1,10-phenanthroline is due to it or to one of its metal complexes. Some workers (Zsolnai, 1961; Albert, 1973; Berger, Johnson and Skinner, 1975) suggested that the mechanism or action of 1,10-phenanthroline, in contrast to that of oxine, was simply by means of intercellular chelation of the heavy metal components in certain enzymes of the cell, leading to its inactivation and preventing its multiplication. On the other hand, other workers (Dwyer *et al*, 1960; Cade, Cohen and Shulman, 1970) suggested that the active species was the 1,10-phenanthroline-metal ion complex; while others (McNaught and Owen, 1949; Turrian, 1951; Feeney, Petersen and Sahinkaya, 1958; Butler, Hurse, Thursky and Shulman, 1969) reported the activity of both the 1,10-phenanthroline and its metal complexes

3. MEDICAL USES OF CHELATING AGENTS

Many of the biologically active chelating agents are unsuited for internal therapeutic use, because they are not selective enough and therefore quite toxic to the host cells (Beccari, 1941; Dwyer *et al*, 1969; Albert, 1973). For example, 1,10-phenanthroline-Rh(II) complex demonstrated signs of acute toxicity by producing muscular paralysis (Dwyer, Gyarfas, Rogers and Koch, 1952), while oxine injured the islets of Langerhans in experimental animals (Kadota and Abe, 1954).

Recently, iodochlorohydroxyquinoline was found to be closely associated with a neurological syndrome known as SMON (Subacute Myelo-Optico Neuropathy). Its clinical symptoms include gait disturbance, paraesthesiae in both legs, axonal damage and

demylination of the optic nerve, lateral and posterior columns of the spinal cord and peripheral nerves, leading to visual disturbances, abnormality of deep tendon reflexes and psychic disorders (Tsubaki, Honma and Hoshi, 1971).

Nevertheless, metal chelators were successfully used in clinical and veterinary practice for the treatment of a variety of topical infections, caused by pathogenic bacteria and fungi, such as wound, dermatological, vaginal, ear, nose and eye infections (Shulman and Dwyer, 1964; Marples, 1971; Frier, 1971).

There are many other antibiotics and antibacterial agents, commonly used in medicine, that have the ability to chelate metal ions. The tetracyclines, isoniazid, streptomycin and thiacetozone (Fig. 1.2) were found to be chelating agents with great avidity for Fe(III) and Al(III) (Albert and Rees, 1956). All are used in the treatment of tuberculosis. Other antibacterial agents used in medicine, that are known to chelate are p-aminosalicylic acid (Albert, 1961), bacitracin (Adler and Snoke, 1962), antimycin (Farley, Strong and Bydalek, 1965), and novobiocin (Franklin and Snow, 1975).

Fig. (1.12) shows the structure of these compounds.

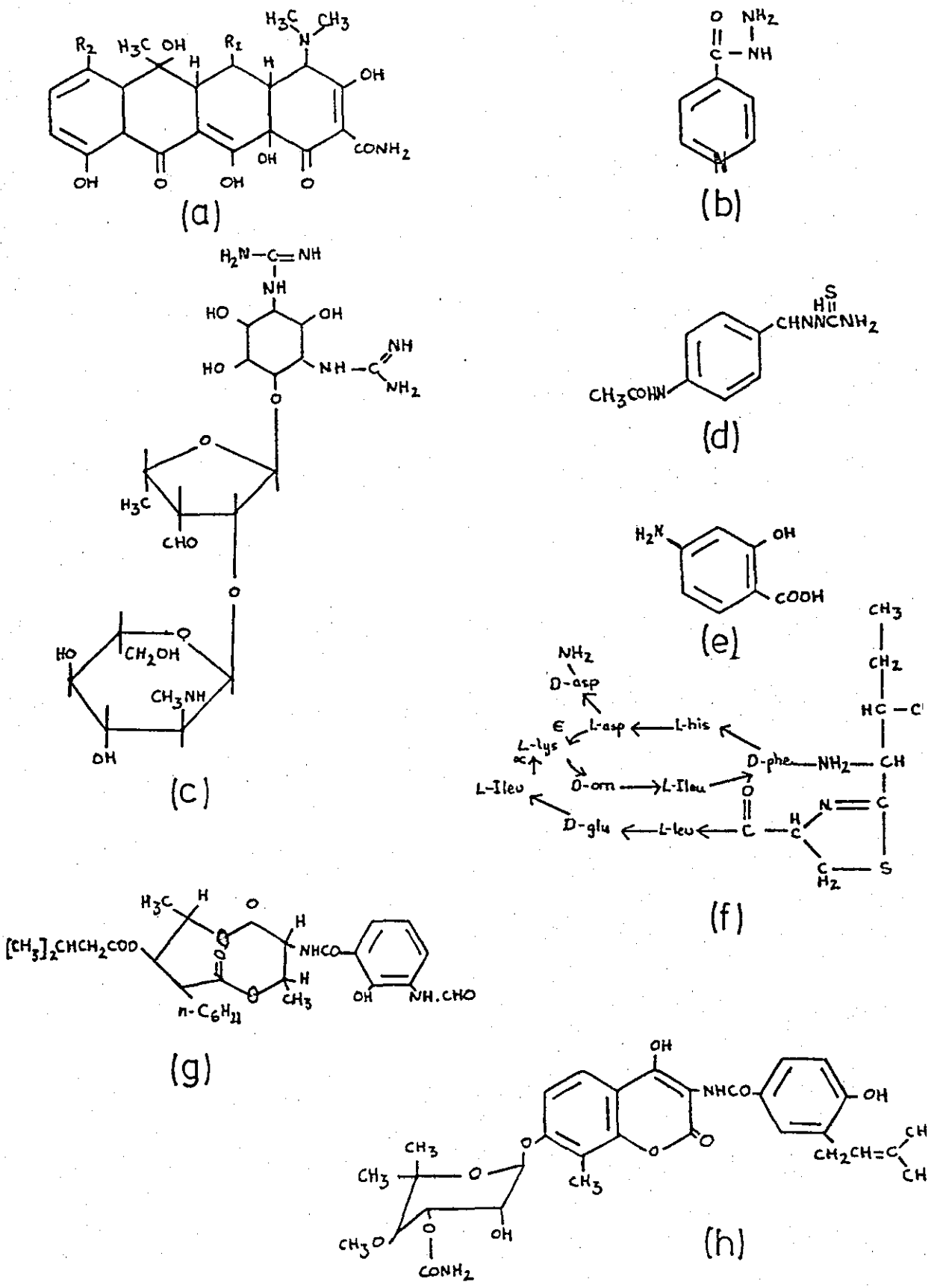


Fig. (1.12)

Structures Of Antibacterial Agents That Chelate

a) tetracyline; b) isoniazid; c) streptomycin; d) thiacetozone; e) p-aminosalicylic acid; f) bacitracin; g) antimycin; h) novobiocin.

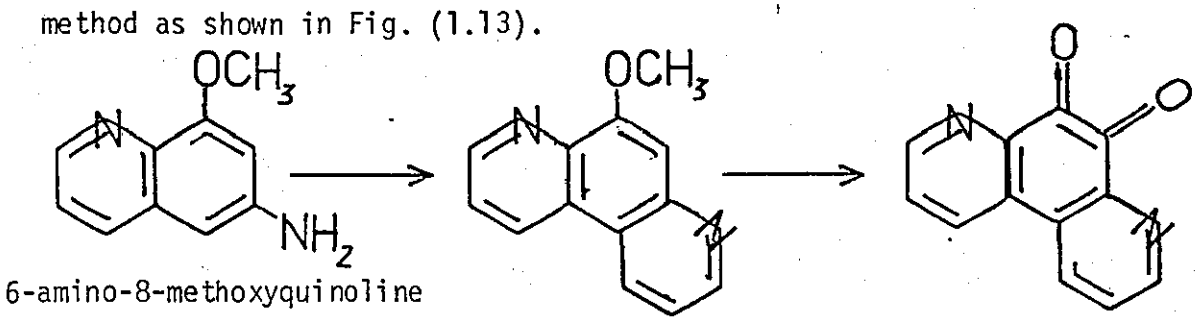
SECTION FOUR

THE ANTIMICROBIAL ACTIVITY OF PHANQUONE

1. THE DISCOVERY AND ASSESSMENT OF PHANQUONE AS AN ANTIMICROBIAL AGENT

Phanquone (4,7-phenanthroline-5,6-quinone; Fig. 1.13) is an orange, odourless, almost tasteless crystaline powder, slightly soluble in water, and almost insoluble in alchohol and chloroform (British Pharmacoepia, 1963; British Pharmaceutical Codex, 1963).

It was synthesized by Druey and Schmidt (1950) using the Skraup's method as shown in Fig. (1.13).



6-amino-8-methoxyquinoline

5-methoxy-4,7-phenanthroline

Phanquone

Fig. (1.13)

The Synthesis Of Phanquone Using The Skraup's Method

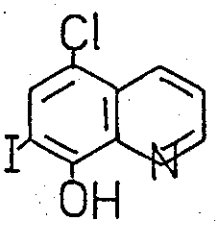
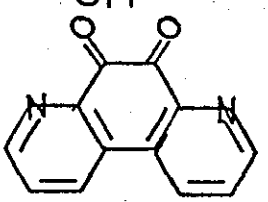
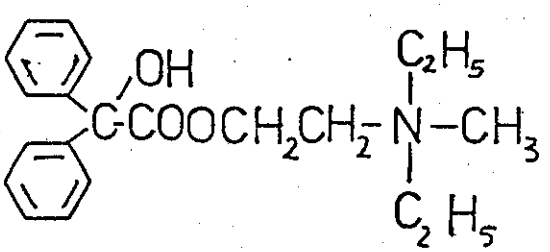
The distinctive amoebicidal effect of Phanquone *in vitro* and *in vivo* (man and rats) has been reported by Schmidt and Druey (1957). This antiamoebic activity, together with the antibacterial activity was further investigated by Kradolfer and Neipp (1958). They concluded that Phanquone was superior against *Entamoeba histolytia* to many other amoebicidal agents, had definite antibacterial activity against *Escherichia coli, Staphylococcus aureus, Streptococcus faecalis, Salmonella typhi, Shigella sonnei* and *Strep. hemolyticus*

and exhibited very little mammalian toxicty (the ratio of effective to toxic dose was thirty times more favourable than that of emetine hydrochloride). However, they concluded that its mode of action was "difficult to define because of its wide range of effect".

Soon after, Phanquone was marketed by Ciba-Geigy (Basle, Switzerland), under the trade name of Entobex. It was also marketed as a component of Mexaform (composition and structure of components shown in Table 1.2).

Table 1.2

COMPOSITION OF MEXAFORM*

Component	Composition	Structure
Vioform	90.09%	
Entobex	9.01%	
Antrenyl (antispasmodic)	0.90%	

* Eisman, Weerts, Jaconia and Barkulis (1960).

2. FURTHER STUDIES ON PHANQUONE

2.1 Pharmacology and Toxicity

Phanquone was absorbed from the intestines and excreted in an active form in the bile and urine (Kradolfer and Neipp, 1958). The total amount of unchanged Phanquone excreted in the human urine between 8 and 24 hours was 12.5% and 11.2% of the dose, while the amount in plasma after a 100 mg dose dropped from 850 µg/ml after one hour, to 200 µg/ml after 24 hours (Degen, Breckbuhler, Schaublin and Riess (1976).

Although nausea, vomiting and transient dizziness could occur (British Pharmaceutical Codex, 1963; Extra Pharmacoepia, 1972), Phanquone was well tolerated by patients (Carter, 1961), and had a LD_{50} of 3 g/kg for rats (Clarke, 1969). Mexaform was found to be well tolerated as well (Konzert, 1971; Madanogopalan and Rao, 1975).

2.2 Stimulation of Coliform Bacteria in the Intestines of Rats

Phanquone was found to increase the number of coliform bacteria in the intestines of rats (Kradolfer and Neipp, 1958), following a short period of reduced bacterial counts (Kradolfer, Sackmann and Bassil, 1960; Sackmann and Kradolfer, 1961). Mexaform also caused a definite increase of bacterial numbers in the intestinal tract, with a simultaneous significant decrease in the yeast population (Eisman, Weerts, Jaconia and Barkulis, 1960).

This unique finding (the stimulation of the intestinal flora) was assumed to be of special importance in the therapeutic action of such compounds, that should be clarified (Kradolfer and Neipp, 1958).

Eisman, Weerts, Jaconia and Barkulis (1960) proposed that this stimulation played an important role in suppressing the growth of various pathogenic microorganisms, including *C. albicans*. Sackmann and Kradolfer (1961) attributed these changes in faecal counts to the destruction of sensitive bacteria and the subsequent development of strains resistant to the action of both Phanquone and Mexaform.

2.3 *In Vitro* and *In Vivo* Studies

Phanquone was found to be bactericidal to *Proteus mirabilis*, *E. coli* and *S. aureus*, (Bailenger, 1961) as well as *P. vulgaris* and *Ps. auruginose* (Sackmann and Kradolfer, 1961). It was also found to be amoebicidal to *Entamoeba invadens* (Kaushiva, 1964; Krishna Prasad, 1972), *E. moshkoushii*, *Naegleria aerobia*, *Tetramitus rostratus*, *Didascalus thortoni* (Krishna Prasad, 1972) in addition to *E. histolytica* (Kaushiva, 1964; Krishna Prasad, 1972; Yadava and Dutta, 1973), antifungal towards *Candida albicans* (Ansel and Thibaut, 1967), and antiprotozoal towards flagellates, ciliates and trematodes (Kradolfer, Sackmann and Bassil, 1960). Moreover, Phanquone was found to inhibit the volatile fatty acids (VFA) production, and to reduce protozoal activity in an *in vitro* rumen fermentation system (O'Conner, Myers, Maplesdan and Noot, 1970).

Mexaform was found to be slightly more active than Phanquone against bacteria, but no synergism was observed between its two antimicrobial components (Sackmann and Kradolfer, 1961)

Carter (1961) reported its effectiveness in treating human patients suffering from an *E. histolytica* infection; Phanquone was found to be active against *E. histolytica* infecting rats, during which *Bacillus subtilis* became the main intestinal microorganism.

Yasutake (1961) used Phanquone to completely eliminate the parasitic infection due to *Hexamita salmonis* in Salmonoid fishes. The *in vivo* activity of Mexaform was also studied. Carter (1960) reported the effectiveness of Mexaform in the treatment of non-specific enteritis; Sackmann and Kradolfer (1962) found that its simultaneous administration inhibited the changes produced by tetracycline in the intestinal flora of rats; while Catteneo, Lucchelli, Bona and Maccacaro (1966) showed that it reduced abdominal symptoms which followed surgery of the biliary tract. Also, Mexaform was used in the prophylaxis of infectious diarrhoeas (Villaregos, Rodriguez-Aragones, Nickle and Duron, 1971), in all forms of dyspepsia of babies (Konzert, 1971) and in the treatment of non-parasitic diarrhoeas (Madanogoplan and Rao, 1975).

3. THE AIM OF THE PROJECT

The aim of this project is to study the mechanism(s) by which Phanquone causes damage and - consequently - death to the bacterial cell. The investigation (and elucidation) of its mode of action may help in the evaluation of this antimicrobial agent, thus broadening or restricting its practical applications.

PART II

MATERIALS AND METHODS

SECTION ONE

MATERIALS

1. PHANQUONE

Phanquone (structure shown in fig. 1.13) was supplied by Ciba Laboratories Limited, Horsham, Sussex. It was found to be almost insoluble in chloroform and ether, very slightly soluble in acetone and methanol, but slightly soluble in warm water. It was moderately soluble in dilute acids such as hydrochloric, sulphuric and acetic acids.

Throughout this project, Phanquone was either dissolved in warm water (up to 1 mg/ml) to give a brilliant yellow solution, or in 0.1N HCl (up to 2.5 mg/ml) to give almost a colourless solution.

The Infra Red (IR) and the Nuclear Magnetic Resonance (NMR) spectra for Phanquone were determined, and are shown in fig. 2.1.

2. RELATED COMPOUNDS

2.1 Phenanthrene - was purchased from Aldrich Chemical Company Limited, Gillingham, Dorset, and was dissolved in acetone (up to 2 mg/ml).

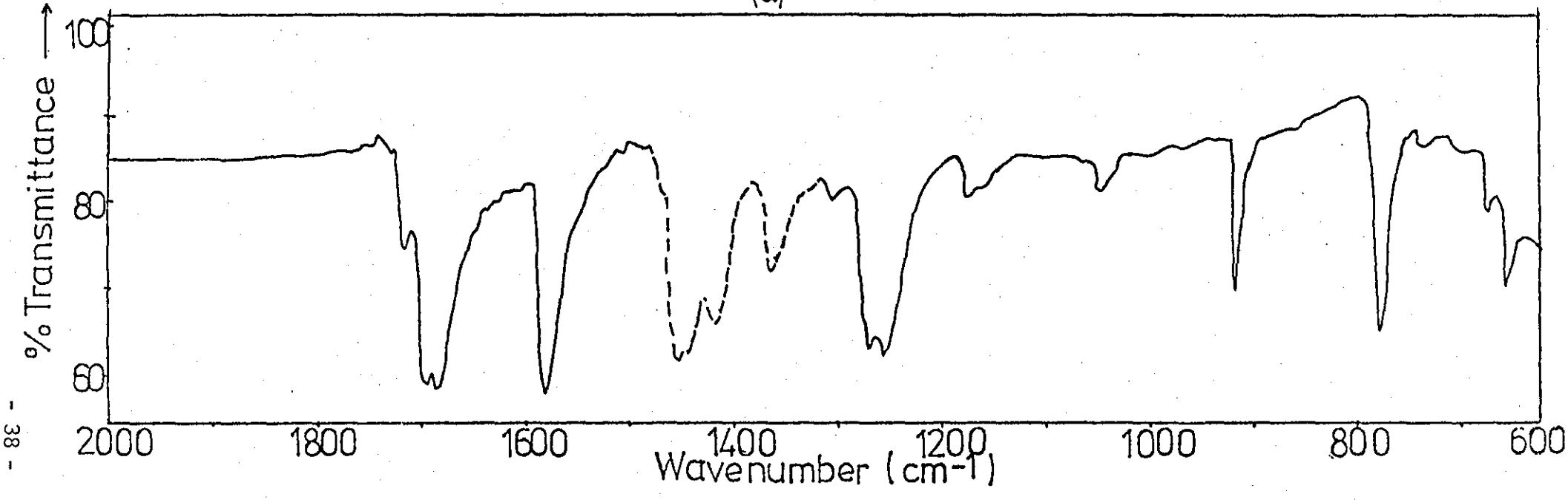
2.2 Phenanthridine - was purchased from Fluka A.G., Chemische Fabrik, Switzerland, and was dissolved in 50% v/v acetone/water (up to 2 mg/ml).

2.3 8-Hydroxyquinoline (hemisulphate salt) - was purchased from Sigma, St. Louis, U.S.A., and was readily soluble in water.

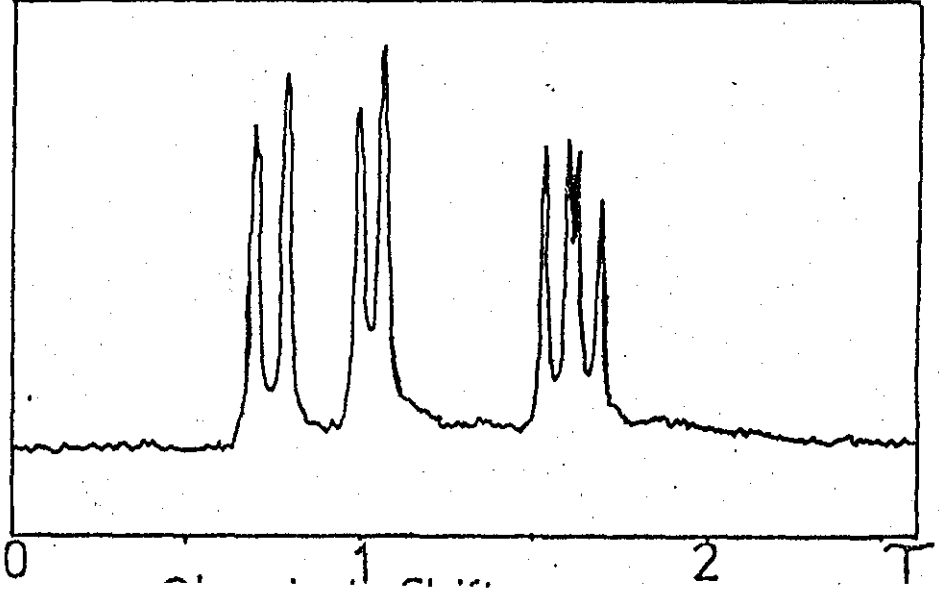
Fig. (2.1)

The Infra Red (IR) and the Nuclear Magnetic Resonance (NMR) Spectra for Phanquone.

(a)


% Transmittance
100
80
60
2000
1800
1600
1400
1200
1000
800
600
Wavenumber (cm-1)

(b)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2.4 4,7-Phenanthroline - was purchased from Kodak Limited, Liverpool, and was soluble in warm water (up to 2 mg/ml).

2.5 9,10-Phenanthrenequinone - was purchased from Aldrich Chemical Company Inc., U.S.A., and was dissolved in acetone (up to 2 mg/ml).

2.6 1,10-Phenanthroline (hydrochloride) - was purchased from Fluka A.G., Chemische Fabrik, Switzerland, and was readily soluble in water.

2.7 1,10-Phenanthroline-5,6-quinone - was purchased from ICN, K and K Labs., Inc., California, U.S.A., and was dissolved in 50% v/v acetone/water (up to 2 mg/ml).

Fig. 2.2 shows the structures of those related compounds.

3. RADIOACTIVE CHEMICALS

3.1 L-(U-C-14)-Glutamic acid - 50 µci Pll (aqueous solution containing 2% ethanol, sterile) - activity on 13/12/77 : > 250 µci/mmol.

3.2 L-(U-C-14)-Proline - 50 µci Pll (aqueous solution containing 2% ethanol, sterile) - activity on 1/2/78 : > 250 µci/mmol.

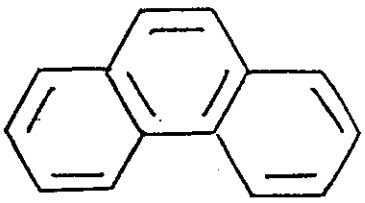
3.3 Phosphorous-32 - (orthophosphate in HCl solution, sterile, pH 2-3) - activity on 25/5/77: 1-9 Mci/ml.

The above radiochemicals were all purchased from the Radiochemical Centre Limited, Amersham.

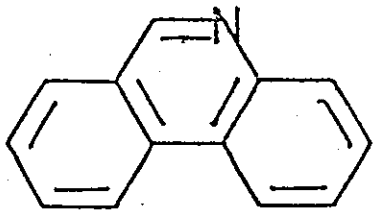
Fig. (2.2)

The structures of compounds related to Phanquone

a) phenanthrene; b) phenanthridine;
c) 8-hydroxyquinoline; d) 4,7-phenanthroline;
e) 9,10-phenanthrenequinone; f) 1,10- phenanthroline;
g) 1,10-phenanthroline-5,6-quinone.



(a)



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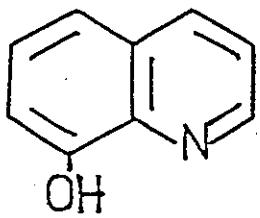

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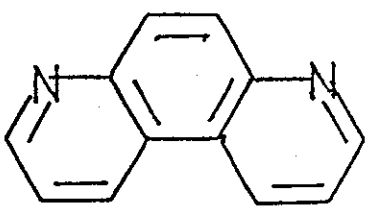

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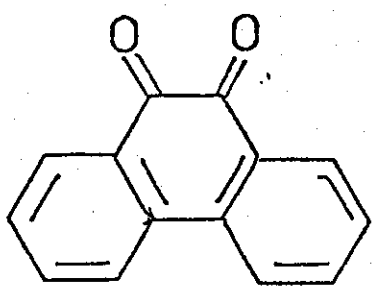
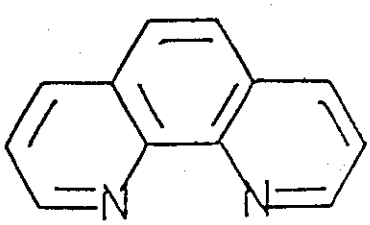
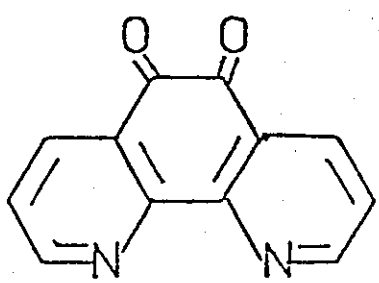


(e)



(f)



(g)

4. OTHER CHEMICALS

Adenosine Triphosphate-dipotassium salt (from equine muscle), D-Biotin, Glucose-6-Phosphate, Nicotinamide Adenine Dinucleotide Phosphate (sodium salt), and 20-Methylcholanthrene were all purchased from Sigma Limited, St. Louis, U.S.A.

Lysozyme (from egg white) was obtained from BHD Chemicals Limited, Poole, Dorset.

Octan-1-ol, SLR, was purchased from Fisons, Loughborough, Leicestershire.

1-Thioglycerol (90% solution) was purchased from Koch-Light Labs. Limited, Colnbrook, Buckinghamshire.

All other chemicals used throughout the project - unless otherwise stated - were of AR grade.

5. CULTURE MEDIA

5.1 Nutrient Broth:

	g/l
Bacteriological Peptone (Oxoid)	10.0
Lab Lemco (Oxoid)	10.0
NaCl	5.0

The pH was adjusted to 7.2-7.4, and the broth was sterilized by standard autoclaving (for 20 mins. at 121^{o}C and 15 psi pressure).

5.2 Nutrient Agar:

	g/l
Bacteriological Peptone (Oxoid)	10.0
Lab Lemco (Oxoid)	10.0
NaCl	5.0
Agar no.3 (Oxoid)	15.0

The pH was adjusted to 7.2-7.4, and the agar was sterilized by standard autoclaving.

5.3 Winkler-de Haan Medium (1948):

	per litre
KH_2PO_4	6.0 g
K_2HPO_4	6.0 g
NH_4Cl	2.0 g
$MgSO_4.7H_2O$	50 mg
$FeSO_4.7H_2O$	5 mg
D-Glucose	20.0 g

$FeSO_4.7H_2O$ was sterilised separately by membrane filtration, while D-glucose was separately sterilized by autoclaving at 115°C and 10 psi for 10 mins.. The pH of the medium was adjusted to 6.8-7.0.

5.4 Staphylococcal Medium (Stretton, 1965):

	per litre
Casamino acids (Difco)	2.00 g
D-Glucose	2.00 g
Yeast Extract (Oxoid)	1.00 g
K_2HPO_4	2.60 g
KH_2PO_4	2.00 g
$CaCl_2.6H_2O$	21.7 mg
$FeSO_4.7H_2O$	13.9 mg
$MnSO_4.7H_2O$	16.9 mg
$MgSO_4.7H_2O$	49.3 mg
$NaHCO_3$	1.70 g

Casamino acids and $FeSO_4.7H_2O$ were sterilized separately by filtration; while D-glucose was sterilized separately by autoclaving at 115°C and 10 psi for 10 mins..

5.5 Vogel-Bonner Medium E-Modified (Vogel and Bonne, 1956; Ames, McCann and Yamasaki, 1975):

	g/l
$MgSO_4.7H_2O$	0.2
Citric acid.H_2O	2.0
K_2HPO_4	10.0
$NaNH_4HPO_4.4H_2O$	3.5
D-Glucose	20.0
Agar no.3 (Oxoid)	15.0

Glucose was separately sterilized by autoclaving at 115°C and 10 psi for 10 mins..

6. BUFFERS

6.1 Phosphate-Buffered Saline (Dulbecco and Vogt, 1954):

	g/l
NaCl	8.00
KCl	0.20
KH_2PO_4	0.20
Na_2HPO_4	1.15

The pH was adjusted to 7.3, and the buffer was sterilized by autoclaving at 115°C and 10 psi for 10 mins..

6.2 Sodium Phosphate Buffer

0.1M solutions of NaH_2PO_4 and Na_2HPO_4 were prepared; then, depending on the pH value required, the two solutions were mixed until the specified pH was obtained.

6.3 Potassium Phosphate Buffer

The same procedure used for the preparation of the sodium phosphate buffer, was followed except that 0.1M solutions of KH_2PO_4 and K_2HPO_4 were used.

6.4 Tris-HCl Buffer (Bates and Bower, 1956):

	ml
0.1M Tris (hydroxymethyl)aminoethane	500
0.1N HCl	x
Distilled Water	500-x

x, the volume of the 0.1N HCl added, depended on the pH of the buffer required.

7. ORGANISMS

Escherichia coli NCTC 9001 and *Staphylococcus aureus* NCIB 8625 were used throughout the project, and will be referred to as *E.coli* and *S.aureus* respectively. Other organisms used in the project were: *Bacillus cereus* NCTC 11755, *Bacillus megaterium* NCIB 8291, *Bacillus subtilis* NCIB 3610, *Clostridium sporogenes* NCIB 532, *Escherichia coli* K12 NCIB 8797, *Escherichia coli* Texas NCIB 10097, *Klebsiella aerogenes* NCIB 8267, *Mycobacterium phlei* NCTC 8151, *Proteus vulgaris* NCIB 8067, *Pseudomonas aeruginosa* NCIB 8295, *Salmonella abony* NCTC 6017, *Salmonella abortus ovis* NCTC 10241, *Sarcina lutea* NCIB 196, *Staphylococcus aureus* Oxford NCTC 6571, and *Streptococcus faecalis* NCIB 6783.

All the organisms - unless otherwise stated - were maintained on nutrient agar slopes, and subcultured in nutrient broth whenever required, with the exception of *Cl. sporogenes*, which was maintained on Reinforced Clostridial Agar (Oxoid), and subcultured in Reinforced Clostridial Medium (Oxoid) whenever required.

S E C T I O N T W O

GENERAL PROCEDURES

1. STERILIZATION OF SOLUTIONS AND EQUIPMENT

All solutions were sterilized either by standard autoclaving (at 121^{o}C and 15 psi for 20 mins.)–unless otherwise stated, or by membrane filtration using Nuflow 0.45 µm Millipore filters.

In most cases, equipment was sterilized by standard autoclaving. In some instances, however, equipment was sterilized in the oven at 140^{o}C for 2 hrs..

2. PREPARATION AND STANDARDIZATION OF BACTERIAL SUSPENSIONS

Roux bottles, containing 150 ml of nutrient agar, were inoculated with a 6 hrs. old nutrient broth culture, and grown overnight at 37^{o}C. Cells were then washed off the agar with 0.1M sodium phosphate buffer, pH 7.4, centrifuged at 150xg (5 mins., 4^{o}C) to separate the cells from the washed-off agar, harvested by further centrifugation at 2,000xg (15 mins., 4^{o}C), washed twice with, and suspended in, 0.1M sodium phosphate buffer, pH 7.4, to give the required bacterial concentration.

The bacterial concentration was estimated by suitably diluting 0.1 ml of the bacterial suspension to obtain an optical density reading of 0.0 - 0.4 at 420 nm, using a Unicam SP500 Spectrophotometer, as the turbidity was found to be a linear function of the cell number at optical density values between 0.0 and 0.4 (De Moss and Bard, 1957).

This was followed either by obtaining the dry weight of the cells by pipetting 1.0 ml of the cell suspension into a preweighed planchette, which was then placed in a dessicator and left to dry over $CaCl_2$ for three days, then weighed again; or by estimating the viable number of cells using the Miles and Misra (1938) technique.

3. DETERMINATION OF THE MINIMUM INHIBITORY AND MINIMUM CIDAL CONCENTRATIONS

Graded concentrations of the compound under investigation, were prepared in 5 ml of nutrient broth, and inoculated with 0.06 ml (2 drops from a standardized Pasteur pipette) from a fresh overnight bacterial culture. Due to the use of acetone as a solvent for some compounds, positive controls were always performed, containing amounts of acetone equal to those used in the minimum inhibitory concentration (MIC) and minimum cidal concentration (MCC) determinations. Moreover, the pH of the nutrient broth, containing the different compounds under study, was always kept as near 7.2 - 7.4 as possible.

The tubes were then incubated at 37°C (with the exception of *B.cereus* and *M.phlei*, which were incubated at 30°C), for 24 hrs. (except for *M.phlei* which was incubated for 4 days), after which the presence or absence of growth was noted, and the MIC determined.

The MCC values were determined by subculturing a loopful of the broth from tubes showing no visible growth, on a nutrient agar plate.

4. VIABLE COUNT DETERMINATIONS

The viable number of a culture was determined using the technique of Miles and Misra (1938), in which a sample from the bacterial culture was withdrawn, and serially diluted in phosphate-buffered saline (PBS), or 0.9% saline. One drop of each dilution was then applied on the surface of a nutrient agar plate using a standardized Pasteur pipette; the plates were incubated at 37^{o}C overnight, and the colonies were then counted. Three replicates were used for each dilution.

5. QUALITATIVE DETERMINATION OF THE INTERACTION BETWEEN TWO COMPOUNDS

The method reviewed by Maccacaro (1961) was utilized in the investigation of synergistic or antagonistic interactions between any two compounds. Rectangular strips (4.0 cm long, 0.8 cm wide) were cut from Whatmann no.1 filter paper, and sterilized in the oven at 140^{o}C for two hours in a glass petri dish. One strip was soaked in a solution of one compound, and placed over the surface of a nutrient agar plate (20 ml/plate) that was already seeded with the required organism. A second strip, soaked in a solution of the other compound, was placed at right angles to the other strip, and with their ends overlapping. After incubating the plate for 24 hrs., the shape of the zone(s) of inhibition around the strip(s) was noted.

6. OXYGEN CONSUMPTION DETERMINATIONS

The oxygen consumption by a bacterial suspension was measured using the Warburg apparatus utilizing the general methods outlined by Umbreit, Burris and Stauffer (1957) and Dawes (1967). The main compartment of each Warburg flask contained the cell suspension, and 0.1M potassium phosphate buffer, pH 7.4; the side arm contained the substrate to be oxidized as well as the inhibitor under investigation; while the centre well contained 0.2 ml of 20% w/v KOH solution and a fluted filter paper. The total volume in each flask was made up to 3.0 ml, and the flasks were always left to equilibrate oscillating in the Warburg bath for around 15 mins., afterwhich all the taps were closed, and the contents of the side arms were immediately tipped into the main compartment.

All the determinations were performed at $37^{o}C$, with a shaking rate of 100 oscillations per minute.

7. RADIOACTIVITY DETERMINATIONS

Samples, whose radioactivity to be determined, were rapidly filtered through a Nuflow 0.45 μm Millipore filter discs, washed twice with the appropriate butter (or solution), and transferred into scintillation vials containing 10 ml of a scintillation liquid. The radioactivity in the vials was counted using a Beckman CPM-100 Liquid Scintillation System for either 10 minutes or 1% error each.

8. DUPLICATION OF EXPERIMENTS

All experiments - unless otherwise stated - were performed in duplicates. The standard deviation was determined whenever required.

SECTION THREE

EXPERIMENTAL METHODS

1. INVESTIGATING THE ANTIBACTERIAL ACTIVITY OF PHANQUONE

1.1 Minimum Inhibitory and Minimum Cidal Concentrations

Graded concentrations of Phanquone, prepared in tubes containing 5 ml of nutrient broth, were inoculated with the required organism, the MIC - and subsequently the MCC - values were determined as described earlier in the General Procedures.

1.2 Effect of Bacterial Inoculum Size on the MIC

The MIC values were determined as outlined earlier, using different inoculum sizes, ranging from 10^2 - 10^7 organisms/ml for both *E.coli* and *S.aureus*.

1.3 Effect of Phanquone In Excess of the MIC on Bacterial Cells.

The effect of Phanquone at 500 µg/ml was determined, for both *E.coli* and *S.aureus*, by mixing 5 ml of a Phanquone solution at 1000 µg/ml, with a 5 ml of double-strength nutrient broth, inoculating with the required organism, incubating at 37°C for 24 hrs., and then subculturing a loopful of the broth on a nutrient agar plate.

1.4 Mean Single Survivor Times

A sterile Phanquone solution was mixed with 10^7 cells/ml of either *E.coli* or *S.aureus* suspended in distilled water, to give the desired final concentration. Aliquots of 0.18 ml (6 drops from a standardized Pasteur pipette) of the reaction mixture (the Phanquone-bacteria mixture) were quickly withdrawn, transferred to sterile test

tubes, and incubated at 37°C, using the technique described by Berry and Bean (1954). At regular intervals, the reaction in 20 tubes was quenched with 10 ml of nutrient broth containing 1% w/v lactose and 0.012% w/v bromocresolpurple. At the end of each experiment, the tubes were transferred to an incubator at 37°C, and were left for 48 hrs.. The change in the colour of the nutrient broth (from purple to yellow) indicated growth.

The Mean Single Survivor Times (MSST) were calculated by the method of Mather (1949).

1.5 Effect on Non-Growing Suspensions

A flask containing 100 ml of PBS was inoculated with a bacterial cell suspension (either *E.coli* or *S.aureus*) to give a final concentration of 10^7 - 10^8 cells/ml, and transferred to a shaking water bath at 37°C. At regular intervals, samples were withdrawn, and the viable count was determined as described earlier. The inoculated PBS was then subdivided into two (or more) flasks, of which one served as a control, while to the other(s), Phanquone was added to give the desired final concentration(s). Once again, and at regular intervals, samples were withdrawn and the viable count determined.

1.6 Effect on Growing Cultures

A flask containing 100 ml of nutrient broth was inoculated with a bacterial cell suspension (either *E.coli* or *S.aureus*) to give a final concentration of around 10^5 organisms/ml, and transferred to a shaking water bath at 37°C. At regular intervals, thereafter, samples were withdrawn, and the viable count determined as outlined earlier. After 90 mins., the "seeded" broth was subdivided into

two (or more) flasks, of which one served as a control, while the other(s), solutions of Phanquone were suitably diluted, so that aliquots of 0.5 ml or 1.0 ml, added to the "seeded" broth, yielded the desired final concentration(s). There again, and at regular intervals, samples were withdrawn, and the viable population estimated.

1.7 Effect of pH on the Size of the Zone of Inhibition.

A sterilized Phanquone solution was poured over sterile Whatmann AA discs (0.6 mm in diameter), to give a final concentration of 50 µg/disc.

Nutrient agar plates (20 ml/plate), prepared at different pH values ranging between pH 6-8, were seeded with either *E.coli* or *S.aureus*; Phanquone discs (50 µg/disc) were placed over the agar plates, with a flamed forceps, and gently pressed to ensure contact. The plates were then left for 2 hrs. before incubating at $37^{o}C$ for 24 hrs., to allow for diffusion. The zones of inhibition around the discs were then measured with calipers, averaged and compared.

2. INVESTIGATING THE ADSORPTION OF PHANQUONE TO THE BACTERIAL CELL

2.1 Construction of a Reference Curve

When dissolved in warm distilled water, Phanquone gave a bright yellow colour, that absorbed well at a wavelength of 302 nm. Therefore, varying amounts of Phanquone were dissolved in 0.1M potassium phosphate buffer, pH 7.4, to give the required concentrations; the optical density was then read at 302 nm using a Unicam SP500 spectrophotometer, and a reference curve was constructed.

2.2 Measurement of the Adsorption - and Uptake - of Phanquone

Following the procedures described by Salton (1951), Hugo and Frier (1969), and Hugo and Bloomfield (1971(a)), cells (*E.coli* or *S.aureus*), suspended in 0.1M potassium phosphate buffer, pH 7.4, were treated with varying concentrations of Phanquone, and for varying lengths of time. After the prescribed contact periods, the cells were removed by centrifugation at 2,000xg (15 mins., 4°C). The supernatant liquid was further clarified by further centrifugation at 12,000xg (10 mins., 4°C). The optical density of the clarified solutions was read at 302 nm, and the concentration of the remaining Phanquone was estimated using the reference curve.

3. INVESTIGATING THE ANTIBACTERIAL ACTIVITY OF COMPOUNDS RELATED TO PHANQUONE

3.1 Minimum Inhibitory and Minimum Cidal Concentrations

As outlined earlier, the MIC and MCC values were determined for the following compounds:- phenanthrene, phenanthridine, 8-hydroxyquinoline (oxine), 4,7-phenanthroline and 1,10-phenanthroline-5,6-quinone.

It is worth mentioning that 9,10-phenanthrenequinone tended to come out of solution when mixed with the nutrient broth, at 200 µg/ml and 100 µg/ml; hence, it was treated as a suspension at those two concentrations.

3.2 Effect on Growing Cultures

The effect of the related compounds (with the exception of 8-hydroxyquinoline) on growing *E.coli* and *S.aureus* cells, was determined as outlined earlier for Phanquone.

3.3 Effect of 9,10-Phenanthrenequinone on Non-Growing Suspensions

The effect of 9,10-phenanthrenequinone on non-growing *E.coli* and *S.aureus* cells was determined as described earlier for Phanquone.

3.4 Effect of 9,10-Phenanthrenequinone on EDTA - Pretreated *E.coli* Cells

Following the method outlined by Leive (1965, 1968, 1974) and Muschel and Gustafson (1968), *E.coli* cells were harvested at the late log phase by centrifugation at 2,000xg (15 mins., 25°C), washed twice on the centrifuge at 2,000xg (15 mins., 25°C), and resuspended in 0.12M Tris-Cl buffer, pH 8.0, to give around 10^9 organisms/ml. The cell suspension was then subdivided into two equal volumes - to one ethylenediaminetetracetic acid - disodium salt (EDTA.2Na) was added to give a final concentration of 2.5×10^{-4}M. Two minutes later, aliquots of the EDTA-treated suspension were added to two flasks, one containing 9,10-phenanthrenequinone (30 µg/ml) in 50 ml of nutrient broth, while the other contained 50 ml of nutrient broth only. At the same time, an equal aliquot of the untreated suspension was added to a third flask containing 9,10-phenanthrenequinone (30 µg/ml) in 50 ml of nutrient broth. The multiplication of the cells was monitored by the viable count method described earlier.

4. INVESTIGATING THE INTERACTION OF SOME ANTIBACTERIAL AGENTS AND ANTIBIOTICS WITH PHANQUONE.

The right-angled strips method reviewed by Maccacaro (1961), and outlined in Section Two was utilized, in which one strip was soaked with a sterile Phanquone solution, while the other strip, soaked in the desired antibacterial agent was placed at right-angles to the Phanquone strip over a nutrient agar plate (20 ml/plate) already seeded with either *E.coli* or *S.aureus*. After incubation at 37°C for 24 hrs., the shapes of the zones of inhibition, around the strips, were noted.

5. INVESTIGATING THE EFFECT OF THIOL-CONTAINING COMPOUNDS ON THE ACTIVITY OF PHANQUONE.

5.1 Assessing the Interaction Between Thiol-Containing Compounds and Phanquone.

The right-angled strips method outlined earlier was used, in which one strip was soaked with a sterile solution of Phanquone, while the other was soaked with the thiol-containing compound. Both were laid at right angles over a nutritent agar plate (20 ml/plate) seeded with either *E.coli* or *S.aureus*.

The thiol-containing compounds investigated were: thioglycollic acid, sodium thioglycollate, 2-mercaptoethanol, 1-thioglycerol, and L-cysteine.

5.2 Effect of Thioglycollic Acid on the Activity of Phanquone Against Non-Growing *E.coli* Cells.

A flask containing 100 ml of PBS was inoculated with *E.coli* to give a final concentration of 10^7 cells/ml, and transferred to a shaking water bath at 37°C. At regular intervals, samples were withdrawn, and the viable count was determined as described earlier. After three determinations, the "seeded" PBS was subdivided into two flasks; to one, Phanquone was added to give a final concentration of 1 µg/ml, while the other served as a control. After three more determinations, the flask containing the Phanquone was further subdivided into two flasks, to one of which thioglycollic acid (neutralized, pH 6.0) was added to give a final concentration of 2×10^{-3}M, while to the other, an equal amount of distilled water (pH 6.0) was added. Thereafter, more samples were withdrawn, and the viable count determined.

6. INVESTIGATING THE EFFECT OF PHANQUONE ON SOME METABOLIC ACTIVITIES OF THE BACTERIAL CELL.

6.1 Effect on the Oxygen Consumption

Following the outline in the General Procedures, 1.0 ml of the cell suspension (either *E.coli* or *S.aureus*) containing 8×10^{12} organisms, was transferred into each main compartment of a number of Warburg flasks, which also contained 1.5 ml of sodium phosphate buffer, pH 7.4. Side arms contained 0.25 ml of either D-glucose (0.02M), or sodium succinate (0.02M or 0.06M), as well as 0.25 ml of Phanquone at the desired concentrations (except in the control flasks, where 0.25 ml of distilled water was added instead).

6.2 Effect on the Activity of Some Dehydrogenases

Following the procedure outlined by Hugo (1954) and Stretton and Manson (1973), tubes were prepared each containing the following: PBS(pH 7.3), 2.5 ml; substrate, 1.0 ml; cell suspension (either *E.coli* or *S.aureus*, to give a final concentration of 3×10^{12} cells/ml), 0.4 ml; triphenyltetrazolium chloride (TTC)-0.1% w/v, 1.0 ml; and Phanquone (suitably diluted to give the desired concentration), 0.1 ml. Controls, however, did not contain Phanquone but 0.1 ml of distilled water instead. Tubes containing neither Phanquone nor substrate were also prepared to be used as blanks.

All the tubes were immediately placed in a shaking water bath at $37^{o}C$, and at regular intervals thereafter, the reaction in tubes from both test and control series was halted by adding 5 ml of glacial acetic acid to each tube, followed by 5 ml of toluene. The tubes were vigourously mixed to disolve the red formazan formed in the toluene phase, centrifuged at 150xg (5 mins., $4^{o}C$) to separate the toluene from the aqueous phase completely, and the red colour was afterwards read at 495 nm as suggested by Owen (1971), using a Unicam SP500.

The substrates used were 0.02M solutions of D-glucose, sodium pyruvate, sodium malate, sodium succinate, sodium lactate and glucose-6-phosphate.

6.3 Effect of Aerobic and Anaerobic Conditions on the Size of the Zone of Inhibition.

Phanquone discs (50 µg/ml), prepared as described earlier (p. 52) were placed on nutrient agar plates (20 ml/plate), pH 7.2, already seeded with the required organism. Six replicates were incubated aerobically at 37°C, while six other replicates were incubated at 37°C in an anaerobic jar, using Gas Pak Hydrogen plus Carbon Dioxide Generator envelopes (BBL), to create the required anaerobic atmosphere. After 24 hrs., the zone diameters were measured with calipers, averaged and compared.

6.4 Effect on the Uptake of P(32)-Inorganic Phosphate

Following the method outlined by Harold, Harold and Abrams (1965), Harold and Baarda (1966, 1969), and Harold, Baarda, Baron and Abrams (1969), 2.5 ml of D-glucose (0.02M) were added, together with 1.0 ml of potassium phosphate solution (600 µg/ml), containing phosphorus-(32) (0.1 µci/ml), to a flask containing 14.5 ml of 0.1M Tris-Cl buffer, pH 7.4 and 10.0 ml of cell suspension (to give a final concentration of 3×10^{12} cells/ml). The flask contents were rapidly divided into two equal volumes, and 1.0 ml of Phanquone solution (suitably diluted to give the desired final concentration) was added to one, while 1.0 ml of distilled water was added to the other. Both flasks were immediately placed in a shaking water bath at 37°C, and at regular intervals thereafter, samples were withdrawn, filtered and washed with 0.1M Tri-HCl buffer, pH 7.4, containing 200 µg/ml K_2PO_4, as outlined in the General Procedures (p. 48). Here, water was used as the scintillation liquid, and therefore, the bacterial cells were washed off the membrane filter discs, into the scintillation vials.

6.5 Effect on the Uptake of C-(14)-L-Glutamic Acid and C-(14)-L-Proline.

To three flasks each containing 7.25 ml of 0.1M Tris-Cl buffer, pH 7.4, 5.0 ml of either *E.coli* or *S.aureus* suspension were added to give a final concentration of 3×10^{12} cells/ml. To two flasks, 1.25 ml of D-glucose (0.02M) were added, while 1.0 ml of a Phanquone solution (suitably diluted to give the desired final concentration) was added to one of the two flasks. The volume in all the flasks was made up to 14.5 ml with distilled water, and immediately after, 0.5 ml of 0.02M solution of L-glutamic acid, or L-proline, containing C-(14) (1.5 µci/ml), was added to all three flasks, which were rapidly placed in a shaking water bath.

At regular intervals, samples were withdrawn, filtered and counted as described earlier. Here, the samples were washed with Tris-Cl buffer, pH 7.4, containing either L-glutamic acid or L-proline (0.06M). The scintillation liquid used was the toluene-based KL372 (Koch Light Labs. Limited, Colnbrook, Buckinghamshire), which dissolved the membrane filter discs.

6.6. Effect on the Uptake of C-(14)-L-Proline by Membrane Vesicles

6.6.1 Preparation of membrane vesciles

As outlined by Kaback (1971, 1974(a), 1974(b)), *E.coli* "K12" was grown in 1,500 ml of nutrient broth in a shaking water bath at $37^{o}C$ for 10 hrs., after which the cells were harvested by centrifugation at 2,000xg (15 mins., $4^{o}C$).

1.0 g of the collected cell paste was later suspended in 30 ml of 50 mM Tris-Cl buffer, pH 8.0, containing 25% sucrose, and

homogenized, at 0-2°C, in a Teflon glass homogenizer to obtain an even suspension. 3.0 ml of 0.25M Tris-Cl, pH 8.0, containing lysozyme (10 mg/ml), was added, mixed thoroughly but gently, and incubated on ice for 5 mins., after which 2.0 ml of 0.2M EDTA - potassium salt, pH 8.0, were added, mixed and incubated on ice for a further 5 mins.. The resultant spheroplasts were harvested by centrifugation at 16,000xg (15 mins., 0°C), homogenized in 40 ml of 50 mM potassium phosphate buffer, pH 6.6, and the whole - or partially lysed cells were then deposited by centrifugation at 1,000xg (30 mins., 0°C). The supernatant liquid was then removed, and the membrane vesicles were harvested by centrifugation at 45,000xg (20 mins., 0°C), washed by vigourous homogenizing for 1 min. in 3.0 ml cold 0.1M potassium phosphate buffer, 10 mM EDTA, pH 6.6, re-centrifuging at 45,000xg (20 mins., 0°C), and decanting.

The vesicles were then suspended in 0.1M potassium phosphate buffer, pH 6.6, placed in capped plastic bottles, and stored in liquid nitrogen at 196°C.

6.6.2 Measurement of the Uptake of C-(14)-L-Proline

Three series of chilled small tubes were prepared each containing 250 µl of membrane vesicles suspension (thawed on the day of use), as well as 250 µl of $MgSO_4$. To two series, either 20 µl of ATP (1.0M) or lithium lactate (0.2M), were added. To one of the two series, 50 µl of Phanquone (to give 50 µg/ml) was added, while to the third, only distilled water was added. Immediately after, 150 µl of 0.02 L-proline containing C-(14)-L-proline (1.5 µci/ml) were added to the three series.

At regular intervals, the reaction in one tube of each series was terminated by adding 2.5 ml of 0.1M LiCl, and immediately pouring the contents of each tube into a Millipore Vacuum filtration apparatus containing a 0.45 µm Millipore filter, washed with a further 2.5 ml of 0.1M LiCl and filtered as outlined earlier (p. 48). The scintillation liquid used was KL 372, in which the filter discs dissolved.

7. INVESTIGATING THE EFFECT OF SOME METAL IONS ON THE ACTIVITY OF PHANQUONE

7.1 Complexing of Metal Ions with Phanquone

To tubes containing 5 ml of 10^{-3}M solution of Phanquone, 5 ml of 10^{-3}M solution of a metal sulphate (or chloride) were added, mixed thoroughly , and left for 15 mins., after which the change in colour and/or the formation of a precipitate in the tubes was noted. The changes in the tubes were noted again after 24 hrs. and 48 hrs..

7.2 Effect of Metal Ions on the Activity of Phanquone on Growing Cells

Maccacaro's right-angled strips method, described earlier, was employed, in which one strip was soaked in a sterile solution of Phanquone, while the other was soaked in a 0.1M solution of a metal sulphate (or chloride), and both were placed over a nutrient agar plate (20 ml/plate), seeded with either *E. coli* or *S. aureus*.

Moreover, the MIC and MCC values were determined for both organisms, in the presence of 2 x 10^{-3}M solution of $FeCl_3$.

7.3 Effect of Metal Ions on the Activity of Phanquone on Non-Growing Suspensions.

Tubes, containing 10^7 organisms/ml of either *E.coli* or *S.aureus* suspended in 8 ml of distilled water, were separated into three groups. To one, only Phanquone was added to give a final concentration of 5×10^{-6}M (~1 µg/ml) for *E.coli*, and 10^{-4}M (~20 µg/ml) for *S.aureus*. To the second group, Phanquone (at the above concentrations) was mixed with metal sulphates (or chlorides) to give a 2:1, 1:1, 1:2, and 1:4 ratios, while to the third group, only the metal ions were added to serve as controls.

The tubes were transferred to a shaking water bath at 37°C, and at 1 hr. and 2 hr. intervals, one drop (from a standardized Pasteur pipette) taken from each of the tubes, was applied on a nutrient agar plate, which was then incubated at 37°C for 24 hrs..

The metal ions used were: Co(II), Cu(II), Fe(II), Fe(III), Mn(II), Ni(II) and Zn(II).

7.4 Construction of Job-Plots for Phanquone-Metal Ion(s) Complexes.

As outlined by Job (1928) and Dangall and West (1961, 1964), tubes were prepared containing 5 ml of Phanquone and 5 ml of metal ions, to give the required Phanquone-metal ion ratios. The tubes were left overnight, after which the optical density was read at different wavelengths, and Job-plots were constructed using the optical density values obtained.

7.5 Effect of Chelating Agents on the Activity of Phanquone

Maccacaro's strip method was employed, in which one strip was soaked in a sterile Phanquone solution, while the other strip was soaked in a 0.1M solution of either ethylendiaminetetracetic acid - disodium salt (EDTA.2Na, pH 6.6), or nitrilotriacetic acid - sodium salt (NTA, pH 6.6).

7.6 Solubility of Phanquone and Its Metal Complexes in Octanol

To a series of quickfit glass tubes containing 5 x 10^{-4}M solution of Phanquone, metal ions (in the form of sulphates of chlorides) were added to give a Phanquone-metal ion ratios of 1:3, 1:2, 1:1, 2:1 and 3:1.

The volume in each tube was made up to 10 ml, mixed thoroughly, and left for 15 mins. at 20°C. 10 ml of octanol were added thereafter; the tubes were then fitted with tight quickfit glass stoppers, placed horizontally on a Winchester bottle shaker specially adapted to hold the tubes, and were vigourously shaken for 1 hr.. The tubes were then placed vertically and the two phases (water and octanol) were allowed to separate. The octanol phase was gently removed, and placed in a silica cuvette, where the absorbance was read using a Unicam SP800 spectrophotometer.

7.7 Effect of Metal Ions on the Inhibition of Oxygen Consumption by Phanquone.

The procedure described earlier (p.48) using the Warburg apparatus was exactly followed, except that 0.25 ml of the metal ion (sulphate) were added to the main compartment to give a Phanquone-metal

ion ratio of 1:1. Phanquone was added to the side arm (except in the case of the controls) as usual.

8. INVESTIGATING THE EFFECT OF PROLONGING THE PREINCUBATION PERIOD ON THE ZONE OF INHIBITION PRODUCED BY PHANQUONE

Phanquone discs (50 µg/disc) were placed over nutrient agar plates (pH 7.7 and pH 8.0), seeded with either *E.coli* or *S.aureus* cultures. The plates were incubated at 37°C for 30 mins., placed in a refrigerator at 4°C for 16 hrs., then incubated again at 37°C for 24 hrs. (as recommended by Feeney, Petersen and Sahinkaya, 1957). The zone of inhibition produced by Phanquone was, then, carefully noted.

9. INVESTIGATING THE EFFECT OF PHANQUONE ON THE BACTERIAL CELL MORPHOLOGY USING SCANNING ELECTRON MICROSCOPY

To a nutrient broth culture of either *E.coli* or *S.aureus*, at mid-log phase, a solution of Phanquone was added to give a final concentration of 50 µg/ml. The "reaction mixture", together with a control, were incubated at 37°C for 90 mins., after which glutarldehyde was added to samples taken from the reation mixture and the control to give a final concentration of 1.5% w/v (as described by Bullman and Stretton, 1974). The cells were removed after 2 mins. contact by centrifugation at 1,500xg (15 mins., 4°C), then suspended in 2 ml of glutaraldehyde (5% w/v) in ¼ strength Ringers solution (Oxoid), for 16 hrs. at 4°C. Thereafter, the cells were collected by centrifugation, washed twice, and

resuspended in distilled water. One drop of that suspension was placed over a cover slip, allowed to dry, and then dehydrated in a desicator over $CaCl_2$ under partial vacuum. The samples were then coated, in a high vacuum unit, with gold palladium and examined in a Cambridge Stereoscan (Mark II A, Cambridge Instruments Company Limited, Cambridge), with a beam specimen angle of 45^{o} and a voltage of 30kV.

10. INVESTIGATING THE EFFECT OF PHANQUONE ON THE RELEASE OF P-(32)-CONTAINING COMPOUNDS FROM THE BACTERIAL CELL

E.coli and *S.aureus* cells were grown on Winkler and de-Haan medium (1948) and Staphyloccacal medium (Stretton, 1965) respectively, in the presence of P-(32)-inorganic phosphate (of known specific activity), for 16 hrs. in a shaking water bath at $37^{o}C$. The cells were then harvested by centrifugation at 2,000xg (15 mins., $4^{o}C$), washed twice, and resuspended in 0.1M sodium phosphate buffer, pH 7.4, to give a final concentration of 3×10^{13} cells/ml.

To each of a series of tubes, the following was added: cell suspension, 1.0 ml; 0.1M sodium phosphate buffer, pH 7.4, 8.0 ml; and Phanquone solution (suitably diluted to give the desired final concentration), 1.0 ml (for the control, however, 1.0 ml of distilled water was added instead).

The contents of each tube were thoroughly mixed by whirling, and left at 20°C. At regular intervals, one tube from each series was centrifuged at 3,000xg (15 mins., 20°C) to deposit the cells (as outlined by Rye and Wiseman, 1964). The supernatant liquids were then gently decanted and transferred into scintillation vials, sonicated for 10 mins. in a Dawe water bath sonicator, after which the radioactivity of both the cells and the supernatant liquids was determined as described earlier in the General Procedures.

11. INVESTIGATING THE EFFECT OF PHANQUONE ON LIVER MONOAMINE OXIDASE

11.1 Preparing the Liver Homogenate

10 g of a fresh guinea-pig liver were homogenized in 0.1M sodium phosphate buffer, pH 7.4, and centrifuged, using a small bench centrifuge, until the solid fragments clearly separated from the supernatant liquid. The supernatant liquid was then discarded, and the solid was resuspended in more buffer, and washed twice on the centrifuge. Finally, the solid was resuspended in 10 ml of buffer (that is, 1 g of liver/ml).

11.2 Measuring the Oxygen Uptake

To each Warburg flask, 1.0 ml of the liver homogenate, together with 1.25 ml of 0.1M sodium phosphate buffer, pH 7.4, were placed in the main compartment. 0.25 ml of Phanquone (suitably diluted to give a final concentration of 100 µg/ml) were placed in the main compartment of one flask, while 0.25 ml of distilled water were added to two others. To the side arms of the flask containing the

Phanquone, as well as to one of the other two, 0.5 ml of benzylamine sulphate (0.04M) were added, while no substrate was added to the third. The flasks were left to equilibrate for 15 mins. after which the oxygen consumption was measured as outlined earlier.

12. INVESTIGATING THE MUTAGENICITY OF PHANQUONE USING THE SALMONELLA/MICROSOME MUTAGENICITY TEST

The method outlined by Ames and Coworkers in many publications (Ames, Lee and Durston, 1973; Ames, Durston, Yamasaki and Lee, 1973; Ames, McCann and Yamasaki, 1975; McCann, Choi, Yamasaki and Ames, 1975; McCann and Ames, 1977) was very closely followed.

12.1 Preparing the Liver Homogenate "S-9" Fraction

A male rat, weighing about 500 g, was injected with a single intraperitoneal injection of 20-methylcholanthrene dissolved in Arachis oil (250 mg/ml), five days before sacrifice. The rat was given drinking water *ad libitum* and Coney Brand Chow (Tyrrel, Byford and Pallett Limited, Norfolk) until 12 hrs. before sacrifice, when the chow was removed. On the fifth day, the rat was stunned by a blow on the head, and then decapitated.

The liver, collected at 0-4^{o}C, was placed in a sterile beaker containing 0.15 ml KCl at 1 ml/g of wet liver. After weighing, the liver was transferred to a beaker containing 3 volumes of 0.15M KCl, minced with sterile scissors, and homogenized in a Teflon homogenizer. The homogenate was centrifuged at 9,000xg (10 mins, 0^{o}C), and the supernatant liquid - designated as the S-9 fraction - was distributed in 2 ml portions in small sterile plastic bottles, quickly frozen

at 20°C, and then stored in liquid nitrogen at 196°C.

12.2 Preparing of the S-9 Mix

The S-9 mix contained (per 20 plates): S-9 fraction, 1.0 ml; 0.2M potassium phosphate buffer, 5.0 ml; NADP-sodium salt, 30 mg; stock salt solution (containing 0.4M $MgCl_2$ and 1.65M KCl), 0.2 ml; 0.1M glucose-6-phosphate, 0.5 ml; and distilled water, 3.3 ml.

12.3 Preparing Plates and Top Agar

Each plate contained 30 ml of modified Vogel-Bonner Medium E (described on p.), while top agar, consisting of 0.6% Oxoid agar no.3, and 0.5% NaCl, was autoclaved and stored in bottles each containing 100 ml of the agar. Before use, the agar was melted by heating in a steam bath, and 10 ml of a sterile solution containing 0.5 mM L-histidine and 0.5 mM D-biotin, were added to the molten agar and mixed gently by swirling.

12.4 Checking the Tester Strains

The tester strains used were *Salmonella typhimurium* LT_2, designated as TA1535, TA1537, TA1538, TA98 and TA100. These strains were checked for their histidine requirement and their other mutations according to the methods outlined by Ames, McCann and Yamasaki (1975).

12.5 Assaying the Mutagenicity of Phanquone

The following were added (in order) to 2.0 ml of molten top agar at 45°C: 0.1 ml of an overnight nutrient broth culture of one of the tester strains; 0.1 ml of Phanquone solution suitably diluted to give

10, 5 and 2.5 µg/plate; and 0.5 ml of the S-9 mix. The top agar and its contents, mixed gently by rotation, were rapidly poured over the modified Vogel-Bonner Medium E plates. As soon as the top agar solidified, the plates were incubated at 37°C for 48 hrs., and the number of the revertant colonies was noted.

PART III

RESULTS

1. THE ANTIBACTERIAL ACTIVITY OF PHANQUONE

1.1 The MIC and MCC Values

Table (3.1) shows the MIC and MCC values obtained for Phanquone against seven Gram-positive, seven Gram-negative, and one Acid-fast bacterium. The MIC values ranged from 3.12 µg/ml for *Salm. abortus ovis*, to 200 µg/ml for *Strep. faecalis*; while the MCC values ranged from 3.12 µg/ml for *Salm. abortus ovis*, to >200 µg/ml for *Strep. faecalis* also.

1.2 The MIC Values for Different Inoculum Sizes

Table (3.2) shows the effect of the inoculum size on the MIC value for *E.coli* and *S.aureus*. For *E.coli*, there was a two and a half fold increase in the MIC for an increase of 10^5 times in the number of cells; while for *S. aureus*, there was a smaller increase in the MIC value (from 50 µg/ml to 60 µg/ml), for the same increase in the cell numbers.

1.3 The Effect of Phanquone in Excess of the MCC on *E.coli* and *S.aureus*.

Phanquone, at 500 µg/ml (five times the MCC value), while totally cidal for the *E.coli* cells, did not have a complete cidal effect on the *S.aureus* culture.

Table (3.1)

The minimum inhibitory concentrations (MIC) and minimum cidal concentrations (MCC) of Phanquone

Organism	MIC (µg/ml)	MCC (µg/ml)
Gram-positive:		
B. cereus	100	200
B. megaterium	25	25
B. subtilis	50	200
Sar. lutea	25	25
S. aureus	50	100
S. aureus "Oxford"	25	100
Strep. faecalis	200	>200
Gram-negative:		
E. coli	25	25
E. coli "Texas"	50	100
K. aerogenes	12·5	12·5
P. vulgaris	25	100
Ps. aeruginosa	50	200
Salm. abortus avis	3·12	3·12
Salm. abony	100	100
Acid-fast:		
M. phlei	50	50

Table (3.2)

The effect of inoculum size on the minimum inhibitory concentrations (MIC) of Phanquone

E. coli		S. aureus	
bacteria/ml.	MIC µg/ml.	bacteria/ml.	MIC µg/ml.
10^2	20	10^2	50
10^3	20	10^3	50
10^4	20	10^4	50
10^5	25	10^5	50
10^6	30	10^6	60
10^7	50	10^7	60

1.4 The Mean Single Survivor Times

Table (3.3) shows a typical tabulation of results, obtained from one run in the determination of the MSST for *E. coli*, following the procedure outlined by Mather (1949).

Fig. (3.1) shows the plots for the determination of the MSST (plotting log(- log P) versus the time of exposure) for Phanquone at 125 µg/ml, 150 µg/ml, 175 µg/ml and 200 µg/ml respectively. In this Fig. the MSST was the point of intersection between the best fit straight line curve and the line y = o. Table (3.4) shows the MSST values for *E. coli* using Phanquone at the four above-mentioned concentrations.

Fig. (3.2) shows the relationship between the MSST values and the Phanquone concentrations for *E. coli*. However, no consistent results could be obtained over the same range of Phanquone concentrations, when *S. aureus* was used.

1.5 The Effect on Non-Growing Suspensions

Figs. (3.3) and (3.4) show the effect of Phanquone on non-growing *E. coli* and *S. aureus* cell suspensions respectively. Phanquone was completely lethal at concentrations as low as 4 µg/ml; even 0.05 µg/ml of Phanquone caused an appreciable drop in the viable number of cells. On the other hand, and although there was a drop in the number of viable cells for *S. aureus*, yet more than 3×10^4 cells/ml continued to survive, in the presence of Phanquone at 10 µg/ml. As can be seen from Table (3.5), Phanquone, at 80 µg/ml, did not cause total cell death after 2 hrs., and more than 3×10^4 cells/ml continued to survive.

Table (3.3)

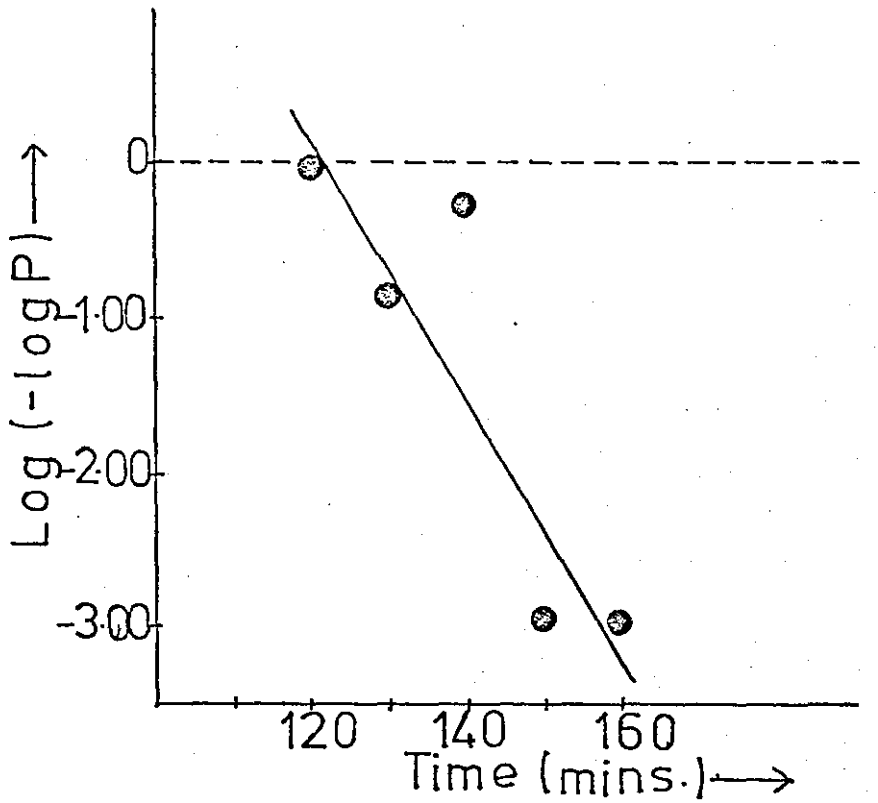
Death times of *E. coli* in Phanquone solution at 175 µg/ml

Tube	Time(min.)						
	60	70	80	90	100	110	120
1	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+
4	+	+	+	+	+	+	−
5	+	+	+	+	+	+	−
6	+	+	+	+	−	−	−
7	+	+	+	+	−	−	−
8	+	+	+	+	−	−	−
9	+	+	+	+	−	−	−
10	+	+	+	+	−	−	−
11	+	+	+	+	−	−	−
12	+	+	+	+	−	−	−
13	+	+	+	+	−	−	−
14	+	+	+	+	−	−	−
15	+	+	+	+	−	−	−
16	+	+	+	+	−	−	−
17	+	+	+	+	−	−	−
18	+	+	+	−	−	−	−
19	+	+	+	−	−	−	−
20	+	+	−	−	−	−	−
+Ve control	+	+	+	+	+	+	+
-ve control	−	−	−	−	−	−	−
P	—	—	0.05	0.15	0.75	0.75	0.85
log(-logP)	—	—	1.097	0.640	-1.246	-1.246	-1.817

Fig. (3.1)

Determination of the mean single survivor times (MSST) of *E. coli* using Phanquone at:

a) 125 µg/ml;
b) 150 µg/ml.


0
-1·00
-2·00
-3·00
Log (-log P) ⟶
120
140
160
Time (mins.) ⟶

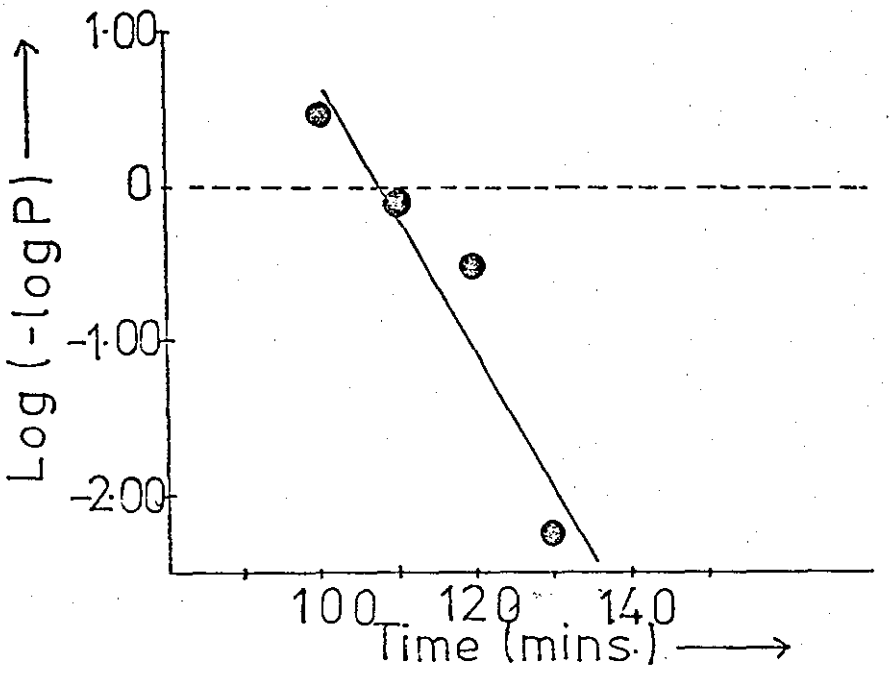
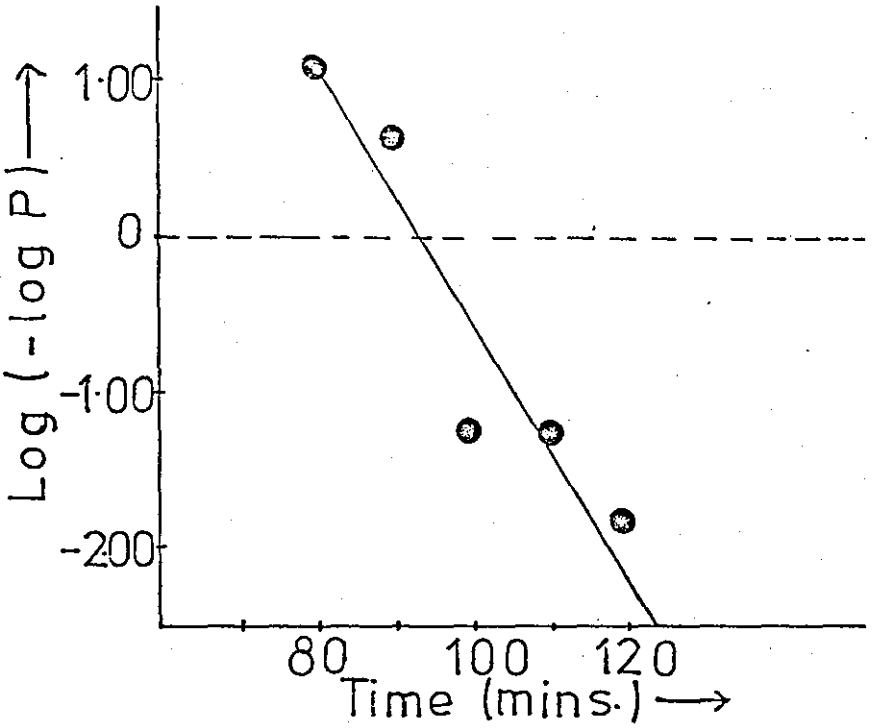

1·00
0
-1·00
-2·00
Log (-log P) ⟶
100
120
140
Time (mins.) ⟶

Fig. (3.1) (continued)

c) 175 µg/ml;
d) 200 µg/ml.


1·00
0
-1·00
-200
Log (-log P) ⟶
80
100
120
Time (mins.) ⟶

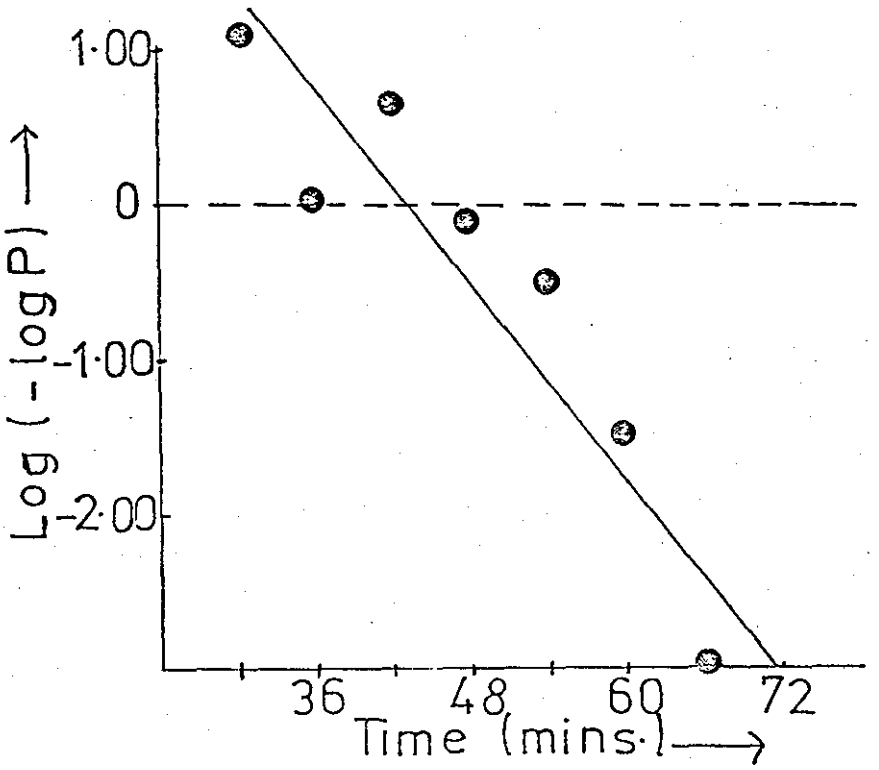

1·00
0
-1·00
-2·00
Log (-log P) ⟶
36
48
60
72
Time (mins.) ⟶

Table (3.4)

The mean single survivor times (MSST) for *E. coli*.

Fig. (3.2)

The relationship between the concentration of Phanquone and the mean single survivor times (MSST)

PQ Concentration	MSST (mins.)
125 ug/ml	122
150 ug/ml	108
175 ug/ml	93
200 ug/ml	43

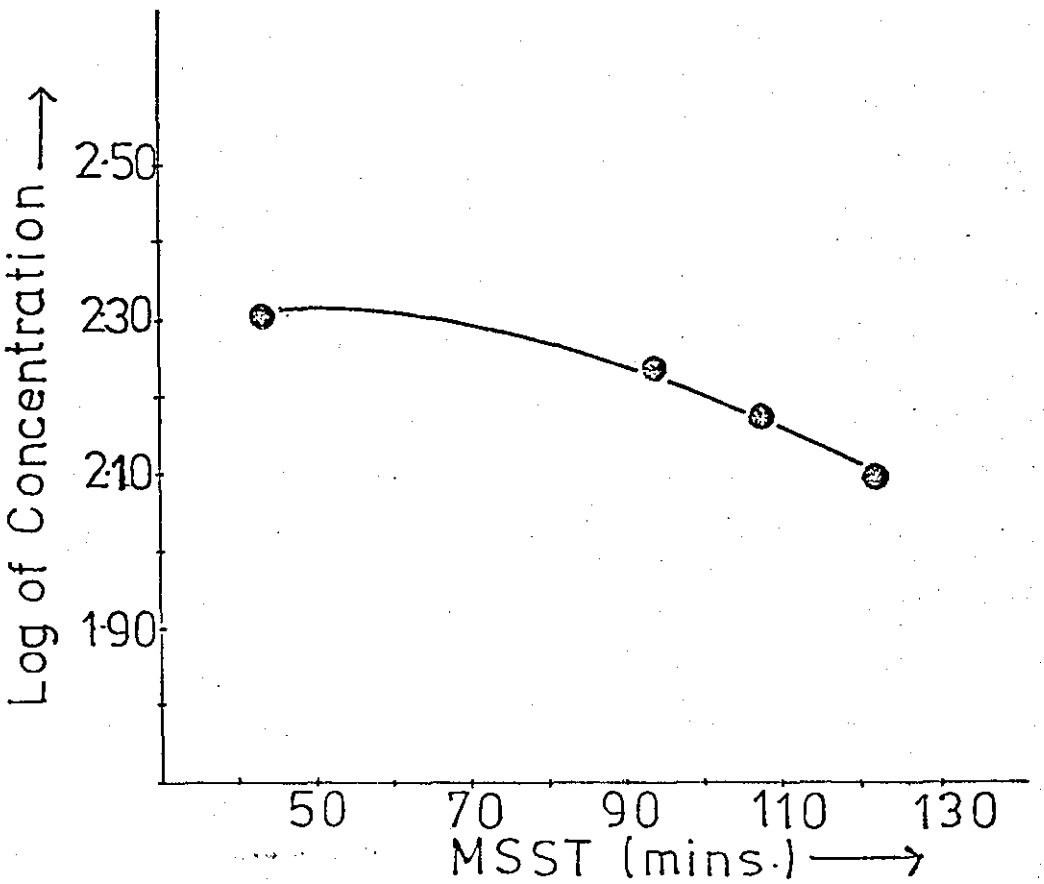


Fig. (3.3)

The effect of Phanquone on non-growing *E. coli* cells suspended in PBS, pH 7.3.

●—● : control
△—△ : 0.05 µg/ml;
■—■ : 0.1 µg/ml;
⬡—⬡ : 2 µg/ml;
▲—▲ : 4 µg/ml;
○—○ : 15 µg/ml;

Arrow indicates addition of Phanquone

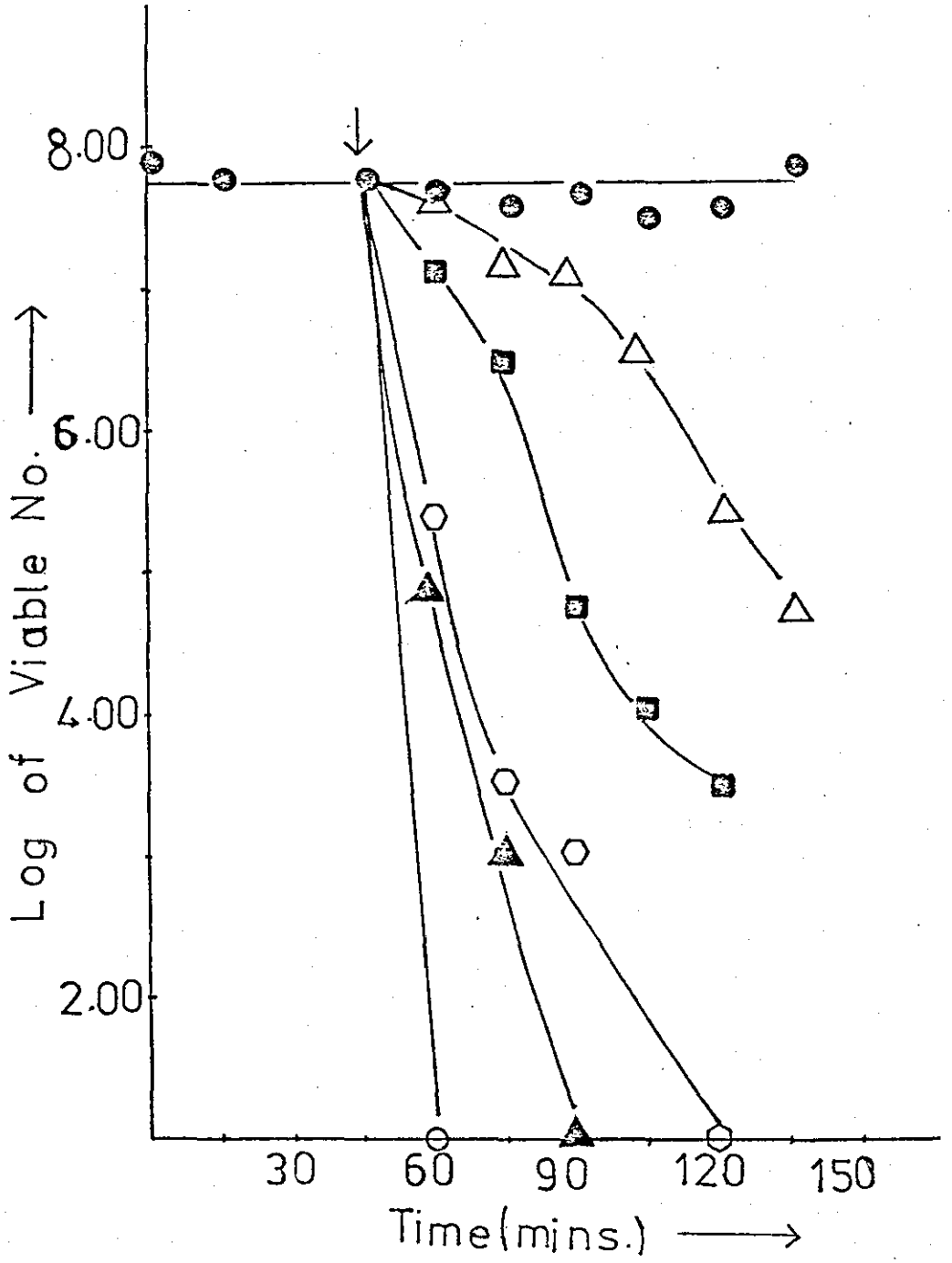

8.00
6.00
4.00
2.00
Log of Viable No.
30
60
90
120
150
Time(mins.)

Fig. (3.4)

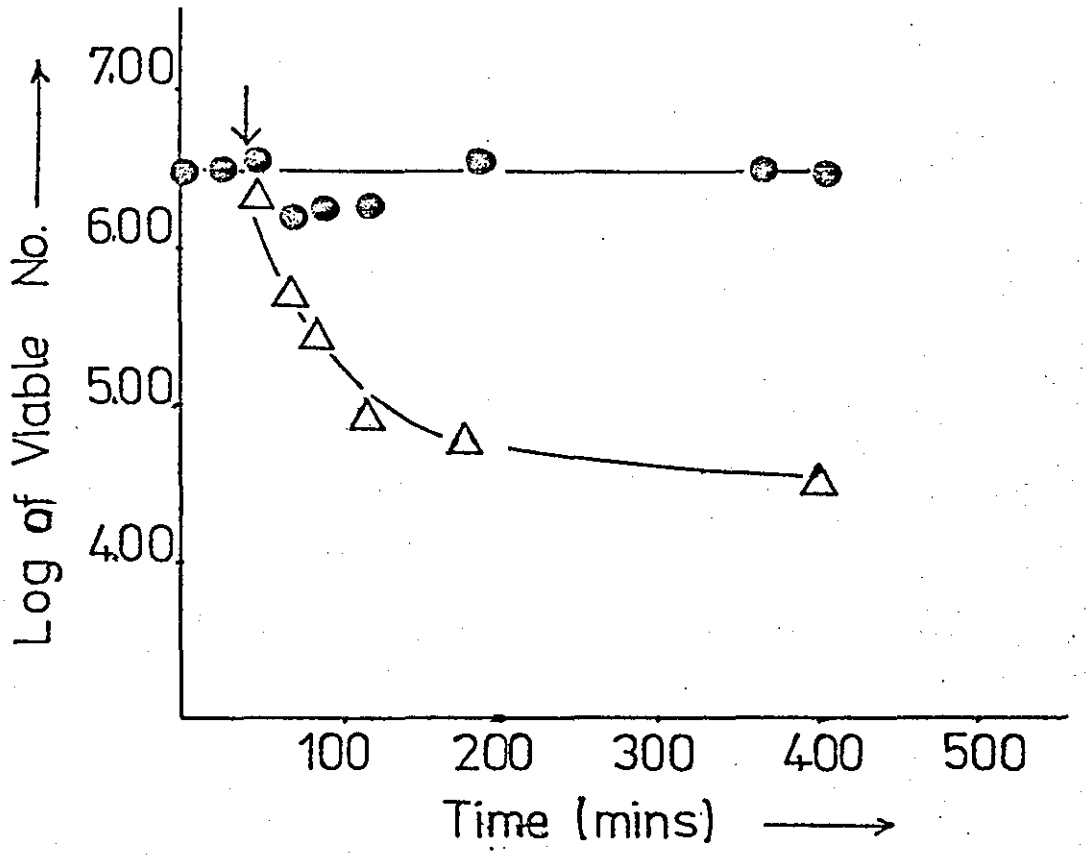
The effect of Phanquone on non-growing *S. aureus* cells suspended in PBS, pH 7.3.

●—● : control;
△—△ : 10 µg/ml

Table (3.5)

The effect of Phanquone on non-growing *S. aureus* cells suspended in distilled water.

++ : ~ 3×10^4 cells/ml;
+++ : > 3×10^4 cells/ml.



Phanquone Concentration (µg/ml)	Exposure Time (mins.)	Viable Count
20	120	+ + +
30	"	+ + +
40	"	+ + +
50	"	+ + +
80	"	+ +

1.6 The Effect on Growing Cultures

Figs. (3.5) and (3.6) show the effect of Phanquone on growing *E. coli* and *S. aureus* cells respectively. For *E. coli* complete kill was achieved with a concentration of 10 µg/ml; a concentration of 4 µg/ml inhibited cell multiplication. For *S. aureus*, complete kill was achieved with 20 µg/ml.

1.7 The Effect of pH on the Size of the Zone of Inhibition

As can be seen from Fig. (3.7), the diameter of the zone of inhibition for *E. coli* linearly decreased as the pH of the medium was increased. For *S. aureus*, however, two zones were produced: a small clear zone that decreased in size as the pH increased (Fig.3.8 (a)), and a larger zone (with many colonies inside). The largest hazy zone was obtained at pH 7.0, decreasing in size with increasing or decreasing pH (Fig. 3.8 (b)).

2. ADSORPTION OF PHANQUONE TO THE BACTERIAL CELL

2.1 The Reference Curve

Fig. (3.9) shows the reference curve used to estimate the unknown Phanquone concentrations at 302 nm. At that wavelength, the optical density values ranged from 0.08 units for 5.5 µg/ml, to 1.40 units for 180 µg/ml. The log-log plot gave the best fit straight line.

Fig. (3.5)

The effect of Phanquone on growing *E. coli* culture in nutrient broth, pH 7.4.

●—● : control
●—● : 4 µg/ml;
□—□ : 10 µg/ml;
▲—▲ : 20 µg/ml.

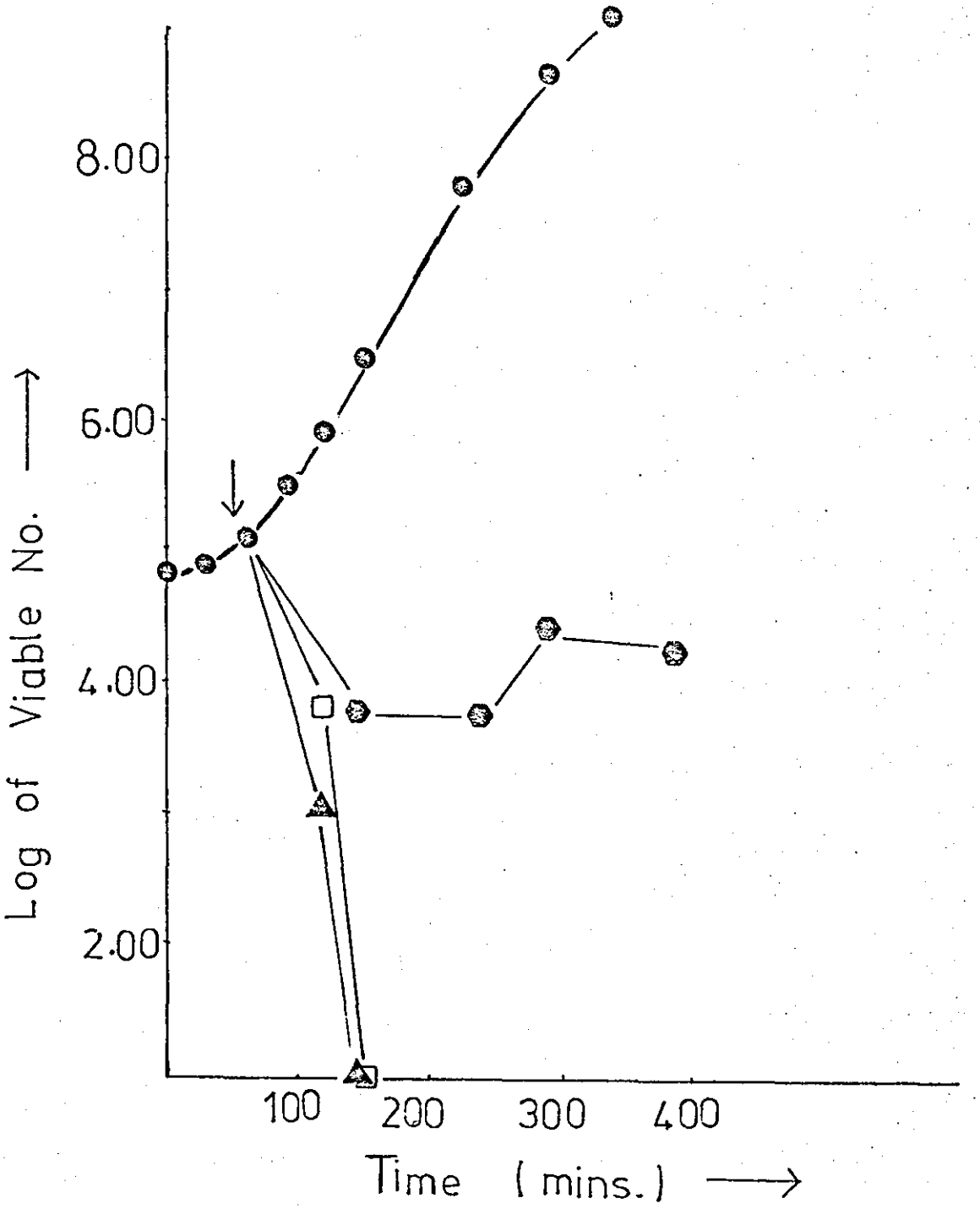

8.00
6.00
4.00
2.00
Log of Viable No. ⟶
100
200
300
400
Time (mins.) ⟶

Fig. (3.6)

The effect of Phanquone on growing *S. aureus* culture in nutrient broth, pH 7.4.

●—● : control;
□—□ : 20 µg/ml.

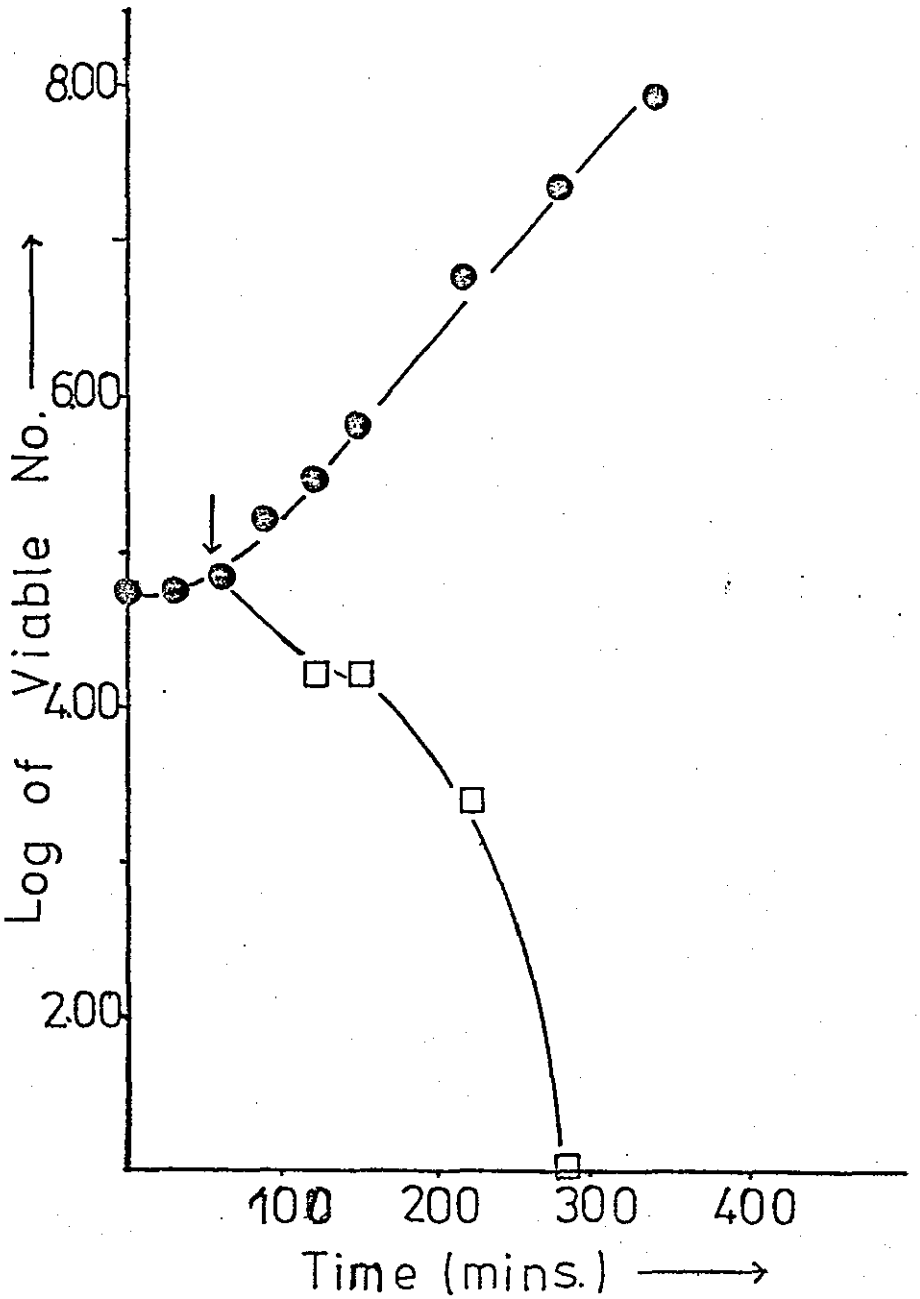

8.00
6.00
4.00
2.00
Log of Viable No.
100
200
300
400
Time (mins.)

Fig. (3.7)

The effect of pH on the size of the zone of inhibition produced by Phanquone against *E. coli* on nutrient agar plates.

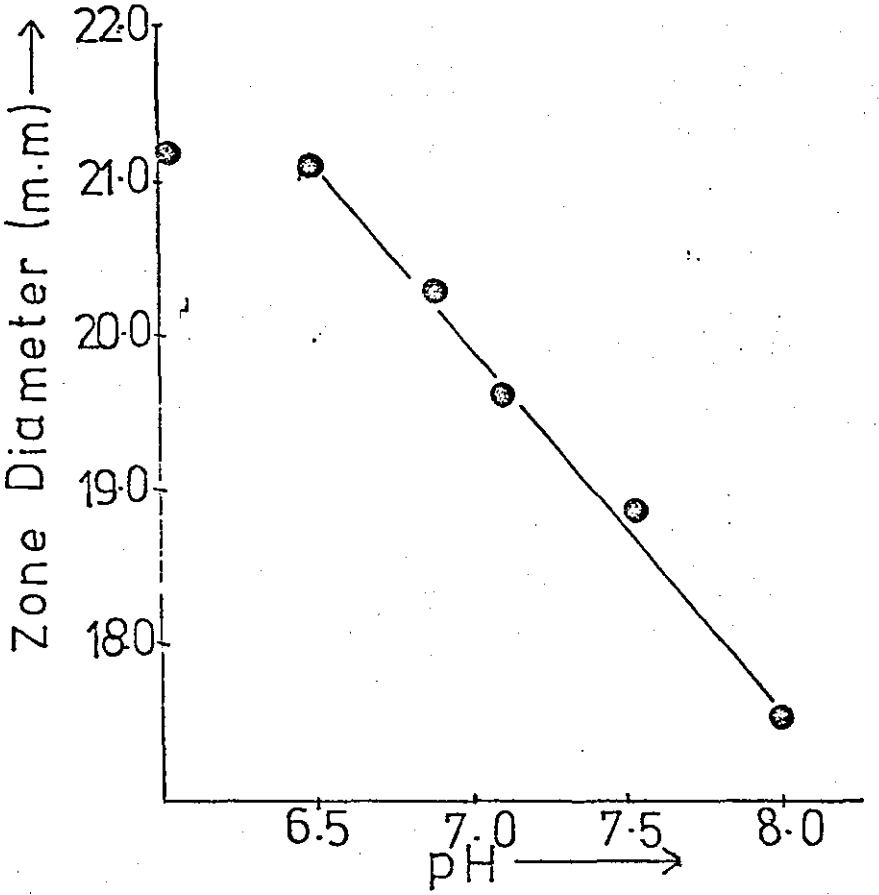
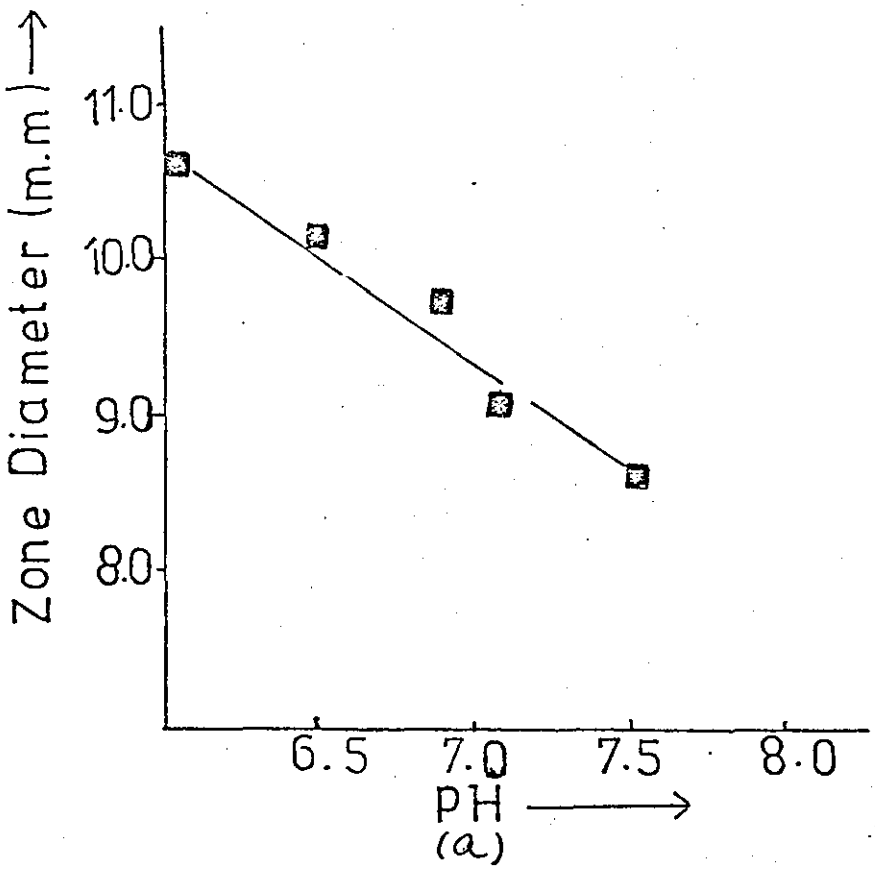

Zone Diameter (m.m) ⟶
22·0
21·0
20·0
19·0
18·0
6·5
7·0
7·5
8·0
pH ⟶

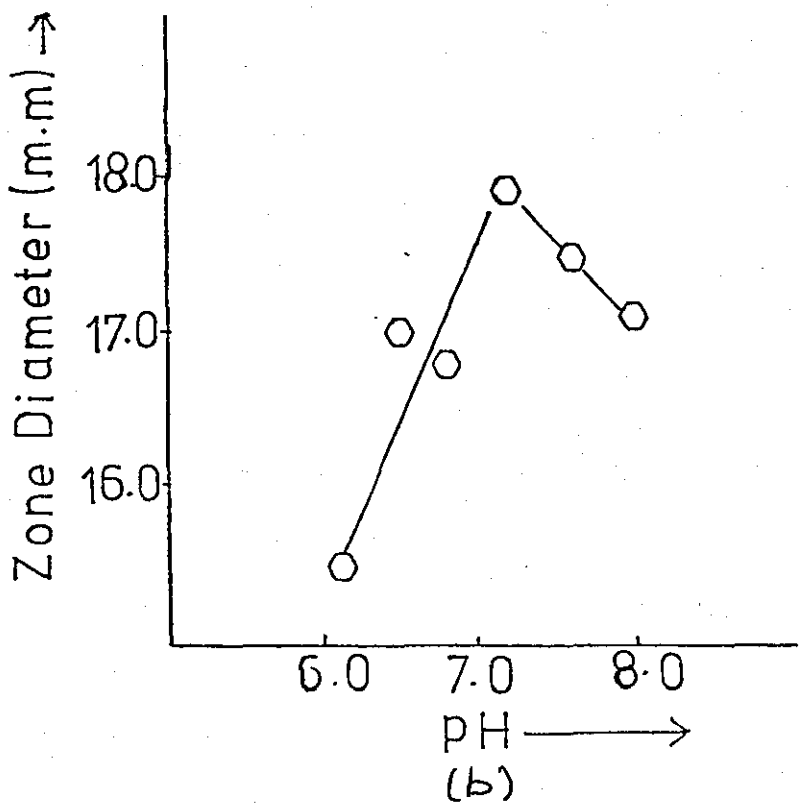
Fig. (3.8)

The effect of pH on the size of the zone of inhibition produced by Phanquone against *S. aureus* on nutrient agar plates.

a) clear zone;
b) total zone (clear and hazy).


Zone Diameter (m.m) →
11.0
10.0
9.0
8.0
6.5
7.0
7.5
8.0
PH →

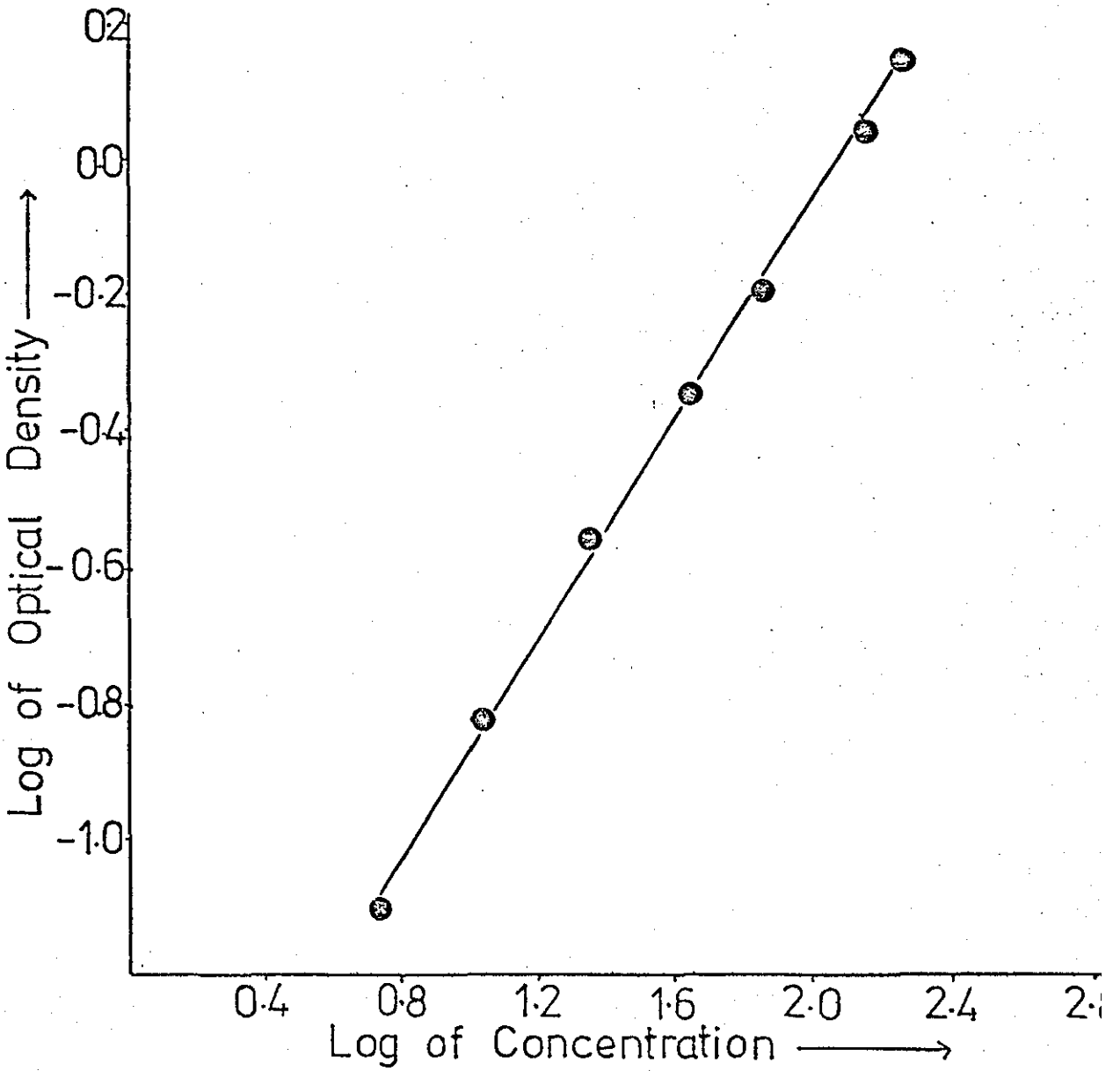
(a)


Zone Diameter (m.m) →
18.0
17.0
16.0
6.0
7.0
8.0
PH →

(b)

Fig. (3.9)

The reference curve for the estimation of unknown concentrations of Phanquone in 0.1M sodium phosphate buffer, pH 7.4.


0·2
0·0
-0·2
-0·4
-0·6
-0·8
-1·0
Log of Optical Density
0·4
0·8
1·2
1·6
2·0
2·4
Log of Concentration

2.2 Adsorption and Uptake of Phanquone

Figs. (3.10) and (3.11) show the amount of Phanquone adsorbed (or taken into) by 1 mg of *E.coli* and *S.aureus* (equivalent to around 4×10^{10} organisms/ml) using different Phanquone concentrations. As can be seen, 1 mg of *S.aureus* cells adsorbed nearly double the amount of Phanquone adsorbed by *E.coli*. The rate of adsorption, in both cases, was higher at the lower concentration of the antibacterial agent, and tended to become constant at 150 µg/ml and above.

Fig. (3.11) related the amount of Phanquone adsorbed by 10^{12} cells to the supernatant liquid concentration. However, the curves obtained were very similar.

Fig. (3.12) shows the amount of Phanquone adsorbed using a constant Phanquone concentration (45 µg/ml), but varying the contact time. Again 1 mg of *S.aureus* cells adsorbed almost double the amount of Phanquone adsorbed by 1 mg of *E.coli* cells.

Another interesting point is that *E.coli* and *S.aureus* cells seemed to adsorb the maximum amounts of Phanquone at 10 and 5 mins. respectively, after which there was a drop in the amount adsorbed for both strains.

Fig. (3.10)

Amount of Phanquone adsorbed by 1 mg of cells suspended in 0.1M sodium phosphate buffer, pH 7.4, using different Phanquone concentrations.

□——□: *S. aureus*
●——●: *E. coli*

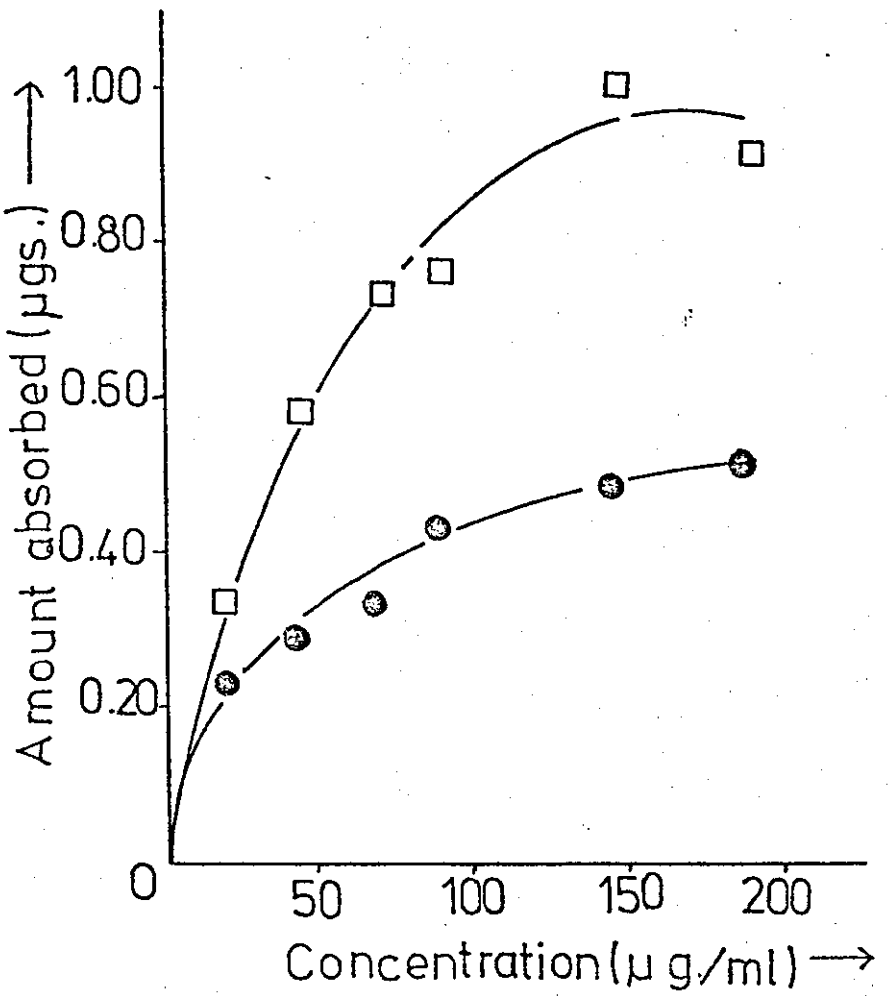

Amount absorbed (µgs.) ⟶
1.00
0.80
0.60
0.40
0.20
0
50
100
150
200
Concentration (µ g./ml) ⟶

Fig. (3.11)

Amount of Phanquone adsorbed by 10^{12} cells/ml suspended in 0.1M sodium phosphate buffer, pH 7.4, plotted against the supernatant liquid concentration

■—■ : *S. aureus*
○—○ : *E. coli*

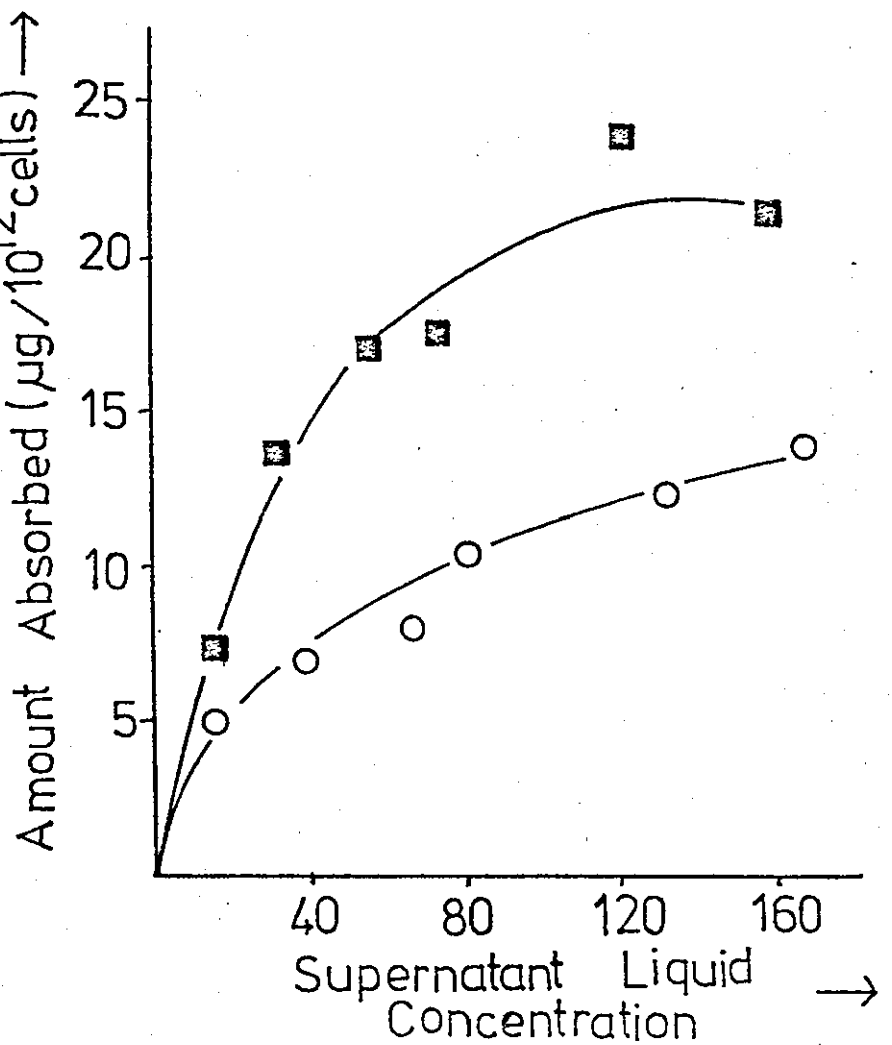
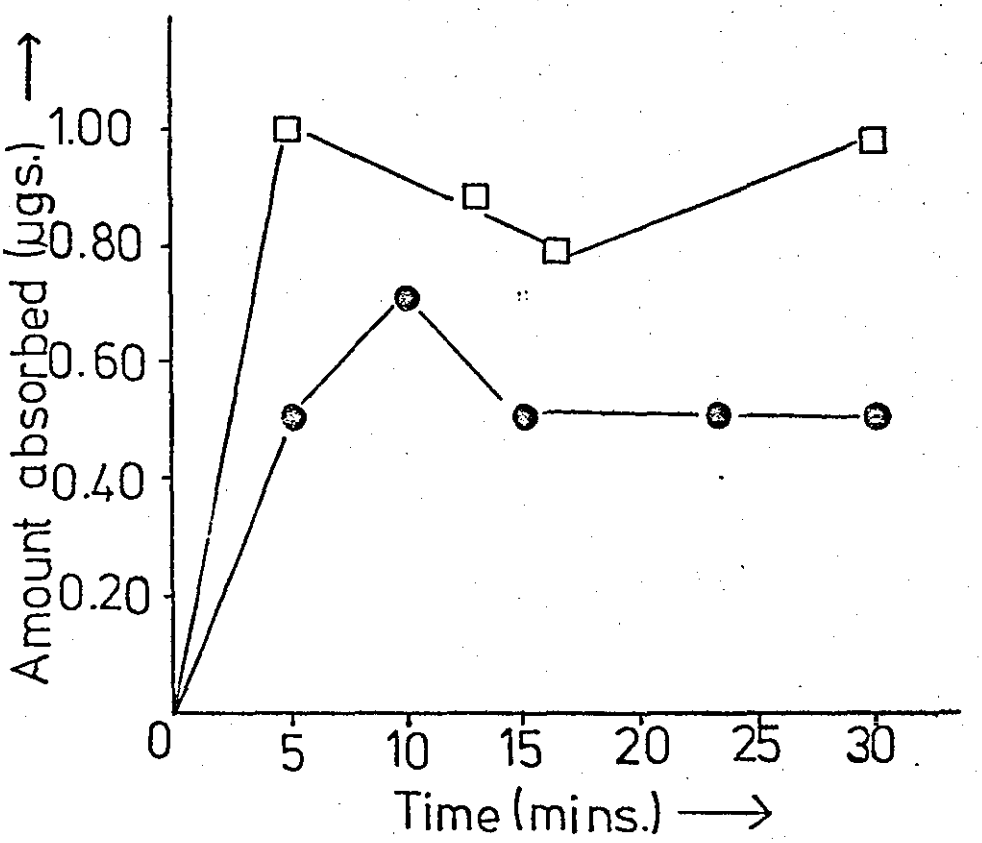

Amount Absorbed (µg/10^12 cells) →
25
20
15
10
5
40
80
120
160
Supernatant Liquid Concentration →

Fig. (3.12)

Amount of Phanquone adsorbed by 1 mg of cells suspended in 0.1M sodium phosphate buffer, pH 7.4, using one Phanquone concentration (45 µg/ml)

□—□ : *S. aureus*

●—● : *E. coli*


Amount absorbed (µgs.) ⟶
1.00
0.80
0.60
0.40
0.20
0
5
10
15
20
25
30
Time (mins.) ⟶

3. THE ANTIBACTERIAL ACTIVITY OF COMPOUNDS RELATED TO PHANQUONE

Tables (3.6) and (3.7) show the MIC and MCC values for phenanthrene, penanthridine, and oxine, on seven Gram-positive, seven Gram-negative, and one Acid-fast bacterium. Phenanthrene showed no activity even at 200 µg/ml; phenanthridine had some activity on some of the Gram-positive and Gram-negative, as well as the Acid-fast bacteria; and although oxine showed no activity on Gram-negative organisms (except *Salm. abortus ovis*), its activity against the Gram-positives ranged from 3.1 µg/ml to 12.5 µg/ml (with the exception of *Strep. faecalis*).

Tables (3.8) and (3.9) show the MIC and MCC values for 4,7-phenanthroline, 9,10-phenanthrenequinone, 1,10-phenanthroline, and 1,10-phenanthroline-5,6-quinone, against the same range of organisms. 9,10-phenanthrenequinone was, in general, more active against Gram-positives, than on Gram-negatives (it had no activity against *E. coli* at 200 µg/ml, while inhibiting *S. aureus* at 2.5 µg/ml). 1,10-phenanthroline showed the same range of activity as Phanquone (but had no activity against *Ps. aeruginosa* at 200 µg/ml). Finally, the MIC values of 1,10-phenanthroline-5,6-quinone ranged from 0.1 µg/ml for *P. vulgaris* to 25 µg/ml for *Ps. aeruginosa* (with the exception of *Sar. lutea*, and *Strep. faecalis*).

3.2 Effect on Growing Cultures

Fig. (3.13) shows the effect of phenanthrene on growing *E. coli* and *S. aureus* cultures, respectively. As can be seen, this compound had no effect on the *E. coli* culture (at 100 µg/ml), while having some activity against *S. aureus* at 50 µg/ml.

Table (3.6)

The MIC and MCC values of phenanthrene, phenanthridine, and oxine for some Gram-positive bacteria.

Organism	phenanthrene		phenanthridine		Oxine	
	MIC µg/ml.	MCC µg/ml.	MIC µg/ml.	MCC µg/ml.	MIC µg/ml.	MCC µg/ml.
B. cereus	>200	>200	100	100	3·1	6·2
B. megaterium	>200	>200	50	50	1·6	1·6
B. subtilis	>200	>200	100	200	6·2	6·2
Sar. lutea	>200	>200	>200	>200	12·5	12·5
S. aureus	>200	>200	100	100	3·1	3·1
S. aureus "Oxford"	>200	>200	100	100	6·2	6·2
Strep. faecalis	>200	>200	>200	>200	200	>200

Table (3.7)

The MIC and MCC values of phenanthrene, phenanthridine, and oxine for some Gram-negative and one Acid-fast bacteria.

Organism	phenanthrene		phenanthridine		Oxine	
	MIC µg/ml.	MCC µg/ml.	MIC µg/ml.	MCC µg/ml.	MIC µg/ml.	MCC µg/ml.
E. coli	>200	>200	>200	>200	>200	>200
E. coli "Texas"	>200	>200	>200	>200	>200	>200
K. aerogenes	>200	>200	50	200	>200	>200
P. vulgaris	>200	>200	50	100	>200	>200
Ps. aeruginosa	>200	>200	>200	>200	>200	>200
Sal. abortus ovis	>200	>200	100	200	25	25
Sal. abony	>200	>200	>200	>200	>200	>200
M. phlei	>200	>200	50	50	25	25

Table (3.8)

The MIC and MCC values of 4,7-phenanthroline, 9,10-phenanthrenequinone, 1,10-phenanthroline, and 1,10-phenanthroline-5,6-quinone for some Gram-positive bacteria.

Organism	4,7-phenanthroline		9,10-phenanthrene-quinone		1,10-phenanthroline		1,10-phenanthroline 5,6-quinone	
	MIC µg/ml.	MCC µg/ml	MIC µg/ml	MCC µg/ml	MIC µg/ml	MCC µg/ml	MIC µg/ml	MCC µg/ml
B. cereus	200	>200	0·4	12·5	100	>200	6·2	200
B. megaterium	100	100	1·6	1·6	25	25	0·8	0·8
B. subtilis	100	200	0·8	3·1	25	25	6·2	50
Sar. lutea	200	>200	100	100	25	25	50	100
S. aureus	100	100	2·5	2·5	50	100	2·5	25
S. aureus "Oxford"	100	200	0·8	1·6	50	100	1·6	50
Strep. faecalis	200	200	3·1	200	50	100	>200	>200

Table (3.9)

The MIC and MCC values of 4,7-phenanthroline, 9,10-phenanthrenequinone, 1,10-phenanthroline, and 1,10-phenanthroline-5,6-quinone for some Gram-negative and one Acid-fast bacteria.

Organism	4,7-phenanthroline		9,10-phenanthrene-quinone		1,10-phenanthroline		1,10-phenanthroline 5,6-quinone	
	MIC µg/ml	MCC µg/ml	MIC µg/ml	MCC µg/ml	MIC µg/ml	MCC µg/ml	MIC µg/ml	MCC µg/ml
E. coli	>200	>200	>200	>200	25	50	1·6	50
E. coli "Texas"	>200	>200	50	100	50	50	0·4	0·4
K. aerogenes	100	100	12·5	25	50	50	1·6	3·1
P. vulgaris	100	>200	0·8	1·6	25	50	0·1	0·8
Ps. aeruginosa	>200	>200	100	100	>200	>200	25	50
Sal. abortus ovis	100	100	25	25	0·8	25	0·4	0·8
Sal. abony	>200	>200	>200	>200	50	>200	0·8	25
M. phlei	100	100	1·6	1·6	12·5	25	6·2	6·2

Fig. (3.13)

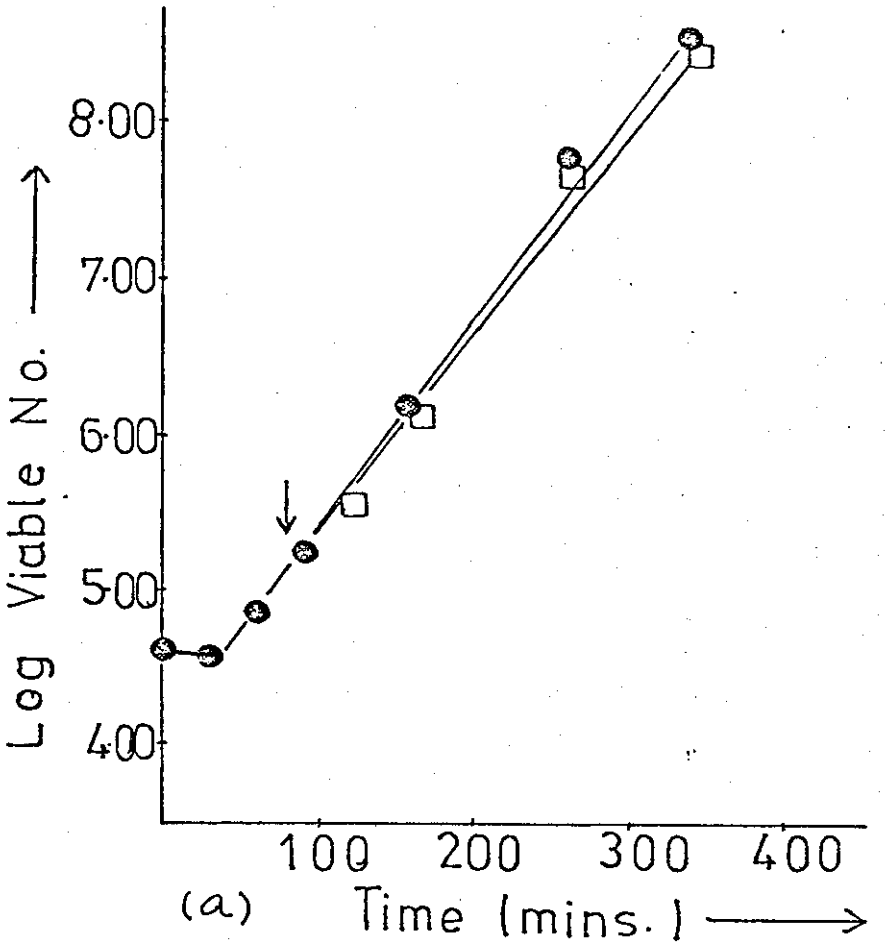
The effect of phenanthrene on growing bacterial cultures in nutrient broth, pH 7.4

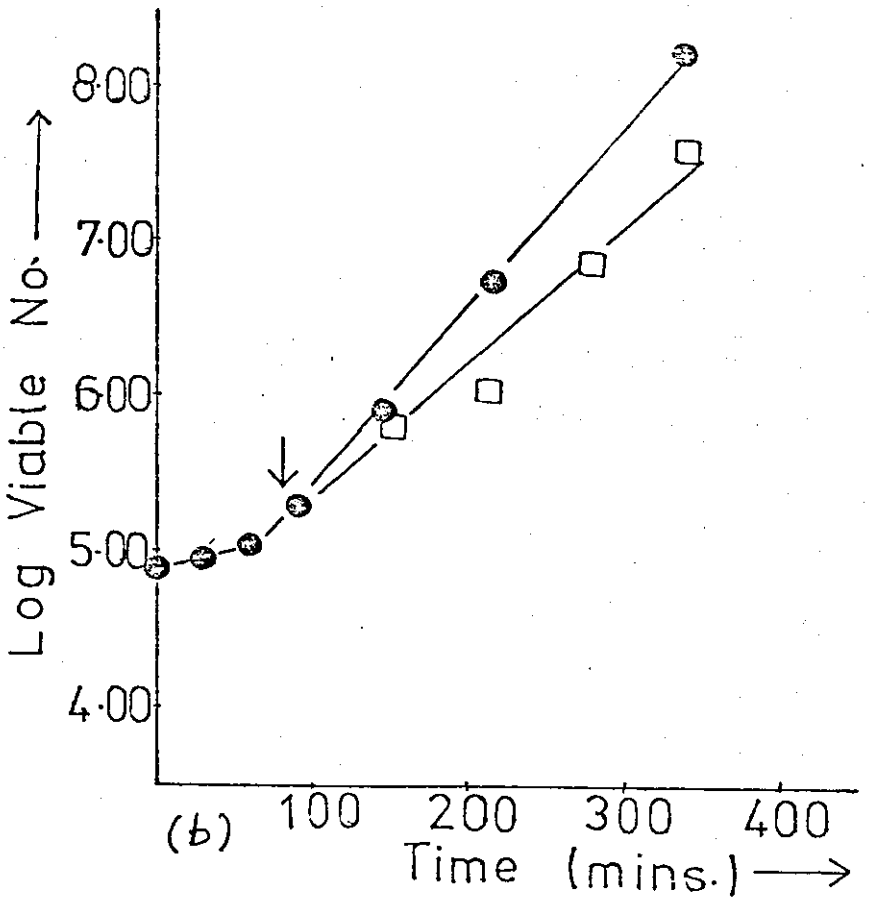
a) *E. coli*

●—●: control;
□—□: 100 µg/ml.

b) *S. aureus*

●—●: control;
□—□: 50 µg/ml.


8·00
7·00
6·00
5·00
4·00
Log Viable No.
100
200
300
400
(a)
Time (mins.)


8·00
7·00
6·00
5·00
4·00
Log Viable No.
100
200
300
400
(b)
Time (mins.)

Phenanthridine, while having no effect on an *E.coli* culture at 80 µg/ml, inhibited the growth of an *S.aureus* culture at the same concentration (Fig. 3.14). Similarly, 4,7-phenanthroline (at 100 µg/ml) inhibited the growth of *S.aureus*, while not affecting the *E.coli* culture. (Fig. 3.15).

9,10-Phenanthenequinone had a slight effect against *E.coli* at 40 µg/ml, but showed a bacteriostatic effect on *S.aureus*, even at 6 µg/ml (Fig. 3.16).

1,10-Phenanthroline had a lethal effect on both *E.coli* and *S.aureus* cells at 20 µg/ml and 40 µg/ml, respectively (Fig. 3.17); while 1,10-phenanthroline-5,6-quinone showed a sharp cidal effect at 4 µg/ml and 10 µg/ml, on both *E.coli* and *S.aureus* respectively, as can be seen from Fig. (3.18).

3.3 The Effect of 9,10-Phenanthrenequinone on Non-Growing Suspensions

As can be seen from Fig. (3.19) 9,10-phenanthrenequinone did not have any bactericidal effect on either *E.coli* or *S.aureus*.

3.4 The Effect of 9,10-Phenanthrenequinone on EDTA - Pretreated *E.coli* Cells

Fig. (3.20) shows the effect of 9,10-phenanthrenequinone at 30 µg/ml, on EDTA - pretreated *E.coli* culture. As can be seen, the quinone was no more active on the pretreated cells, than on the untreated ones.

Fig. (3.14)

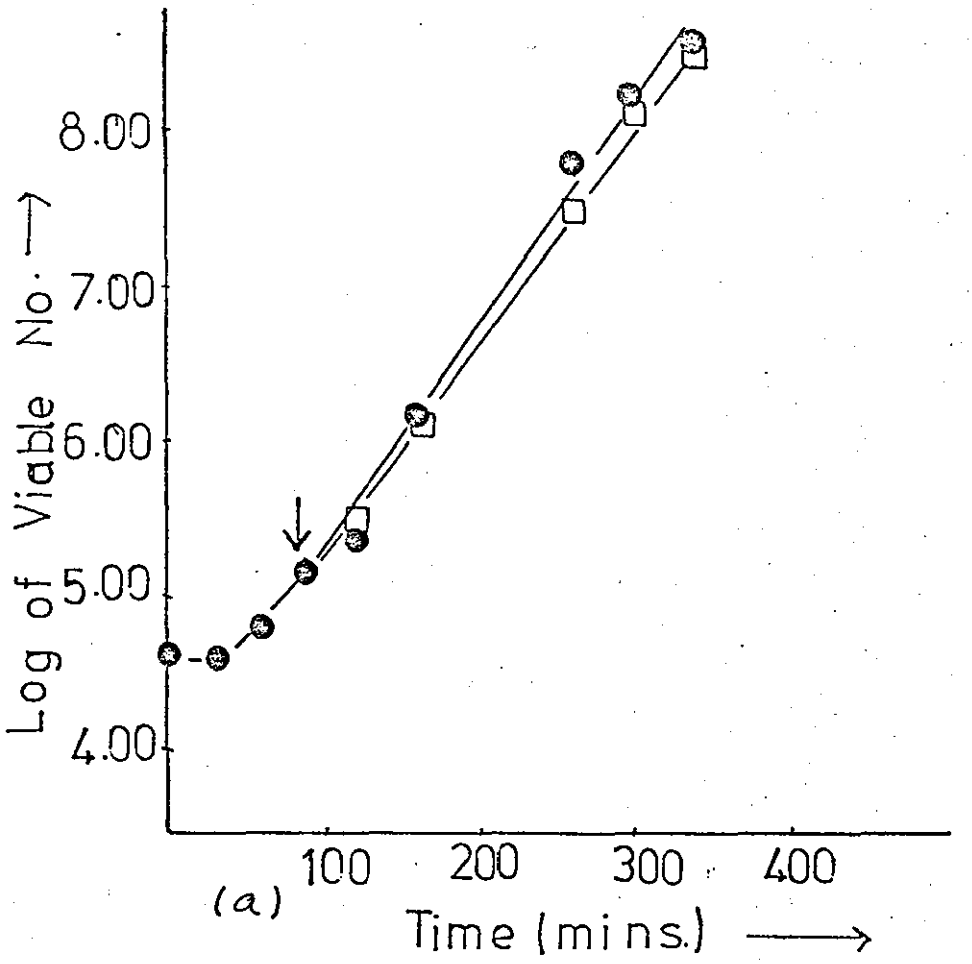
The effect of phenanthridine on growing bacterial cultures in nutrient broth, pH 7.4

a) *E. coli*

●—●: control;
□—□: 80 µg/ml.

b) *S. aureus*

●—●: control;
□—□: 80 µg/ml.


8.00
7.00
6.00
5.00
4.00
Log of Viable No. →
100
200
300
400
(a)
Time (mins.) ⟶

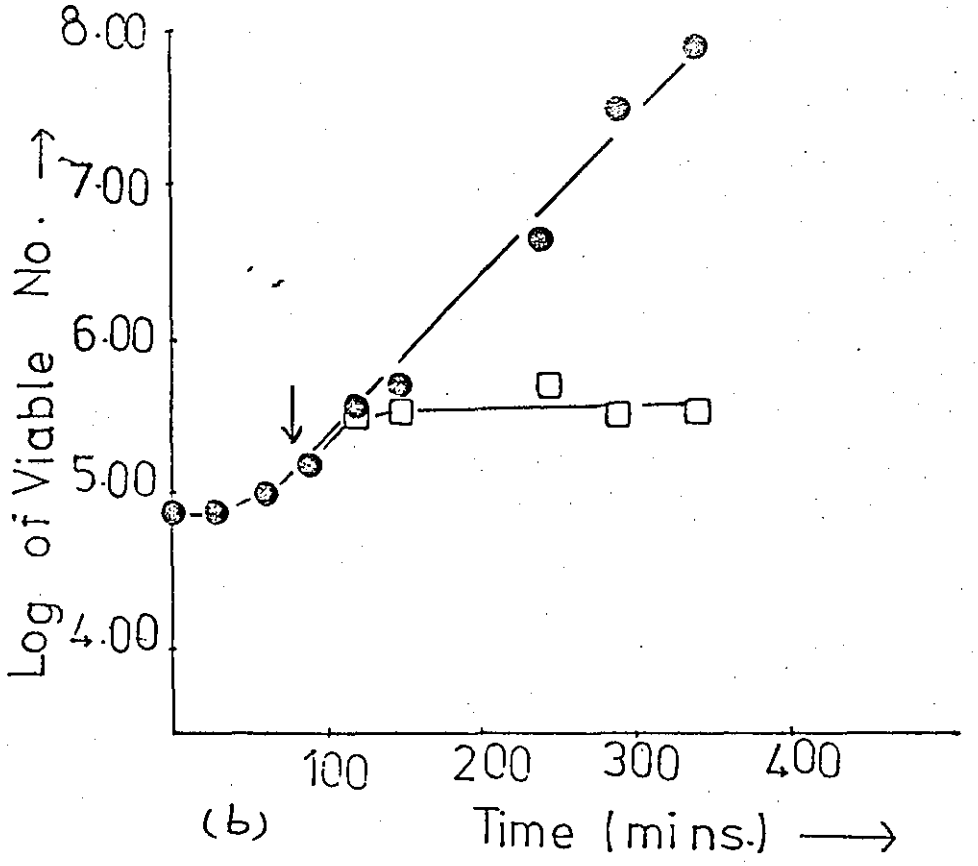

8.00
7.00
6.00
5.00
4.00
Log of Viable No. →
100
200
300
400
(b)
Time (mins.) ⟶

Fig. (3.15)

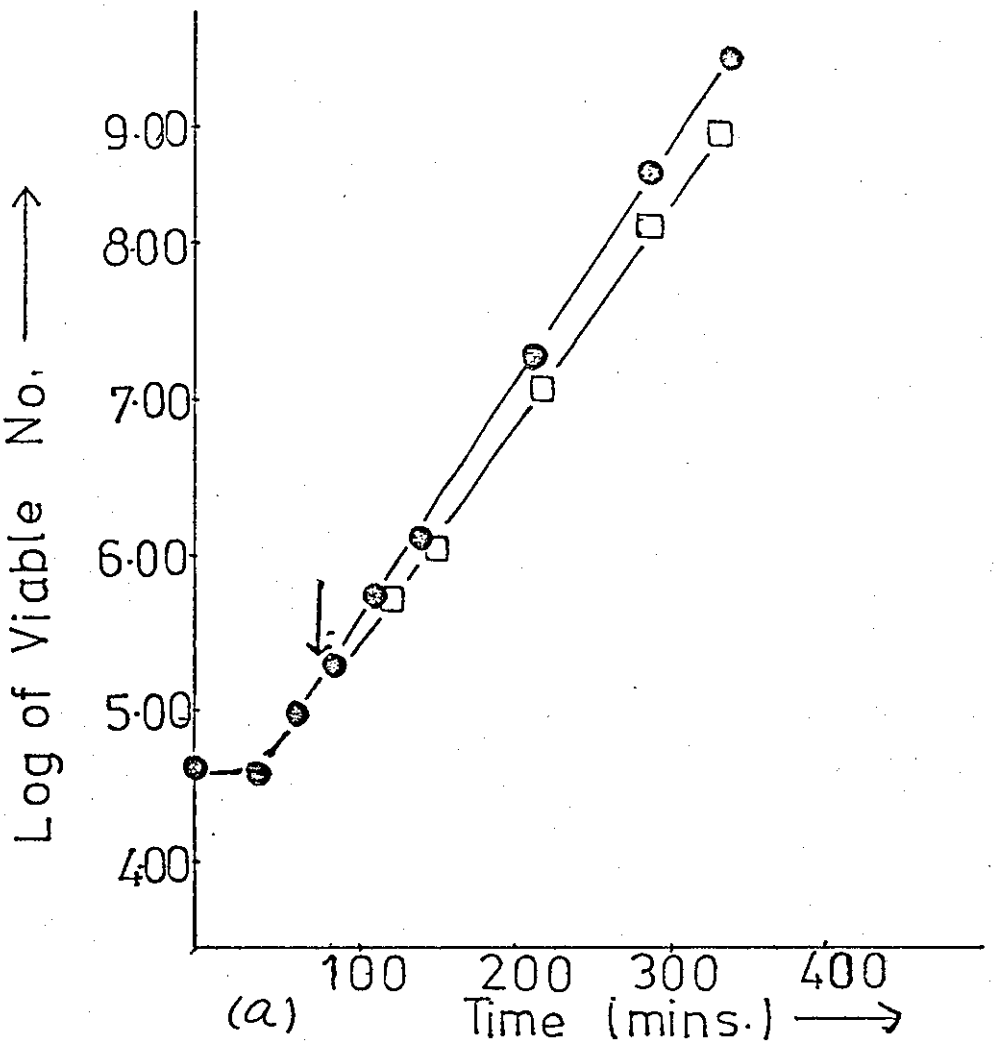
The effect of 4,7-phenanthroline on growing bacterial cultures in nutrient broth, pH 7.4

a) *E. coli*

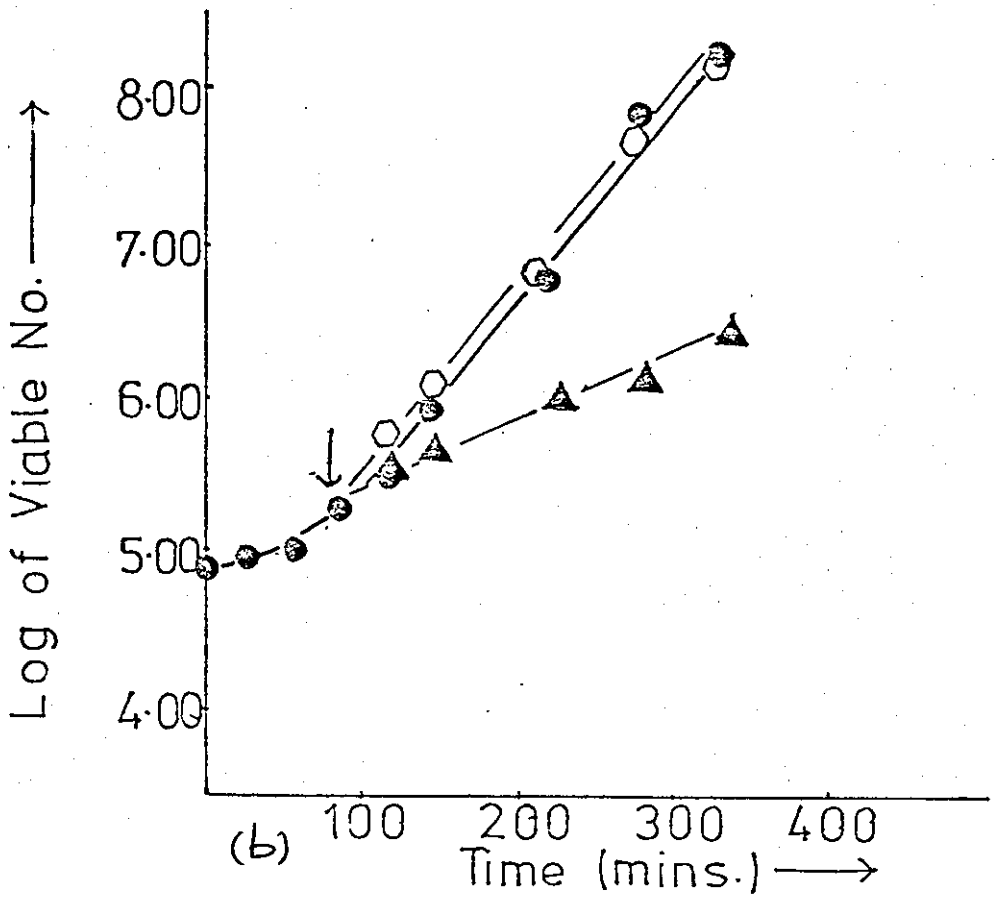
●—●: control;
□—□: 100 µg/ml.

b) *S. aureus*

○—○: control;
⬢—⬢: 50 µg/ml;
▲—▲: 100 µg/ml.


9·00
8·00
7·00
6·00
5·00
4·00
Log of Viable No. ⟶
100
200
300
400
Time (mins.) ⟶

(a)


8·00
7·00
6·00
5·00
4·00
Log of Viable No. ⟶
100
200
300
400
Time (mins.) ⟶

(b)

Fig. (3.16)

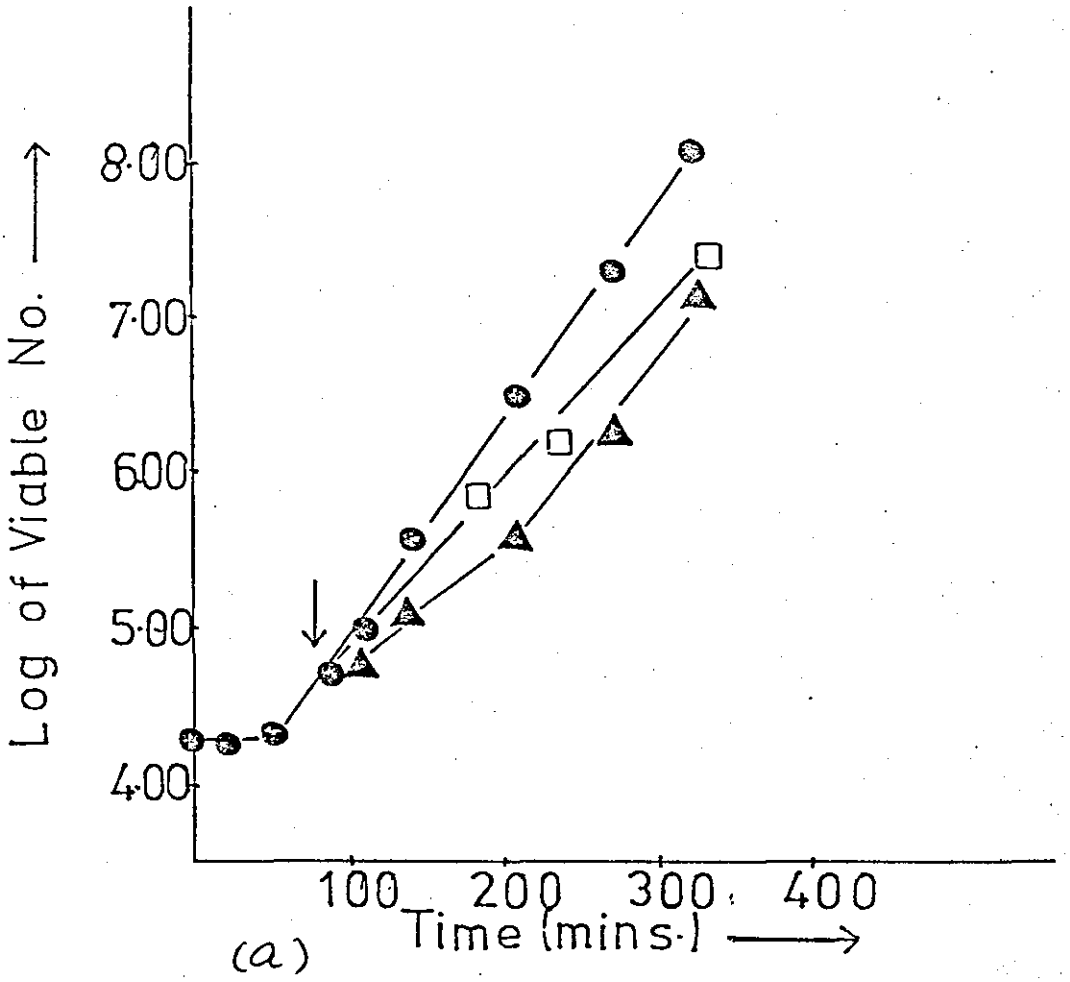
The effect of 9,10-phenanthrenequinone on growing bacterial cultures in nutrient broth, pH 7.4

a) *E. coli*

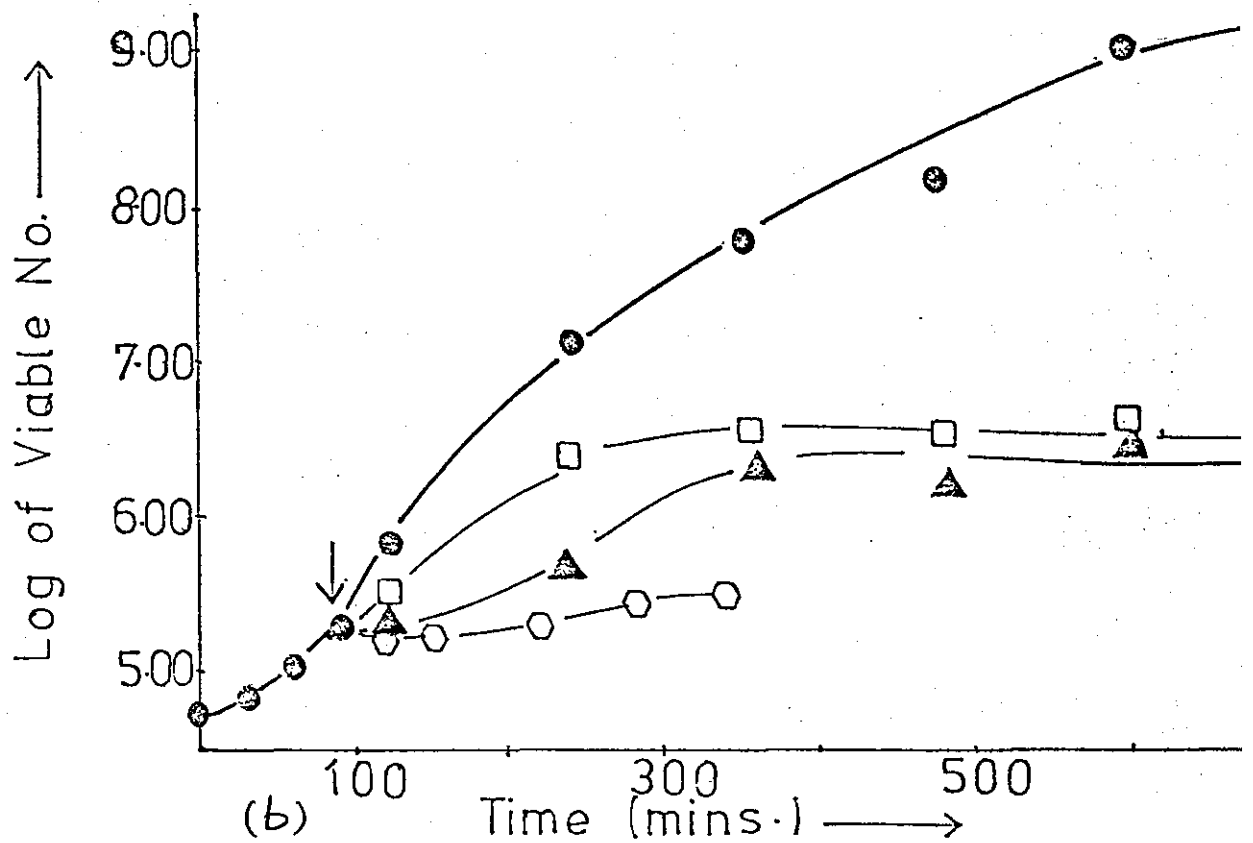
●—●: control;
□—□: 30 µg/ml;
▲—▲: 40 µg/ml.

b) *S. aureus*

●—●: control;
□—□: 6 µg/ml;
▲—▲: 12 µg/ml;
○—○: 20 µg/ml.


Log of Viable No. ⟶
8·00
7·00
6·00
5·00
4·00
100
200
300
400
Time (mins.) ⟶

(a)


Log of Viable No. ⟶
9·00
8·00
7·00
6·00
5·00
100
300
500
Time (mins.) ⟶

(b)

Fig. (3.17)

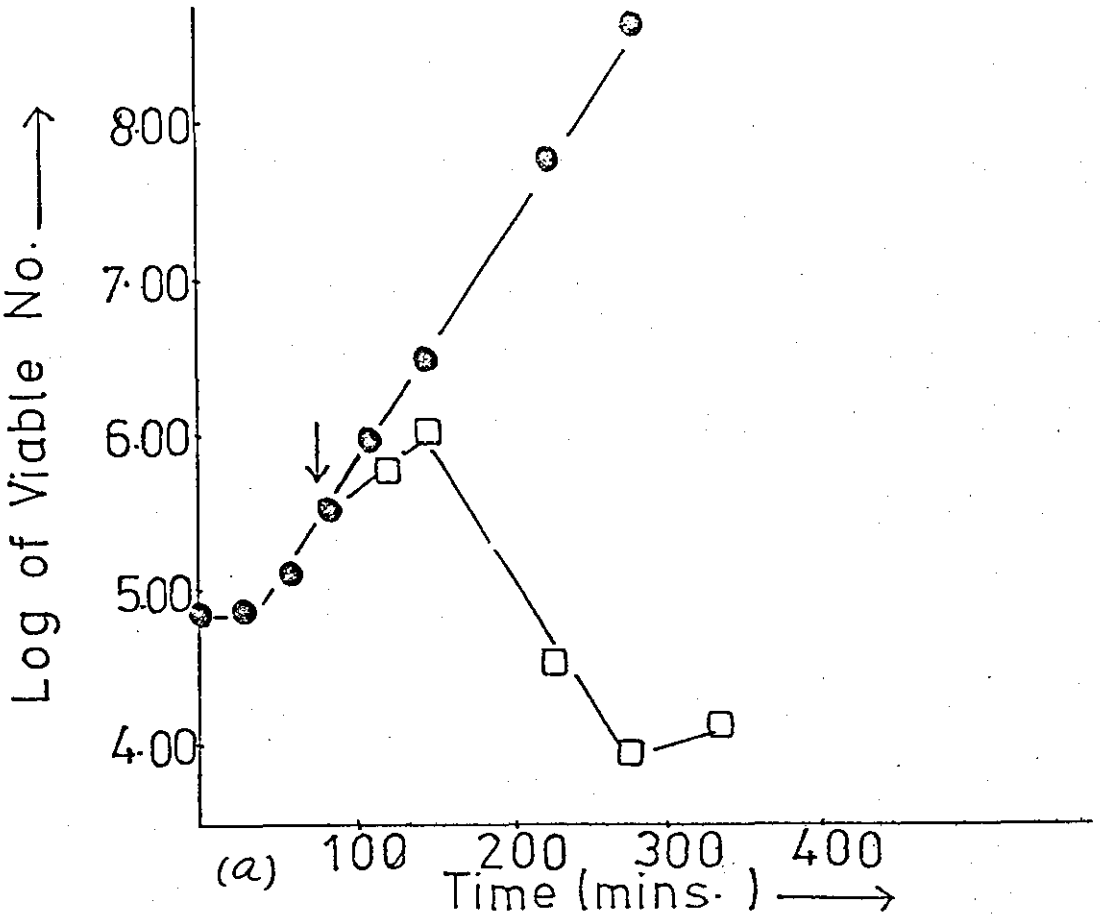
The effect of 1,10-phenanthroline on growing bacterial cultures in nutrient broth, pH 7.4

a) *E. coli*

●—●: control;
□—□: 20 µg/ml.

b) *S. aureus*

●—●: control;
□—□: 20 µg/ml;
▲—▲: 40 µg/ml.


Log of Viable No.
8·00
7·00
6·00
5·00
4·00
100
200
300
400
(a)
Time (mins.)

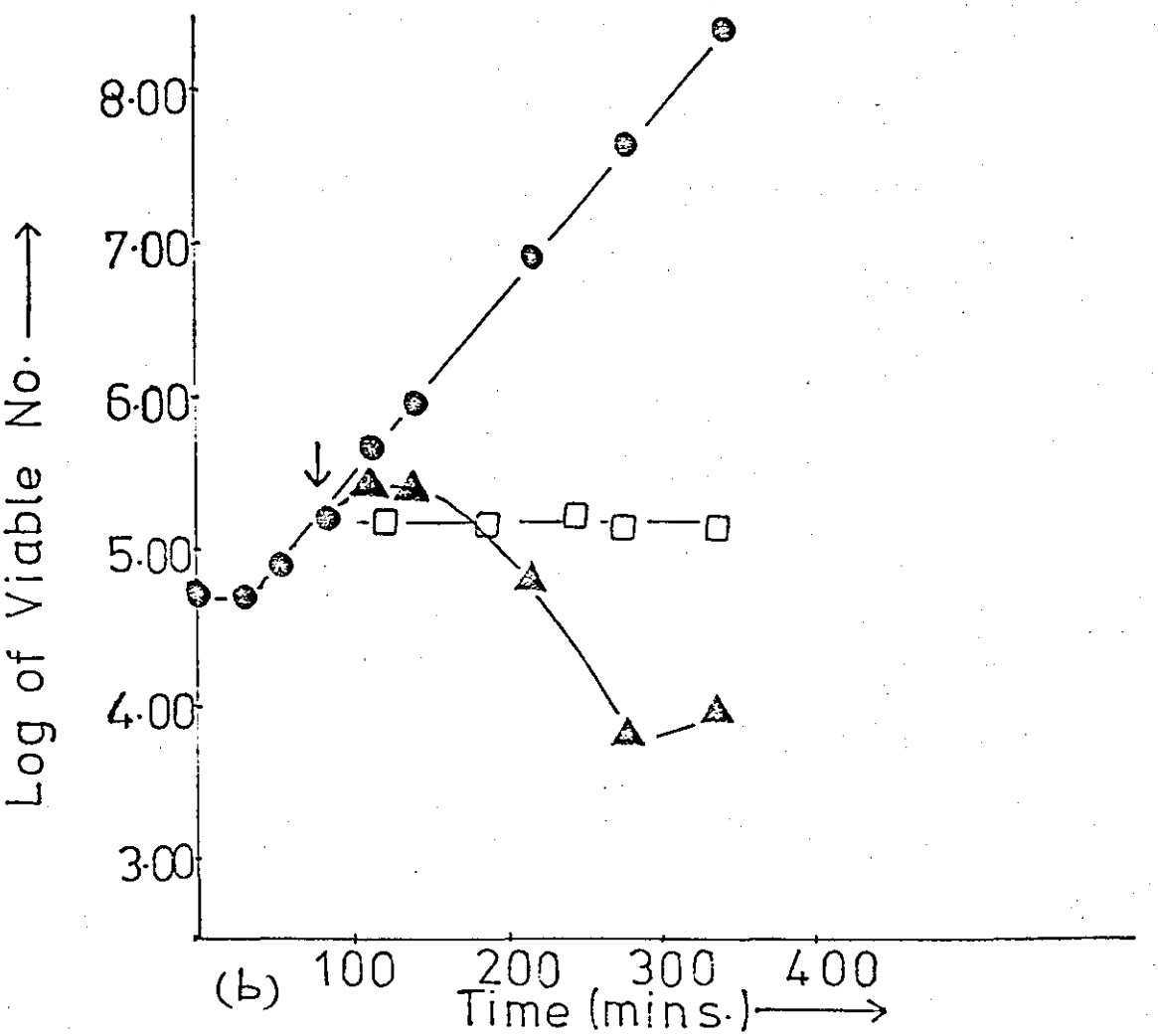

Log of Viable No.
8·00
7·00
6·00
5·00
4·00
3·00
100
200
300
400
(b)
Time (mins.)

Fig. (3.18)

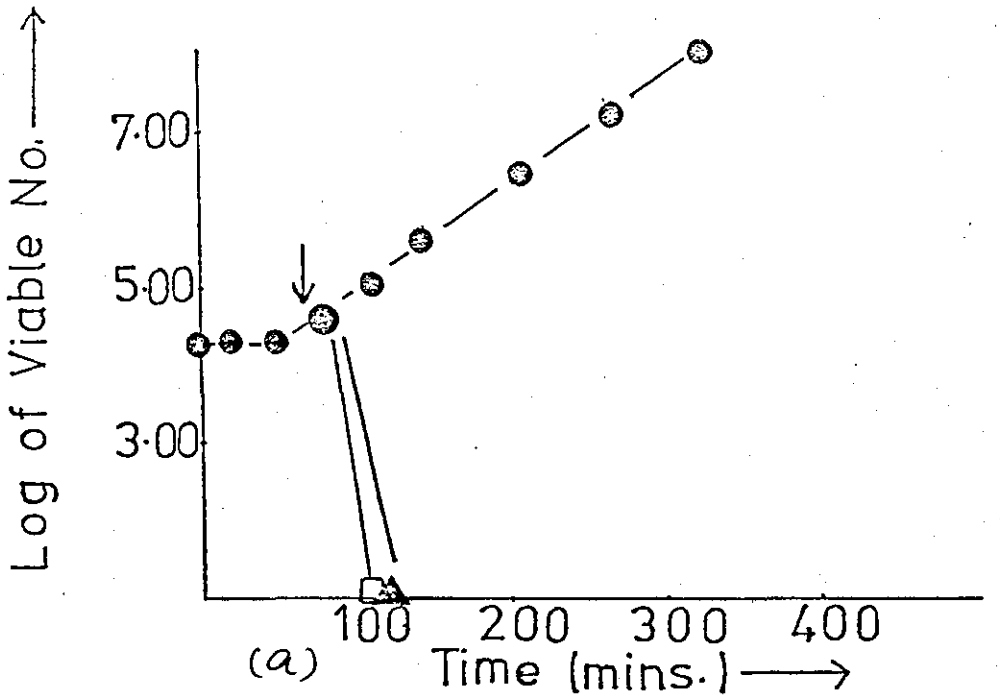
The effect of 1,10-phenanthroline-5,6-quinone on growing bacterial cultures in nutrient broth, pH 7.4

a) *E. coli*

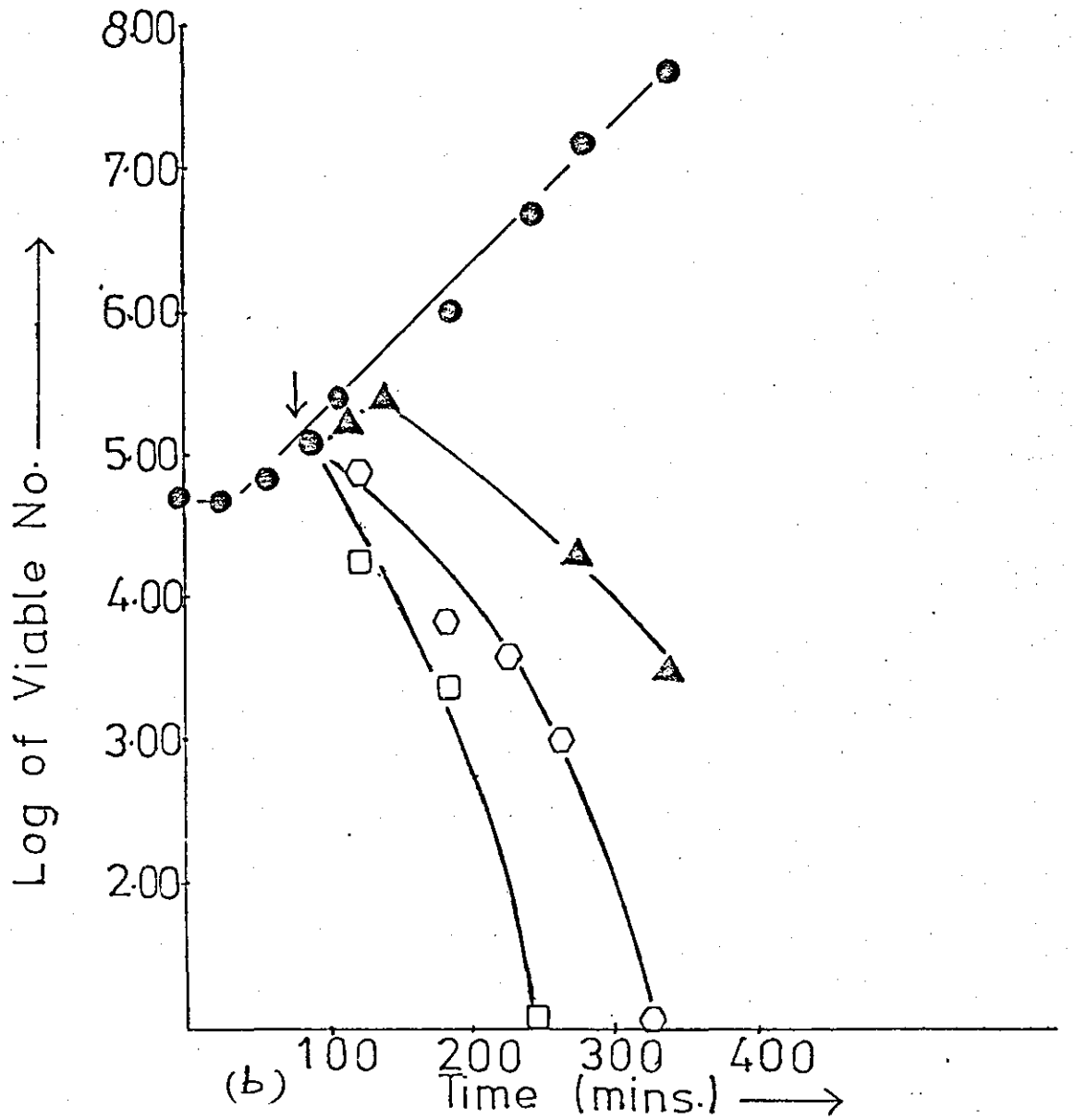
●—●: control;
▲—▲: 4 µg/ml;
□—□: 10 µg/ml.

b) *S. aureus*

●—●: control;
▲—▲: 4 µg/ml;
⬡—⬡: 10 µg/ml;
□—□: 20 µg/ml.


Log of Viable No.
7·00
5·00
3·00
100
200
300
400
Time (mins.)

(a)


Log of Viable No.
8·00
7·00
6·00
5·00
4·00
3·00
2·00
100
200
300
400
Time (mins.)

(b)

Fig. (3.19)

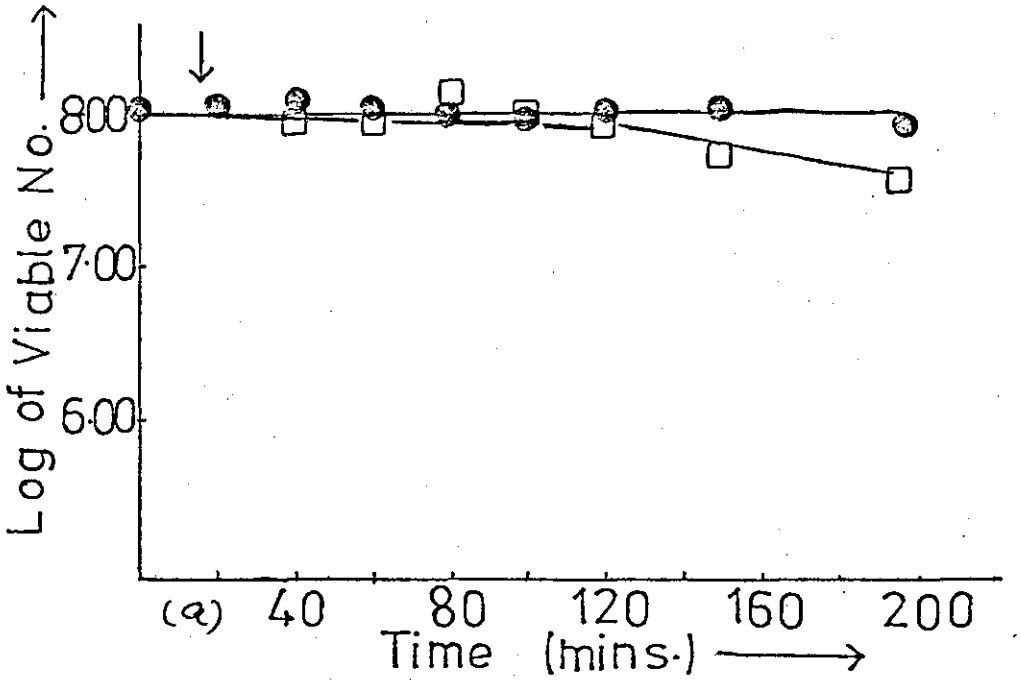
The effect of 9,10-phenanthrenequinone on non-growing bacterial cells suspended in PBS, pH 7.3

a) *E. coli*

●—●: control;
□—□: 40 µg/ml.

b) *S. aureus*

●—●: control;
□—□: 30 µg/ml.


Log of Viable No.
8·00
7·00
6·00
(a)
40
80
120
160
200
Time (mins.)

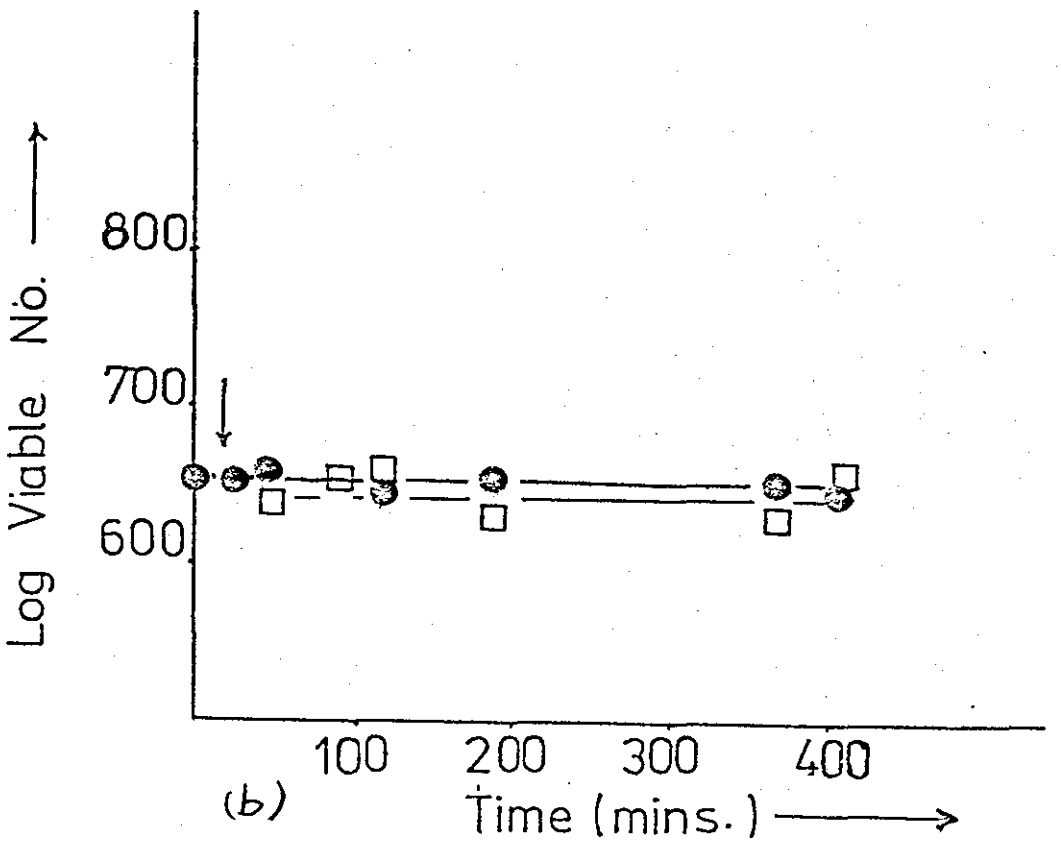
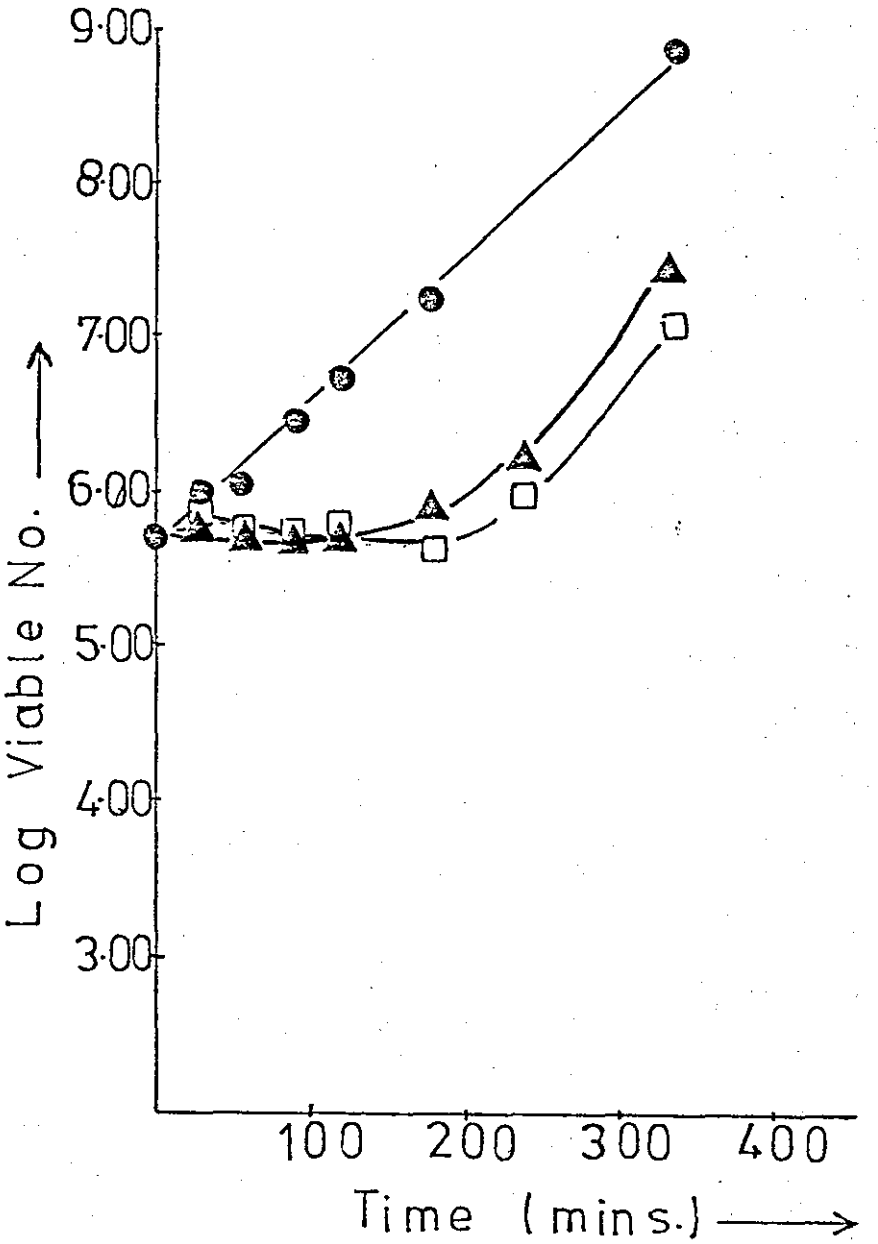

Log Viable No.
800
700
600
(b)
100
200
300
400
Time (mins.)

Fig. (3.20)

The effect of EDTA-pretreatment of *E. coli* on its resistance to 9,10-phenanthrenequinone.

● —— ● : control (EDTA-pretreated cells);
▲ —— ▲ : non-treated cells plus 30 µg/ml;
□ —— □ : EDTA-pretreated cells plus 30 µg/ml.


9·00
8·00
7·00
6·00
5·00
4·00
3·00
Log Viable No.
100
200
300
400
Time (mins.)

4. THE INTERACTION OF SOME ANTIBACTERIAL AGENTS AND ANTIBIOTICS WITH PHANQUONE

As can be seen from Table (3.10), Phanquone interacted neither synergistically nor antagonistically, with the antibacterial agents and the antibiotics tested, with the exception of ethidium bromide and 9,10-phenanthrenequinone where some synergism was noted (Fig. 3.21). In comparison, Figs. (3.22a) and (3.22b) show the additive effect between Phanquone and tetracycline and Vancomycin against *S. aureus* cultures.

Table (3.11) shows the drop in the MIC values of Phanquone, against *B.subtilis* and *B.cereus*, when combined with subinhibitory concentrations of 9,10-phenanthrenequinone, thus confirming the existence of some sort of a synergistic interation. Moreover, Fig. (3.23) shows the destruction of *B.subtilis* cells originally resistant to Phanquone, when a disc containing 9,10-phenanthrenequinone was placed nearby. Here, only the resistant colonies nearer to the 9,10-phenanthrenequinone disc were destroyed, while the confluent growth in between the two zones of inhibition remained intact.

Table (3.10)

The interaction of some common antibacterial agents and antibiotics with Phanquone; using the right-angled strips method.

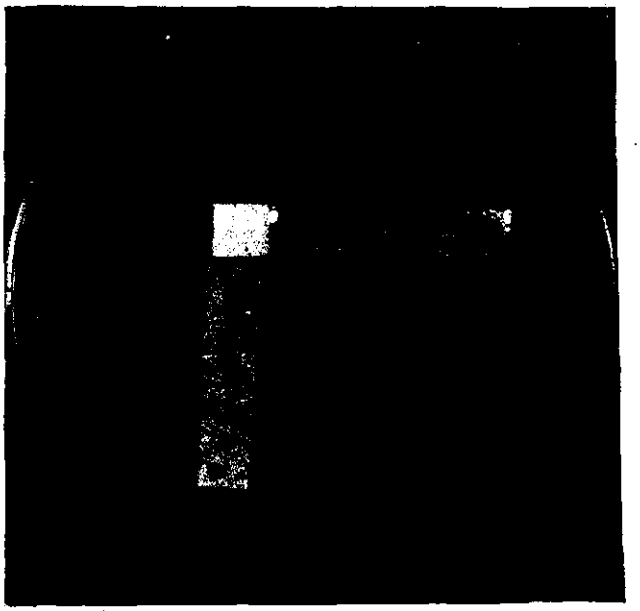
S denotes synergism;
N denotes no effect.

Strip A soaked in	STRIP B soaked in	Type of interaction	
		E. coli	*S. aureus*
Phanquone (2000µg/ml)	Acriflavine	N	N
	Ampicillin	N	N
	Chlorotetracycline	N	N
	Clindomycin	N	N
	Cloxacillin	N	N
	CTAB	N	N
	D-Cycloserine	N	N
	Ethidium Bromide	N	S
	Fosfomycin	N	N
	Gentamycin	N	N
	Lincomycin	N	N
	Methicillin	N	N
	Oxine	N	N
	Penicillin G	N	N
	9,10-phenanthrene-quinone	N	S
	1,10-phenanthroline	N	N
	1,10-phenanthroline-5,6-quinone	N	N
	Rifampicin	N	N
	Streptomycin	N	N
	Vancomycin	N	N

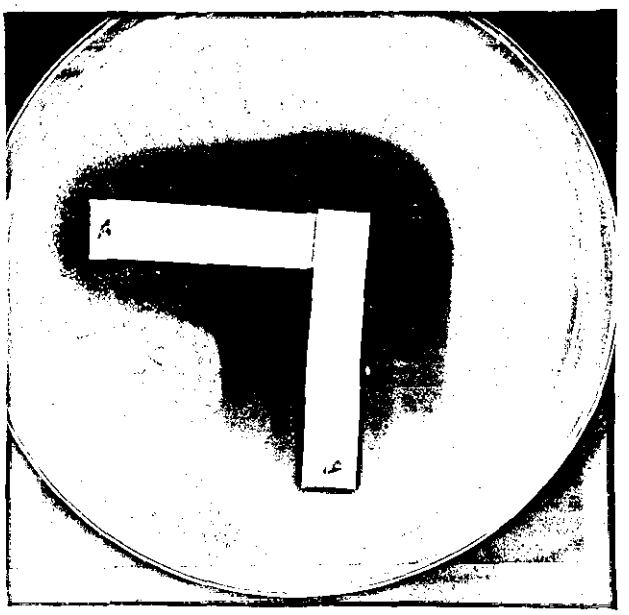
Fig. (3.21)

The synergistic interaction against *S.aureus* between Phanquone (strip A, soaked in 2000 µg/ml) and

a) 9,10-phenanthrenequinone (strip B, soaked in 2000 µg/ml);
b) ethidium bromide (strip B, 1000 µg/ml).



(a)

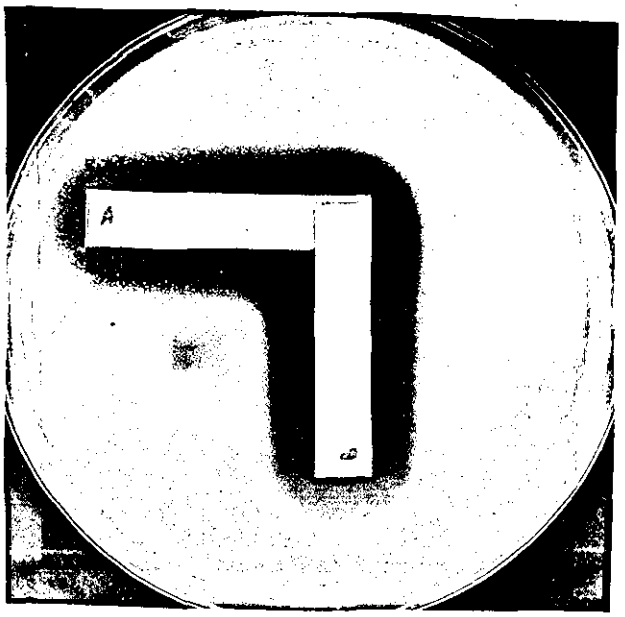


(b)

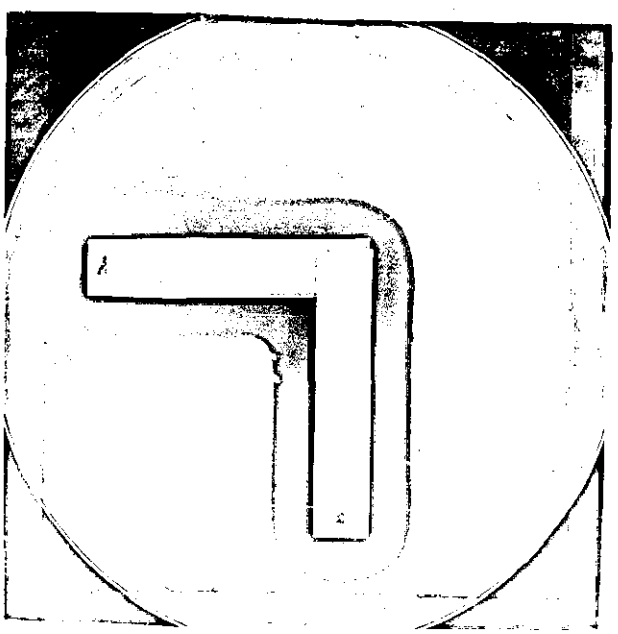
Fig. (3.22)

The no-effect interaction against *S. aureus* between Phanquone (strip A, 2000 µg/ml) and

a) chlorotetracycline (strip B, 1000 µg/ml);
b) vancomycin (strip B, 1000 µg/ml).



(a)



(b)

Table (3.11)

The effect of sub-inhibitory concentrations of 9,10-phenanthrenequinone on the MIC and MCC values of Phanquone for *B. subtilis* and *B. cereus*.

Fig. (3.23)

The synergistic interaction between Phanquone and 9,10-phenanthrenequinone against *B. subtilis* on nutrient agar.

A : Phanquone disc (50 µg);

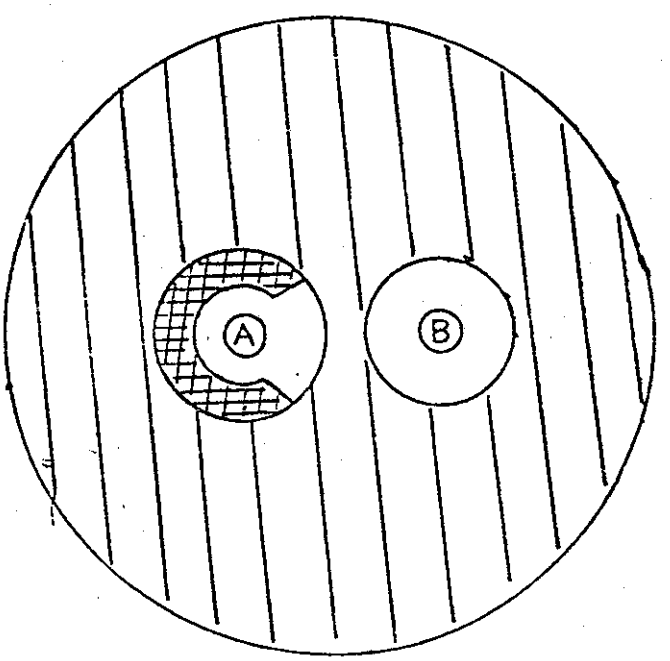
B : 9,10-phenanthrenequinone disc (50 µg);

: confluent growth;

: hazy zone of inhibition (with resistant colonies);

: clear zone of inhibition;

Organism	Antibacterial (s)	MIC µg/ml	MCC µg/ml
B. subtilis	Phanquone(PQ)	50	200
	9,10-phenanthrene-quinone(Phq)	0.8	3.1
	PQ + Phq(0.25µg/ml)	25	50
B. cereus	PQ	100	200
	Phq	0.4	12.5
	PQ+Phq(0.2µg/ml)	25	200



5. THE INTERACTION OF SOME THIOL-CONTAINING COMPOUNDS WITH PHANQUONE

Table (3.12), and Fig. (3.24) show the antagonistic effect exerted by some thiol-containing compounds on the zone of inhibition produced by Phanquone. However, this antagonistic effect was exerted for *E.coli* cultures only, while thiol-containing compounds had no effect on the activity of Phanquone against *S.aureus*.

Moreover, Fig. (3.25) shows the effect of the addition of 2×10^{-3}M thioglycollic acid, on the activity of Phanquone against a non-growing *E.coli* culture; the thiglycollic acid seemed to antagonize further action of Phanquone, thus allowing the *E.coli* cells to survive.

6. THE EFFECT OF PHANQUONE ON SOME METABOLIC ACTIVITIES OF THE BACTERIAL CELL

6.1 The Effect on Oxygen Consumption

Figs. (3.26) and (3.27) show the effect of different concentrations of Phanquone on the oxygen consumption by *E.coli* and *S.aureus*, respectively, using D-glucose (0.02M) as a substrate. Phanquone - while having no effect at 16 µg/ml and 32 µg/ml - inhibited the oxygen consumption by nearly 25%, at 64 µg/ml, for *E.coli*. As for *S.aureus*, there was between 35% and 45% inhibition, with Phanquone at 25 µg/ml, 50 µg/ml and 100 µg/ml.

When succinate (0.02M) was used as a substrate (c/o Fig. 3.28), there was a stimulation in the oxygen consumption by *E.coli* cells, by nearly 80%, 57% and 25%, in the presence of Phanquone at 8 µg/ml, 16 µg/ml and 32 µg/ml, respectively. However, Phanquone, at 64 µg/ml,

Table (3.12)

The effect of thiol-containing compounds on the zone of inhibition produced by Phanquone on nutrient agar using the right-angled strips method.

\+ : antagonism;
\- : no effect.

STRIP A soaked in	STRIP B soaked in	Effect on zone of Inhibition	
		E. coli	S .aureus
Phanquone (2000 µg/ml)	Thioglycollic Acid, pH 6.5, (0.2M)	+	-
	Soduim Thioglycollate (0.2M)	+	-
	2-Mercaptoethanol (0.02M)	+	-
	1-Thioglycerol (0.1M)	+	-
	L-Cysteine (0.02M)	+	-

Fig. (3.24)

The antagonism produced by thiol-containing compounds on the zone of inhibition produced by Phanquone (strip A, 2000 µg/ml) against *E. coli*, on nutrient agar.

a) thioglycollic acid (strip B, 0.2M solution);
b) 1-thioglycerol (strip B, 0.1M solution).

Fig. (3.25)

The effect of thioglycollic acid (neutralized) on the activity of Phanquone against non-growing *E. coli* cells suspended in PBS, pH 7.3.

●——●: thioglycollic acid (pH 6.0, 2 x 10^{-3}M) only;

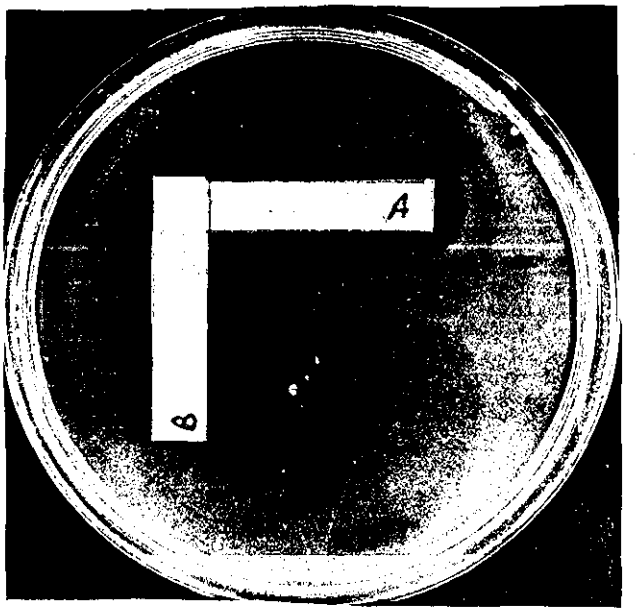
△——△: Phanquone (1 µg/ml) only;

■——■: Phanquone (1 µg/ml) followed by thioglycollic acid (2 x 10^{-3}M);

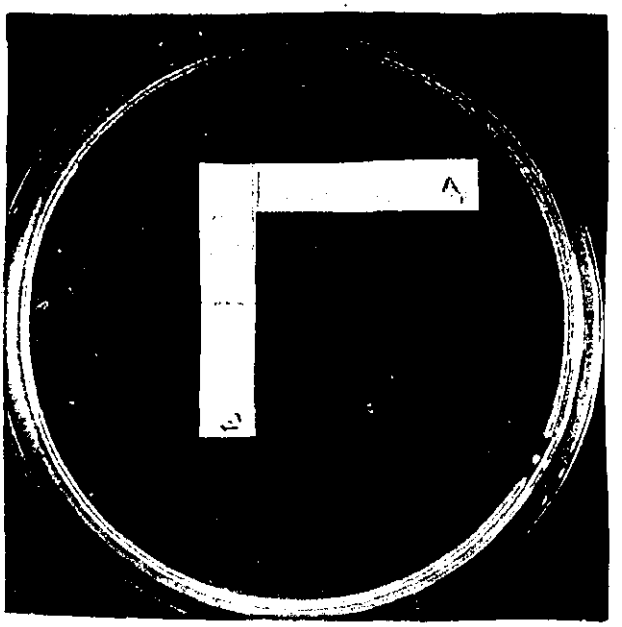
↓ : Phanquone added;

⇣ : thioglycollic acid added.

(a)



(b)



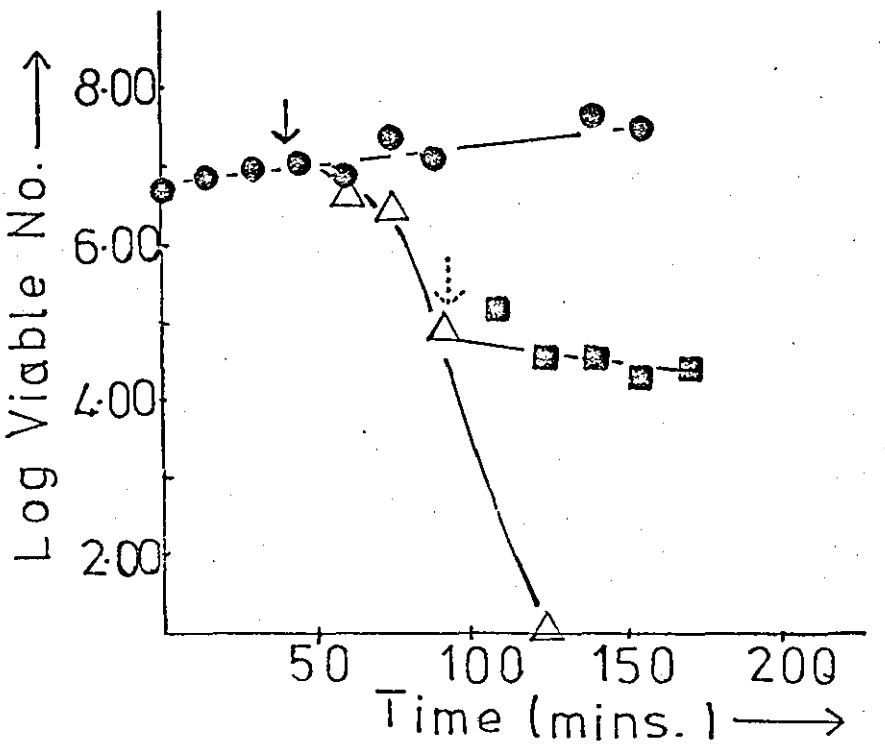


Fig. (3.26)

Effect of Phanquone on oxygen consumption by *E. coli* suspended in 0.1M sodium phosphate buffer, pH 7.4, using D-glucose (0.02M) as substrate.

□—□ : control;
▲—▲ : 16 µg/ml;
⬡—⬡ : 32 µg/ml;
○—○ : 64 µg/ml;
●—● : endogenous respiration.

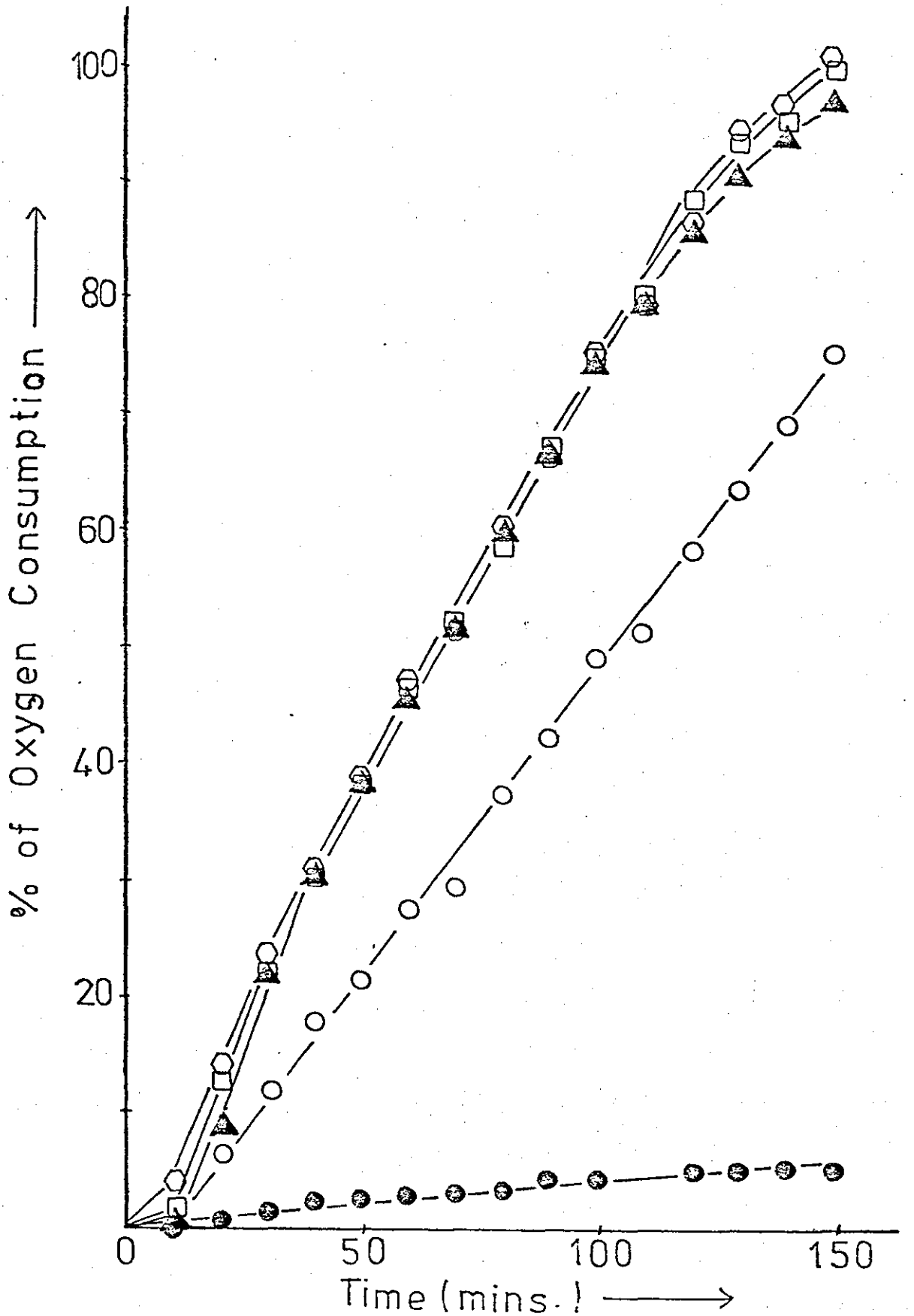
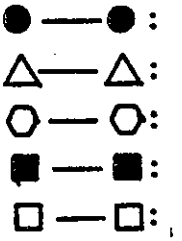

100
80
60
40
20
% of Oxygen Consumption
0
50
100
150
Time (mins.)

Fig. (3.27)

Effect of Phanquone on the oxygen consumption by *S. aureus* suspended in 0.1M sodium phosphate buffer, pH 7.4, using D-glucose (0.02M) as substrate



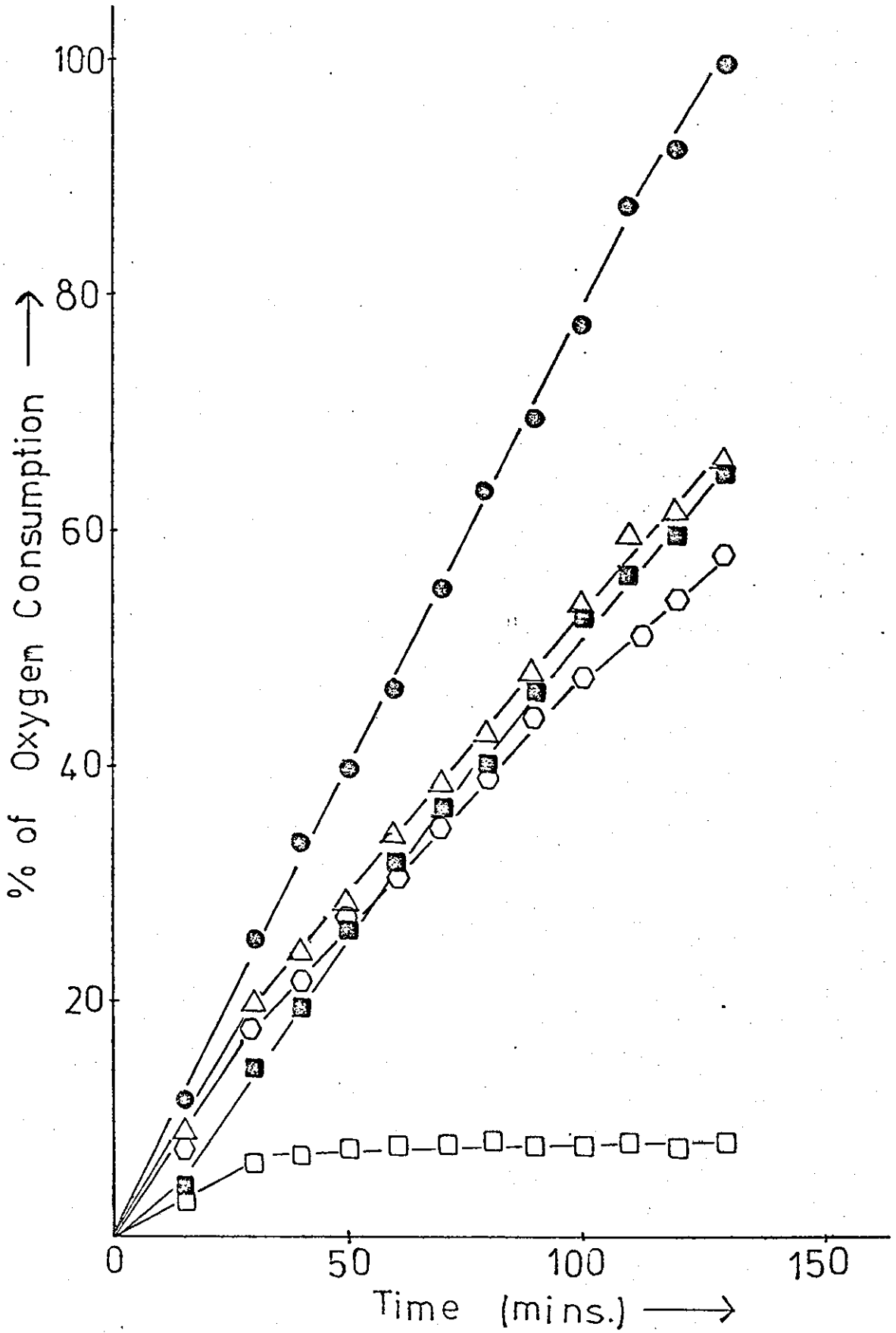
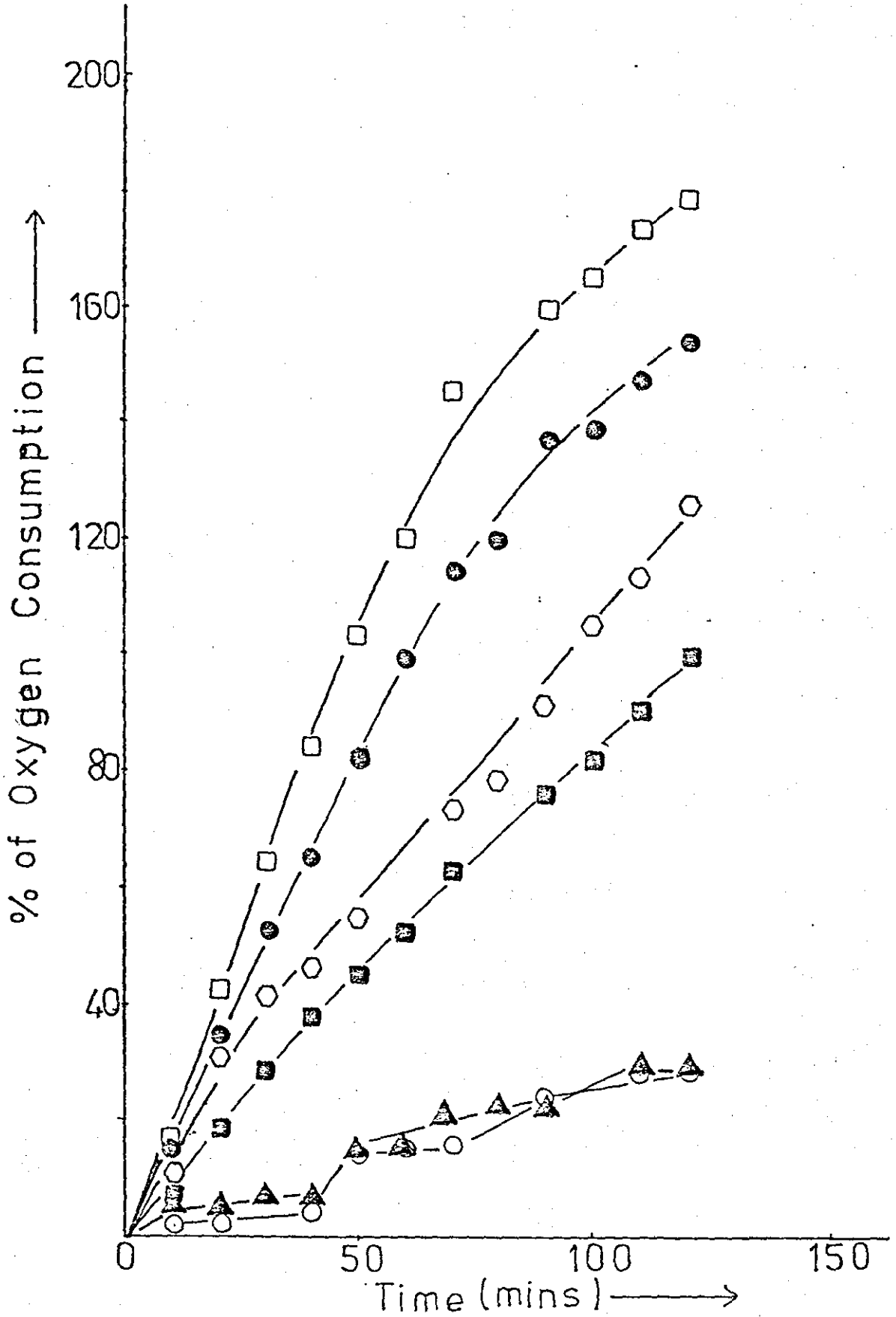

100
80
60
40
20
% of Oxygen Consumption
0
50
100
150
Time (mins.)

Fig. (3.28)

Effect of Phanquone on the oxygen consumption by *E.coli* suspended in 0.1M sodium phosphate buffer, pH 7.4, using sodium succinate (0.02M) as substrate.

■—■ : control;
□—□ : 8 µg/ml;
●—● : 16 µg/ml;
⬡—⬡ : 32 µg/ml;
○—○ : 64 µg/ml;
▲—▲ : endogenous respiration.


200
160
120
80
40
% of Oxygen Consumption
0
50
100
150
Time (mins)

inhibted the oxygen consumption by about 70%.

Figs. (3.29) and (3.30) show the effect of Phanquone on the oxygen consumption, using a 0.06 M succinate solution as a substrate, by *E. coli* and *S. aureus*, respectively. Again, Phanquone, at 16 µg/ml, stimulated the consumption by *E. coli* by about 8%, while inhibiting it (the consumption), by about 17%, 77% and 90%, at 32 µg/ml, 64 µg/ml, and 100 µg/ml, respectively. For *S. aureus*, however, Phanquone at 25 µg/ml, 50 µg/ml, and 100 µg/ml, inhibited the amount of oxygen consumed by around 47%, 60% and 80%, respectively.

Fig. (3.31) shows the effect of Phanquone on the endogeneous respiration of *Ps. aeruginosa*; there was about 23% inhibition in the presence of Phanquone at 32 µg/ml.

6.2 The Effect on Some Dehydrogenases Activities

Figs. (3.22), (3.33), (3.34), (3.35) and (3.36) show the effect of Phanquone - at 16 µg/ml - on *E. coli* dehydrogenases activities using 0.02 M solutions of D-glucose, sodium succinate, sodium malate, sodium pyruvate and sodium lactate as substrates, respectively. On one hand, there was a 20% stimulation of activity when either glucose or pyruvate were used; on the other hand, there was around 32%, 58% and 70% inhibition of the dehydrogenases activities, in the presence of Phanquone, when lactate, succinate and malate were used, respectively. When glucose-6-phosphate was used, there was either little or no red colour formation.

As for *S. aureus*, there was no, or very little, red colour formed (even with higher concentrations of substrate), and therefore meaningful results could not be obtained. However, when a 0.05 M solution of D-glucose was used, enough colour developed to allow for the results to be plotted (Fig. 3.37); the dehydrogenase activity was stimulated

Fig. (3.29)

Effect of Phanquone on the oxygen consumption by *E. coli* suspended in 0.1M sodium phosphate buffer, pH 7.4, using sodium succinate (0.06M) as substrate.

○—○ : control;
■—■ : 16 µg/ml;
▲—▲ : 32 µg/ml;
△—△ : 64 µg/ml;
□—□ : 100 µg/ml.

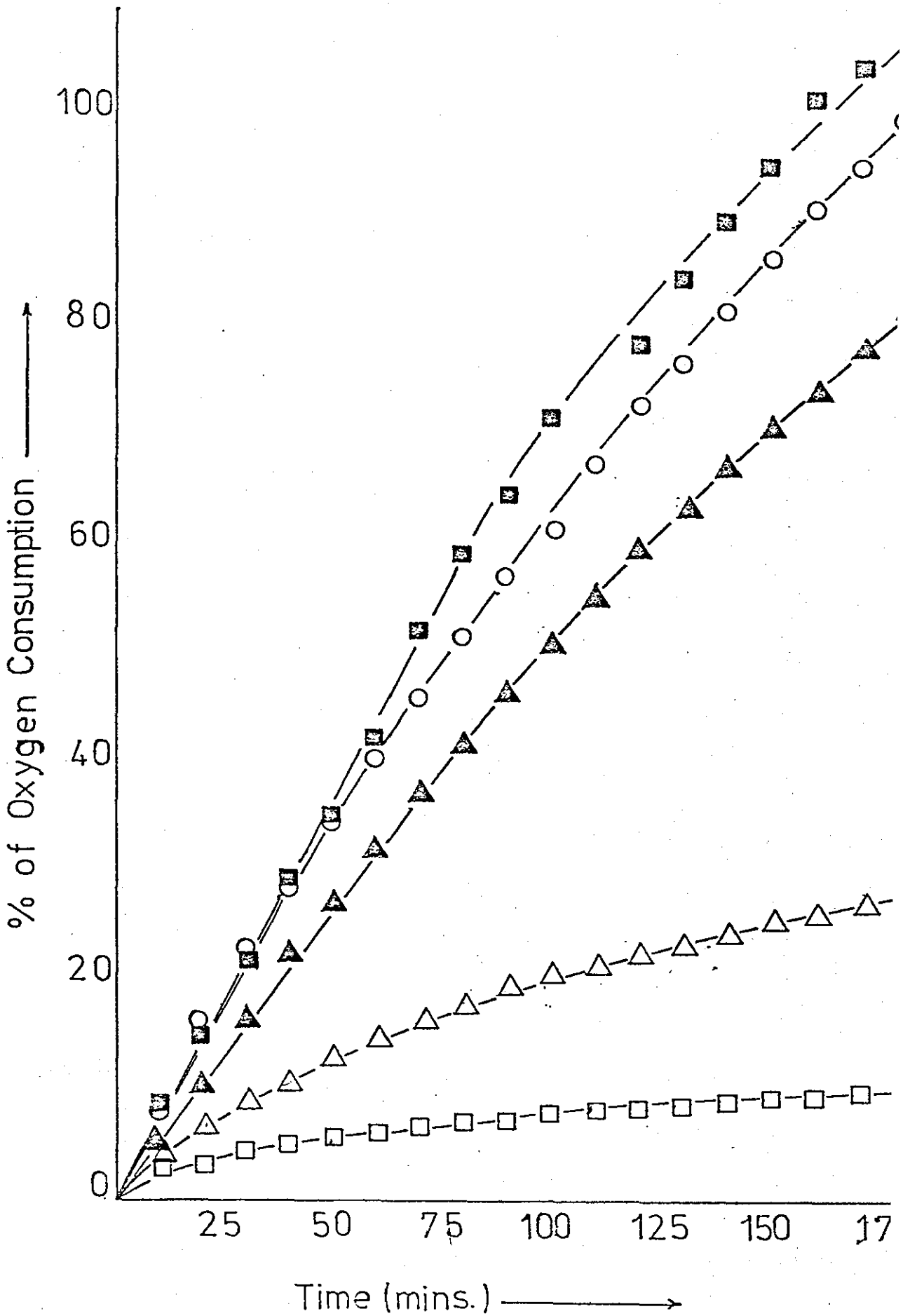

100
80
60
40
20
0
% of Oxygen Consumption
25
50
75
100
125
150
Time (mins.)

Fig. (3.30)

Effect of Phanquone on the oxygen consumption by *S. aureus* suspended in 0.1M sodium phosphate buffer pH 7.4, using sodium succinate (0.06M) as substrate.

△—△ : control;
●—● : 25 µg/ml;
□—□ : 50 µg/ml;
▲—▲ : 100 µg/ml;
○—○ : endogenous respiration.

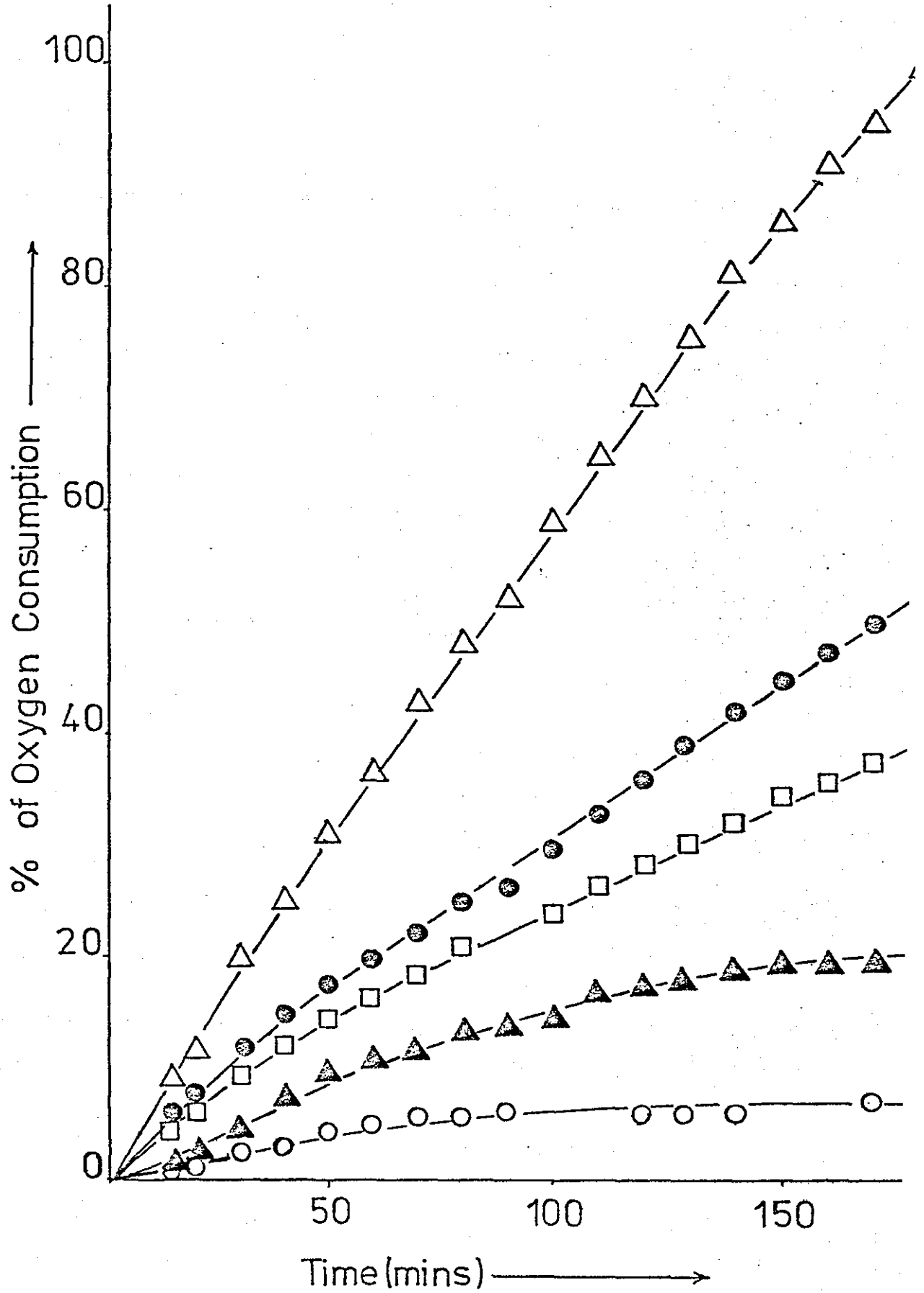

100
80
60
40
20
0
% of Oxygen Consumption
50
100
150
Time (mins)

Fig. (3.31)

Effect of Phanquone on the oxygen consumption by *Ps. aeruginosa* suspended in 0.1M sodium phosphate buffer, pH 7.4.

▲—▲ : endogenous respiration;

O—O : endogenous respiration in presence of 32 µg/ml.

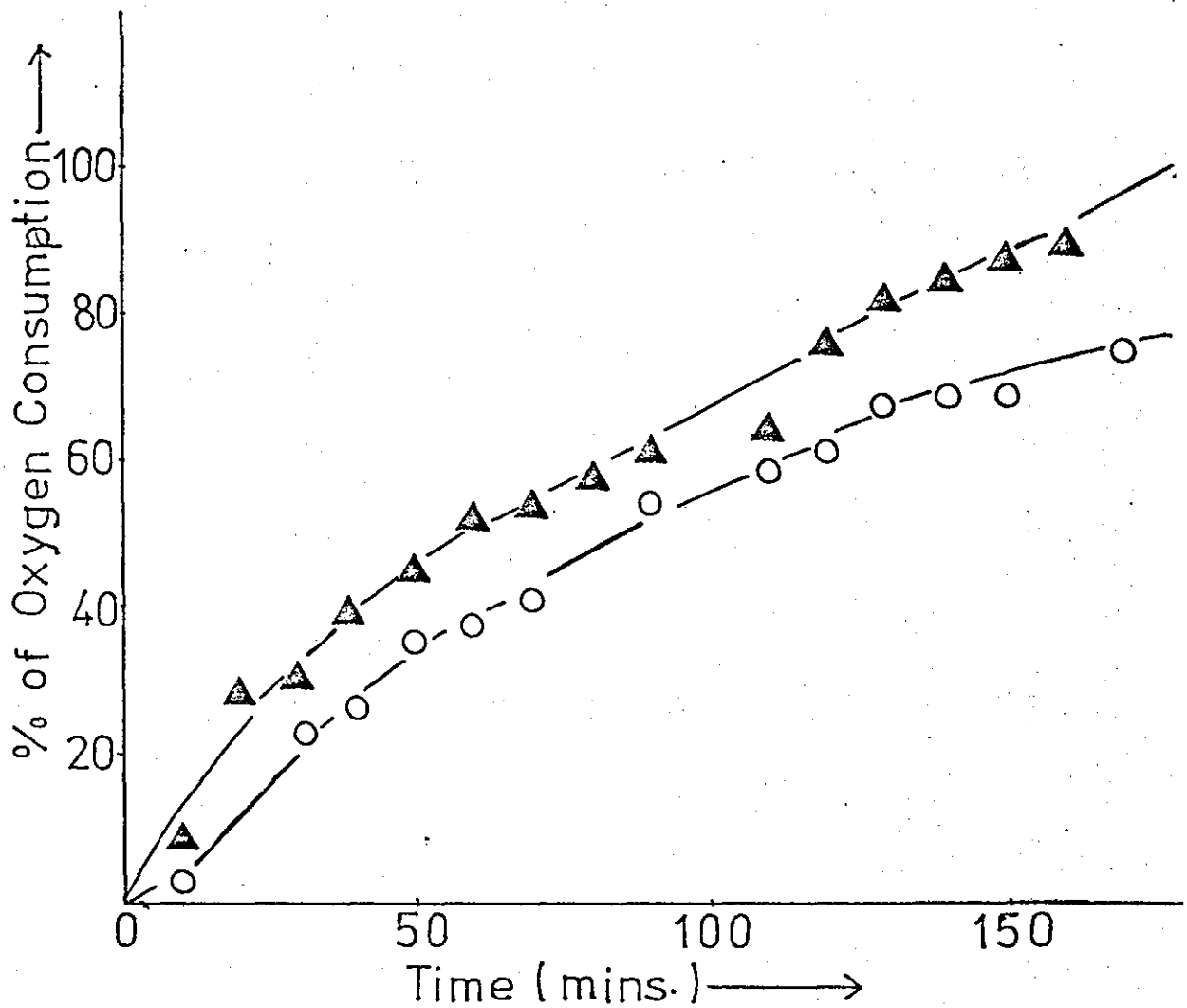

% of Oxygen Consumption
100
80
60
40
20
0
50
100
150
Time (mins.)

Fig. (3.32)

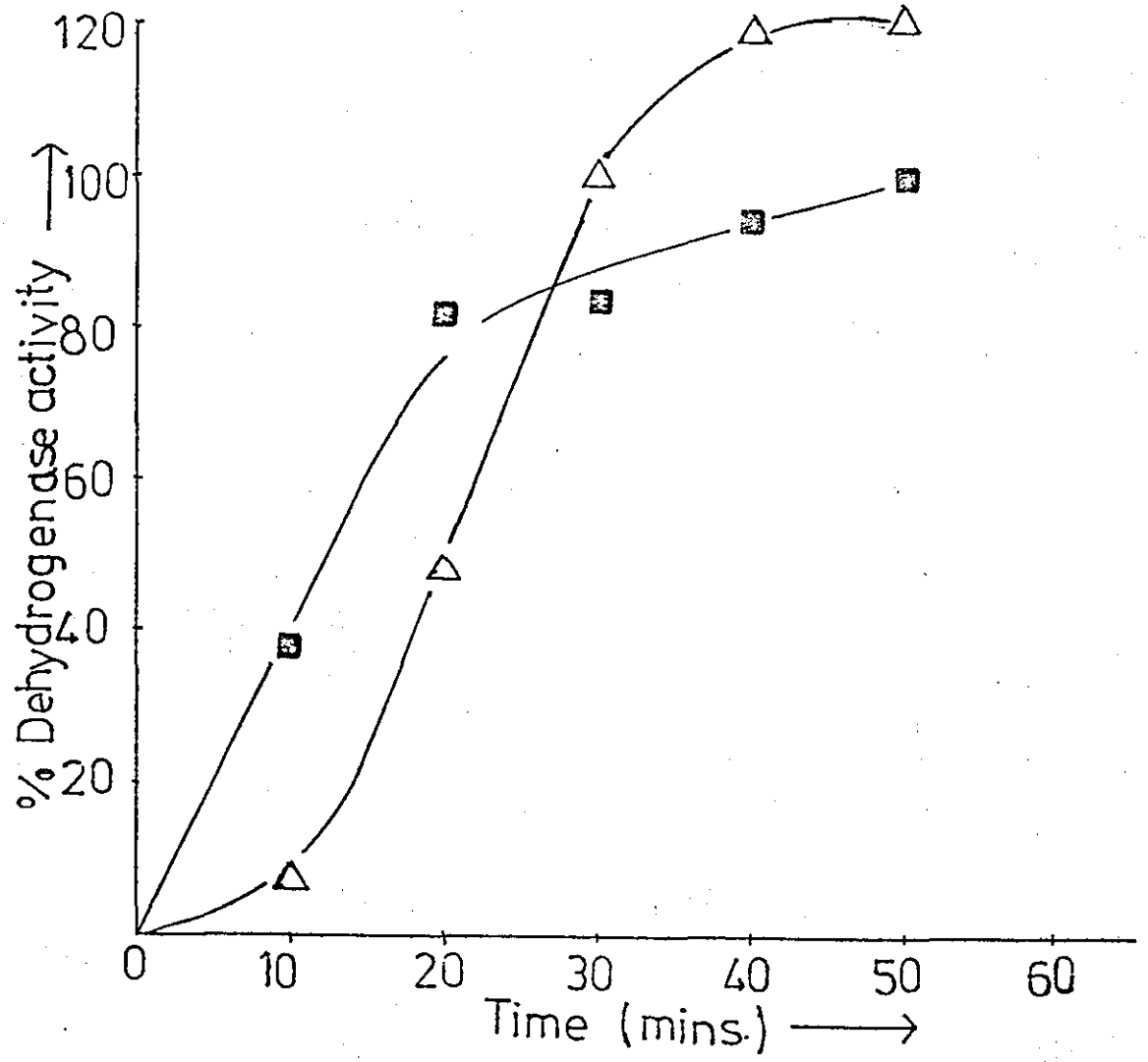
Effect of Phanquone on *E. coli* dehydrogenase activity using D-glucose (0.02M) as substrate

■—■ : control;
△—△: 16 µg/ml;
medium : PBS, pH 7.3

Fig. (3.33)

Effect of Phanquone on *E. coli* dehydrogenase activity using sodium succinate (0.02M) as substrate.

■—■ : control;
△—△: 16 µg/ml;
medium : PBS, pH 7.3.


120
100
80
60
40
20
% Dehydrogenase activity
0
10
20
30
40
50
60
Time (mins.)

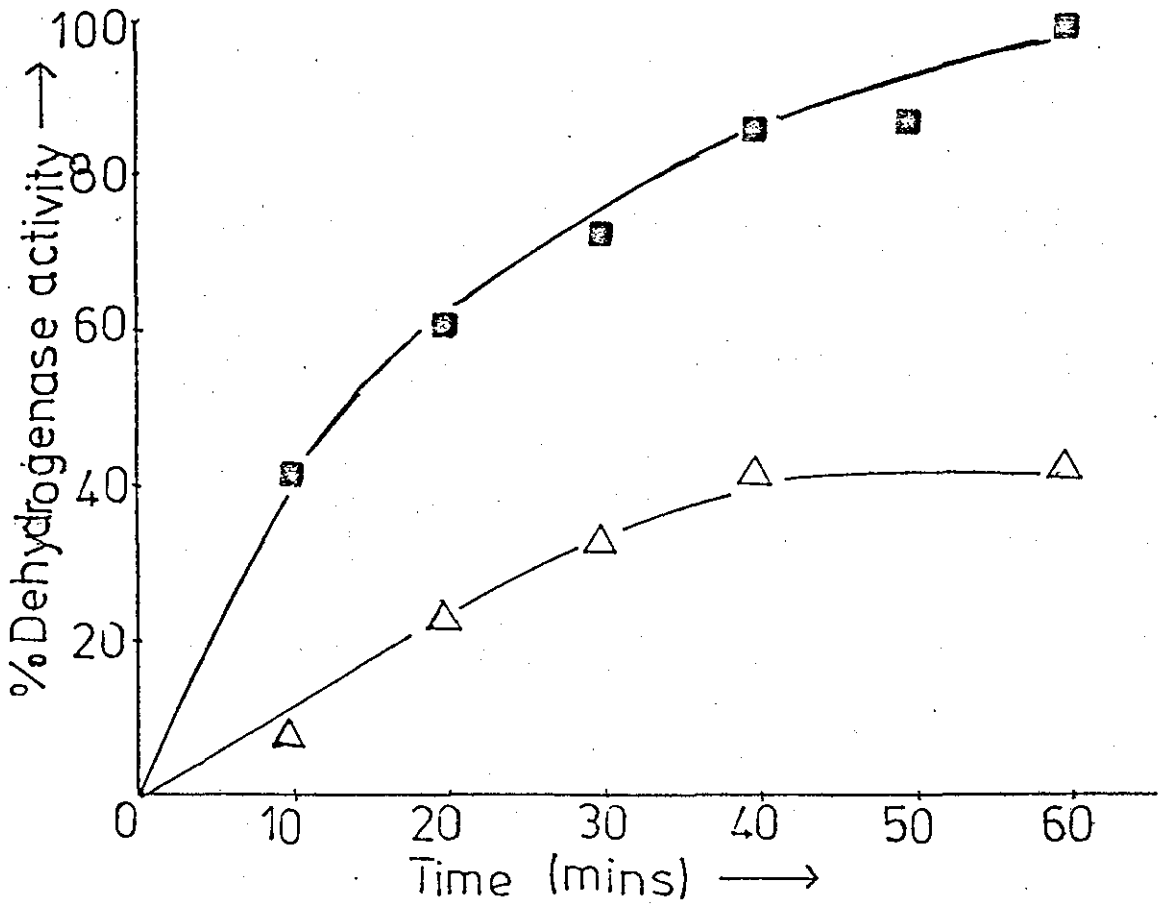

100
80
60
40
20
% Dehydrogenase activity
0
10
20
30
40
50
60
Time (mins)

Fig. (3.34)

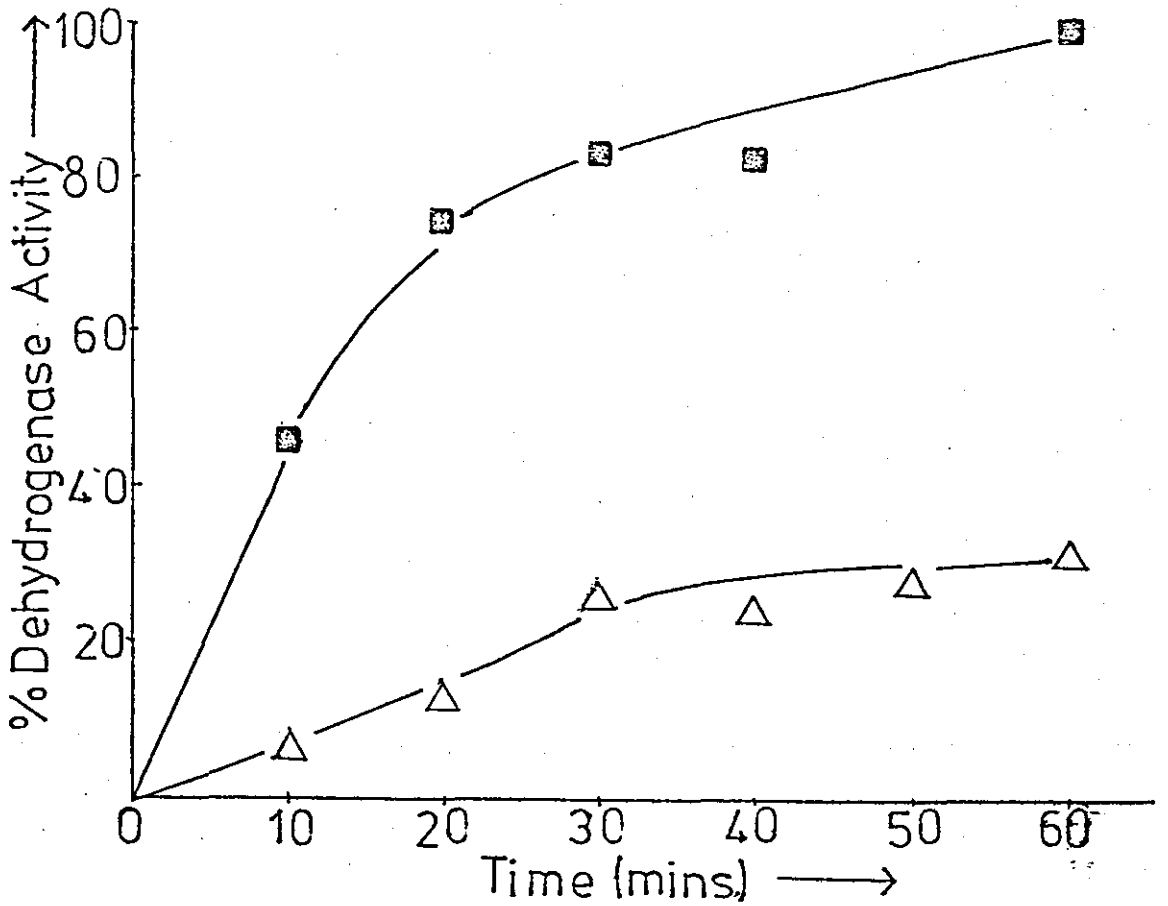
Effect of Phanquone on *E. coli* dehydrogenase activity using sodium malate (0.02M) as substrate

■—■ : control;
△—△: 16 g/ml;
medium : PBS, pH 7.3.

Fig. (3.35)

Effect of Phanquone on *E. coli* dehydrogenase activity using sodium pyruvate (0.02M) as substrate

■—■ : control;
△—△: 16 µg/ml;
medium : PBS, pH 7.3.


% Dehydrogenase Activity
100
80
60
40
20
0
10
20
30
40
50
60
Time (mins.)

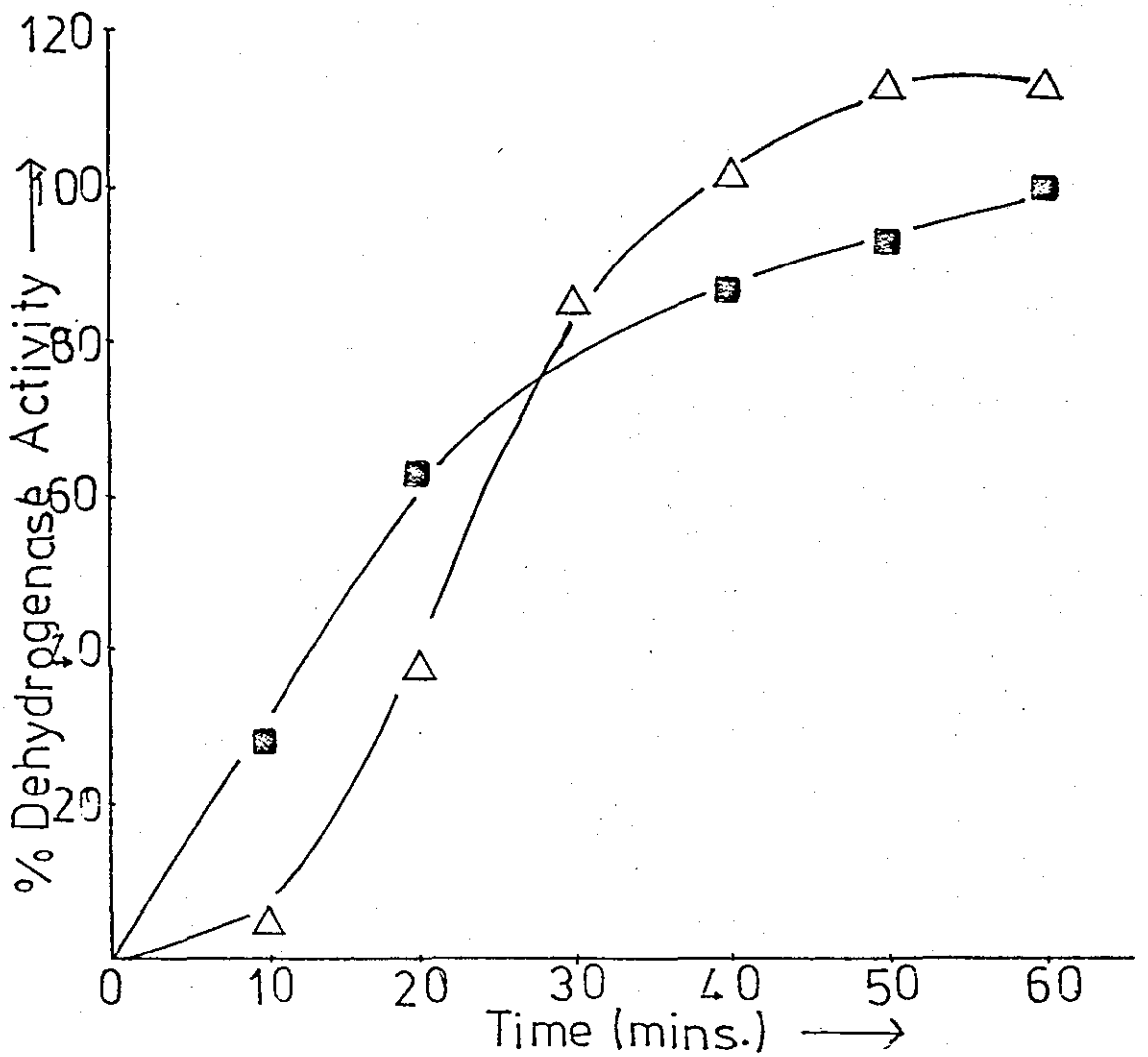

% Dehydrogenase Activity
120
100
80
60
40
20
0
10
20
30
40
50
60
Time (mins.)

Fig. (3.36)

Effect of Phanquone on *E. coli* dehydrogenase activity using sodium lactate (0.02M) as substrate.

■—■ : control;
△—△ : 16 µg/ml;
medium : PBS, pH 7.3

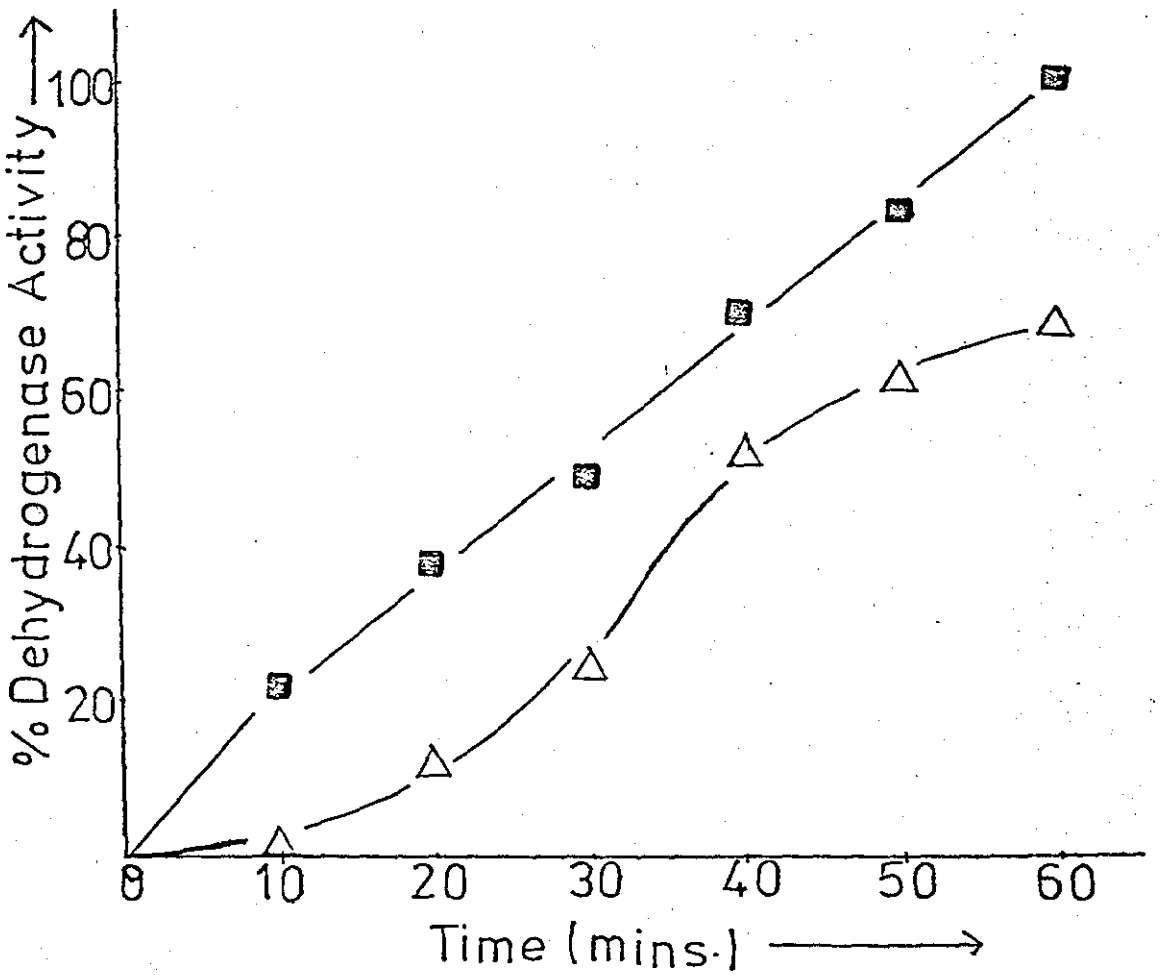
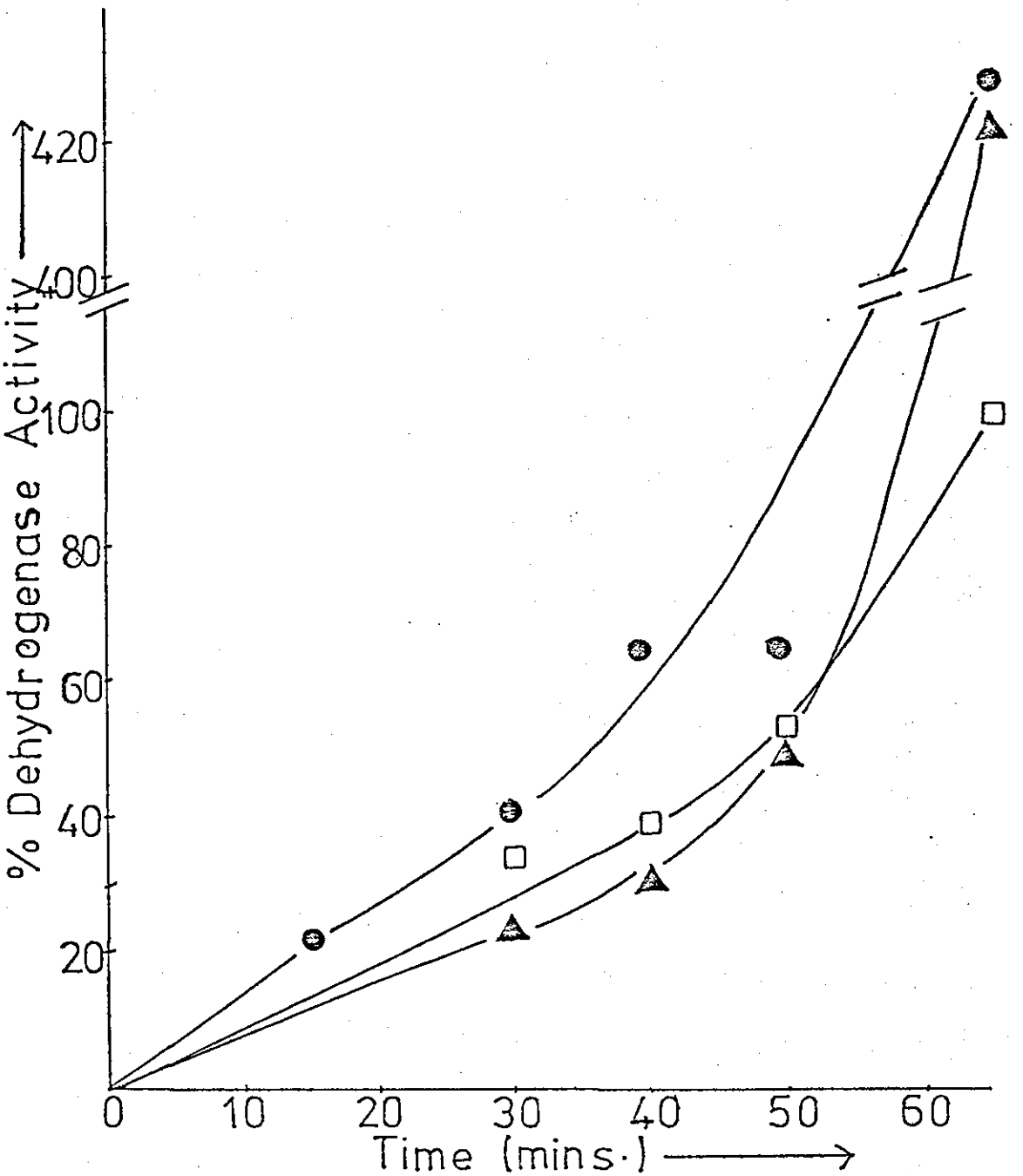

% Dehydrogenase Activity
100
80
60
40
20
0
10
20
30
40
50
60
Time (mins.)

Fig. (3.37)

Effect of Phanquone on *S. aureus* dehydrogenase activity using D-glucose (0.05M) as substrate

□—□ : control;
●—● : 10 µg/ml;
▲—▲ : 25 µg/ml;
medium : PBS, pH 7.3


% Dehydrogenase Activity
420
400
100
80
60
40
20
0
10
20
30
40
50
60
Time (mins.)

by about 400%, in the presence of 10 µg/ml and 25 µg/ml of Phanquone.

Table (3.13) shows the effect of Fe(II) on the activity of Phanquone on the dehydrogenase, using succinate as a substrate. As can be seen, the dehyrogenase activity was stimulated by 11% in the presence of Fe(II), and was stimulated even further, in the presence of both Fe(II) and Phanquone.

6.3 The Effect of Aerobic and Anaerobic Conditions on the Size of the Zone of Inhibition.

Table (3.14) shows the differences in the sizes of the zones of inhibition produced by Phanquone discs (50 µg/disc), under aerobic and anaerobic conditions. As can be seen, there was a marked decrease in the zone size for *B. subtilis*, *E. coli*, *K. aerogenes*, and *S. aureus*, under anaerobic conditions, while there was an increase in the zone size for *Strep. faecalis*.

Moreover, Phanquone was also active against *Cl. sporogenes*, and produced a zone of inhibition of 10.3 mm.

6.4 The Effect on the Uptake of P-(32)-Inorganic Phosphate

The effect of different concentrations of Phanquone on the uptake of P-(32)-inorganic phosphate by *E. coli* and *S. aureus* cells, is shown in Figs. (3.38) and (3.39), respectively. As can be seen, Phanquone - while slightly inducing the phosphate uptake at 16 µg/ml by about 8% - caused an inhibition of the uptake by 25% and 40%, at 32 µg/ml and 64 µg/ml, respectively.

For *S. aureus*, the inhibition of the uptake caused by Phanquone at 50 µg/ml and 100 µg/ml was about 80% and 90% respectively.

Table (3.13)

Effect of Fe(II) on the effect of Phanquone on *E. coli* dehydrogenase activity using sodium succinate (0.02M) as substrate, in PBS, pH 7.3.

Tube No.	Addition	% of Dehydrogenase Activity
1	Succinate (0.02 M) only.	100 %
2	Succinate (0.02 M) plus $FeSO_4$ (2×10^{-5} M).	111 %
3	Succinate (0.02 M) plus Phanquone (16 µg/ml)	42%
4	Succinate (0.02 M) plus $FeSO_4$ (2×10^{-5} M) plus Phanquone (16 µg/ml)	144 %

Table (3.14)

The sizes of the zones of inhibition produced by Phanquone against some facultative anaerobes, under aerobic and anaerobic conditions.

Organism	anaerobic zone(mm.)	SD	aerobic zone(mm.)	SD
B. subtilis	10.6	0.4	16.0	0.5
E. coli	13.3	0.5	16.6	0.4
K. aerogenes	10.6	0.4	16.0	0.0
S. aureus	10.7	0.5	15.6	0.6
Strep. faecalis	10.2	0.4	7.6	0.2
Cl. sporogenes	10.3	0.6	—	—

Fig. (3.38)

Effect of Phanquone on the uptake of P(32)-inorganic phosphate by *E. coli* suspended in 0.1M tris-HCl buffer, pH 7.4, using D-glucose (0.02M) as substrate.

□—□ : control;
●—● : 16 µg/ml;
△—△ : 32 µg/ml;
⬢—⬢ : 64 µg/ml.

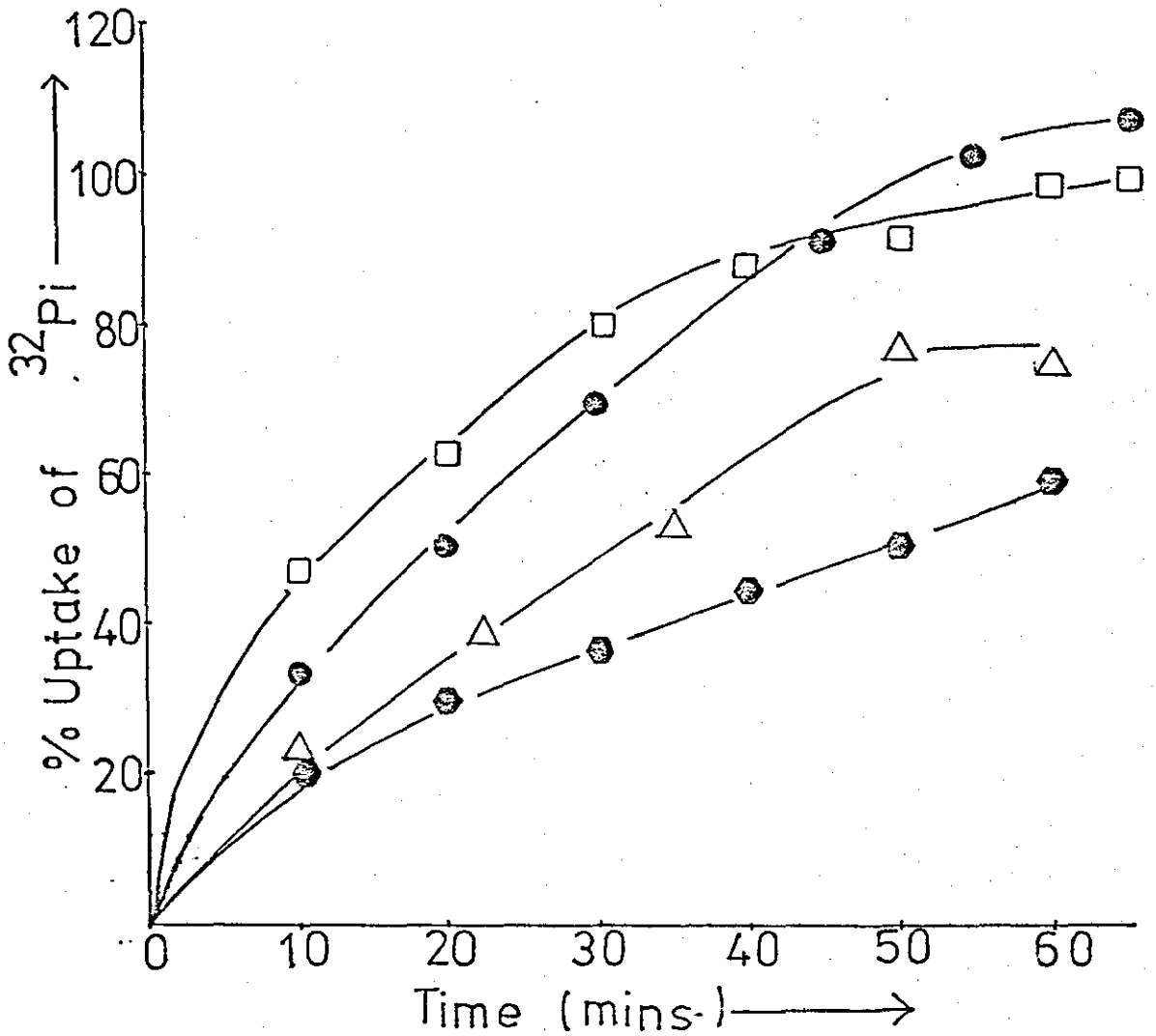
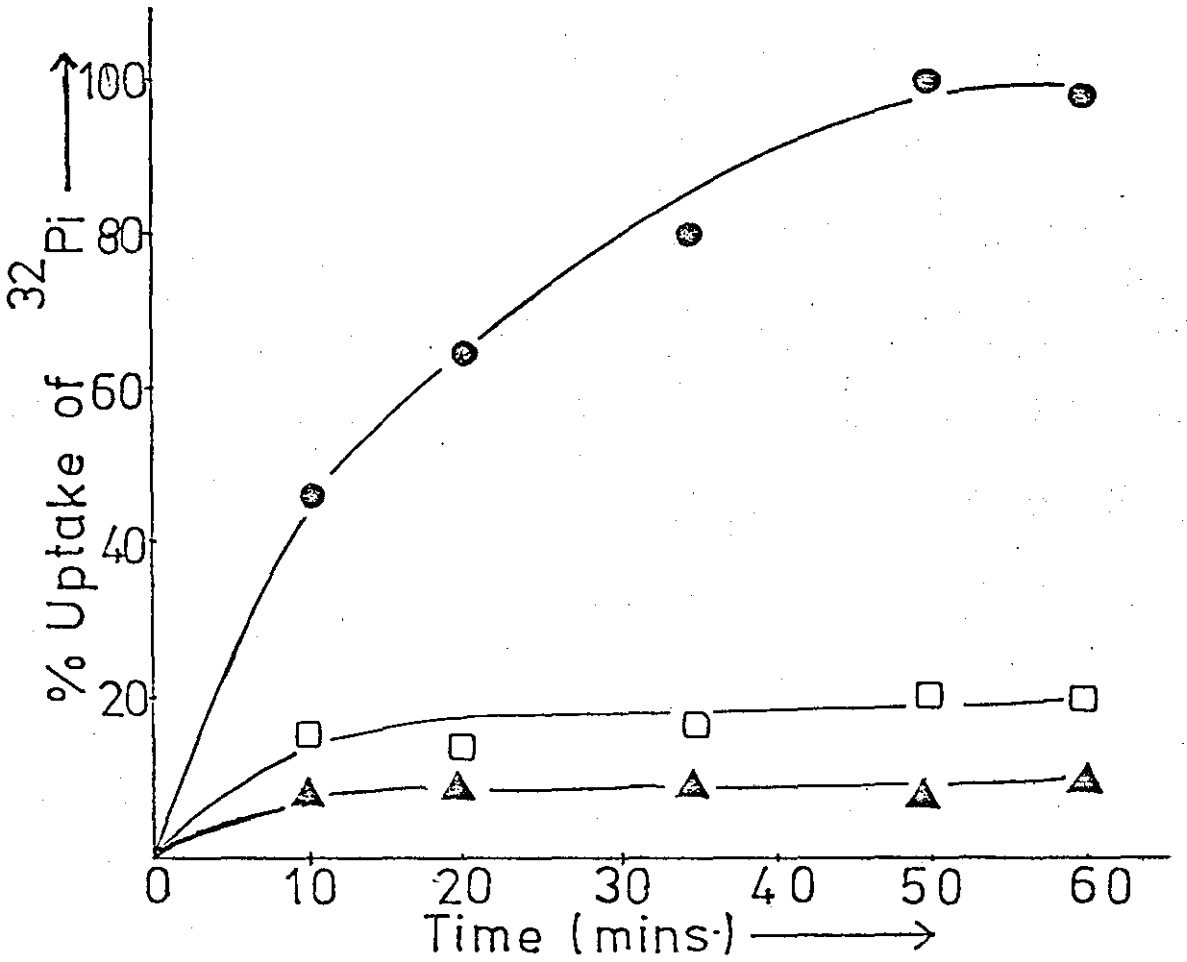

120
100
80
60
40
20
% Uptake of $^{32}P_i$
0
10
20
30
40
50
60
Time (mins.)

Fig. (3.39)

Effect of Phanquone on the uptake of P(32)- inorganic phosphate by *S. aureus* suspended in 0.1M Tris-HCl buffer, pH 7.4, using D-glucose (0.02M) as substrate.

●—●: control;
□—□: 50 µg/ml;
▲—▲: 100 µg/ml.


% Uptake of 32Pi
100
80
60
40
20
0
10
20
30
40
50
60
Time (mins)

6.5 The Effect on the Uptake of C-(14)-L-Glutamic Acid and C-(14)-L-Proline

Figs. (3.40) and (3.41) show the effect of Phanquone on the uptake of C-(14)-L-glutamic acid by *E. coli* and *S. aureus* cells respectively. For *E. coli*, more glutamic acid was taken up in the absence of glucose, than in its presence, although the uptake started slower in the former case. Here, Phanquone, whilst having no real effect at 16 µg/ml, appreciably inhibited the uptake of the amino acid in the presence of glucose (by about 30%). For *S. aureus*, the presence of glucose enhanced the uptake of the glutamic acid by about 45%, and Phanquone, at 16 µg/ml and 50 µg/ml, inhibited the uptake of the amino acid by about 50% for both concentrations.

The effect of Phanquone on the uptake of C-(14)-L-proline by *E. coli* and *S. aureus* cells, is shown in Figs. (3.42) and (3.43). As was the case for the uptake of glutamic acid by *E. coli*, the uptake of proline was greater in the absence of glucose. Nevertheless, Phanquone at 16 µg/ml and 50 µg/ml, inhibited the uptake of proline by about 8% and 33% respectively. As for glutamic acid, the uptake of proline by *S. aureus* cells was enhanced in the presence of glucose. Phanquone, at 16 µg/ml and 50 µg/ml inhibited the amino acid uptake by about 10% and 21% respectively. The rate of uptake of both amino acids by both *E. coli* and *S. aureus*, in the presence of Phanquone showed a distinct pattern: a fast rate of uptake at the beginning, followed by a slower rate, then a fast rate once again.

6.6 The Effect on the Uptake of C-(14)-L-Proline by Membrane Vesicles

The effect of Phanquone on the uptake of C-(14)-L-proline by membrane vesicles prepared from *E.coli* K12, could not be demonstrated, when using either 1.0 M solution of ATP or 0.2 M solution of lithium lactate as substrates (Fig. 3.44).

Fig. (3.40)

Effect of Phanquone on the uptake of L-(U-C14)-glutamic acid by *E. coli* suspended in 0.1M Tris-HCl buffer, pH 7.4.

●—●: glutamic acid only;

□—□: glutamic acid plus D-glucose (0.02M);

▲—▲: glutamic acid plus D-glucose (0.02M) plus Phanquone (16 µg/ml);

△—△: glutamic acid plus glucose (0.02M) plus Phanquone (50 µg/ml).

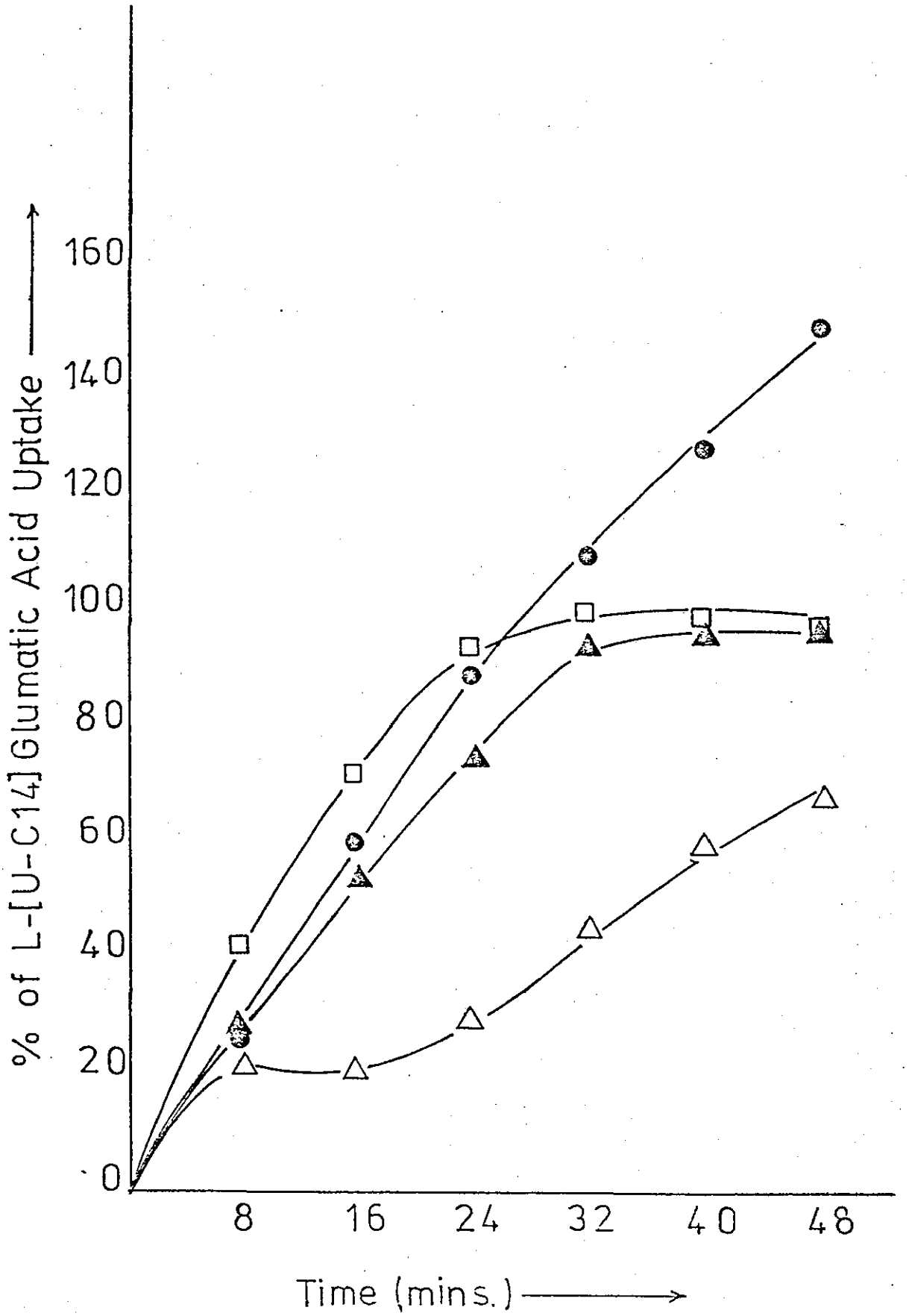

% of L-[U-C14] Glumatic Acid Uptake
160
140
120
100
80
60
40
20
0
8
16
24
32
40
48
Time (mins.)

Fig. (3.41)

Effect of Phanquone on the uptake of L-(U-C14)-glutamic acid by *S. aureus* suspended in 0.1M Tris-HCl buffer, pH 7.4

●——● : glutamic acid only;

■——■ : glutamic acid plus D-glucose (0.02M);

□——□ : glutamic acid plus D-glucose (0.02M) plus Phanquone (16 µg/ml);

△——△ : glutamic acid plus D-glucose (0.02M) plus Phanquone (50 µg/ml).

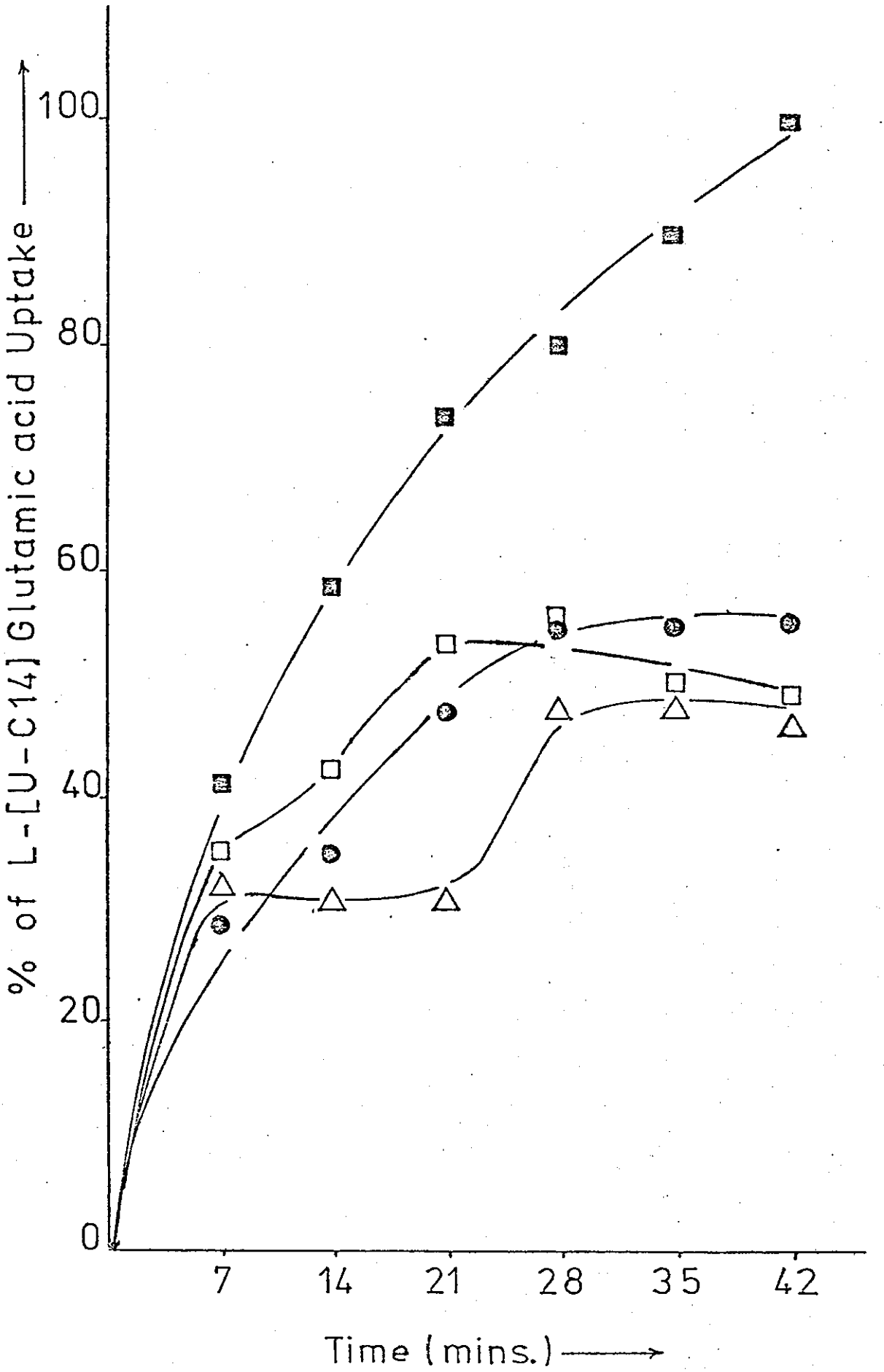

% of L-[U-C14] Glutamic acid Uptake
100
80
60
40
20
0
7
14
21
28
35
42
Time (mins.)

Fig. (3.42)

Effect of Phanquone on the uptake of L-(U-C14)-proline by *E. coli* suspended in 0.1M Tris-HCl buffer, pH 7.4

■—■: proline only;

○—○: proline plus D-glucose (0.02M);

□—□: proline plus D-glucose (0.02M) plus Phanquone (16 µg/ml);

▲—▲: proline plus D-glucose (0.02M) plus Phanquone (50 µg/ml).

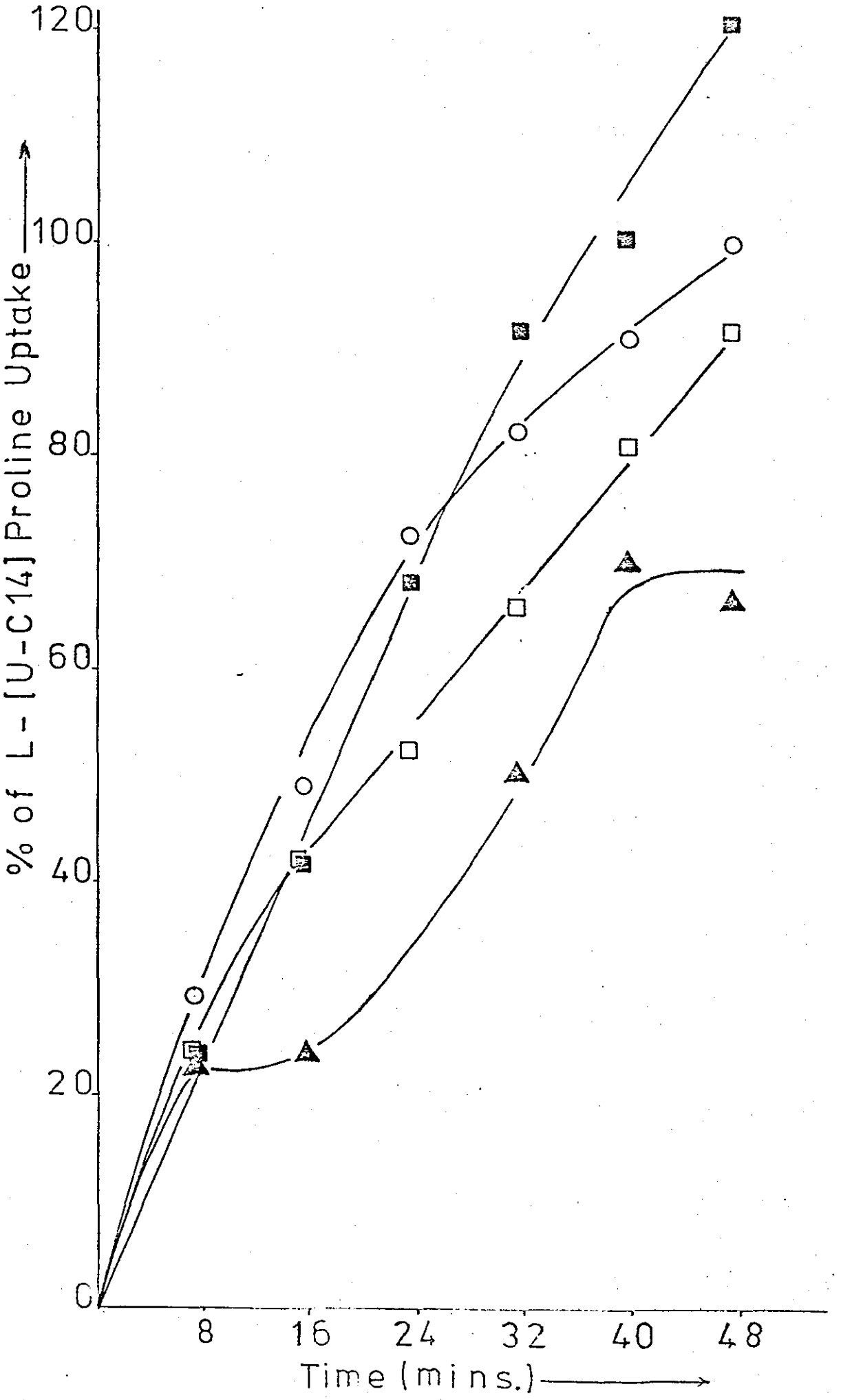

120
100
80
60
40
20
0
% of L-[U-C14] Proline Uptake
8
16
24
32
40
48
Time (mins.)

Fig. (3.43)

Effect of Phanquone on the uptake of L(U-C14)-proline by *S. aureus* suspended in 0.1M Tris-HCl buffer, pH 7.4

△—△ : proline only;

■—■ : proline plus D-glucose (0.02M);

○—○ : proline plus D-glucose (0.02M) plus Phanquone (16 µg/ml);

▲—▲ : proline plus D-glucose (0.02M) plus Phanquone (50 µg/ml).

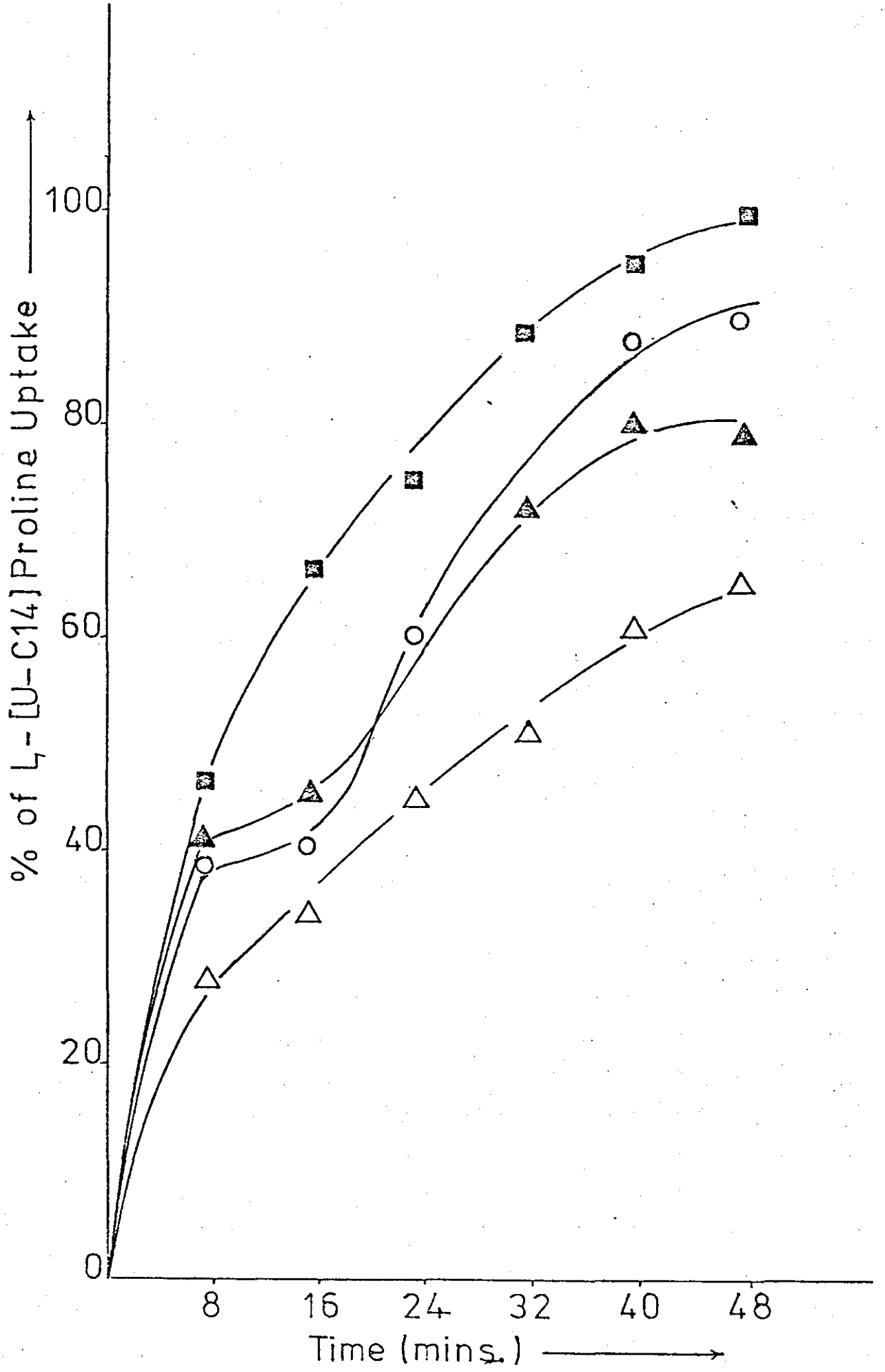

% of L-[U-C14]Proline Uptake
100
80
60
40
20
0
8
16
24
32
40
48
Time (mins.)

Fig. (3.44)

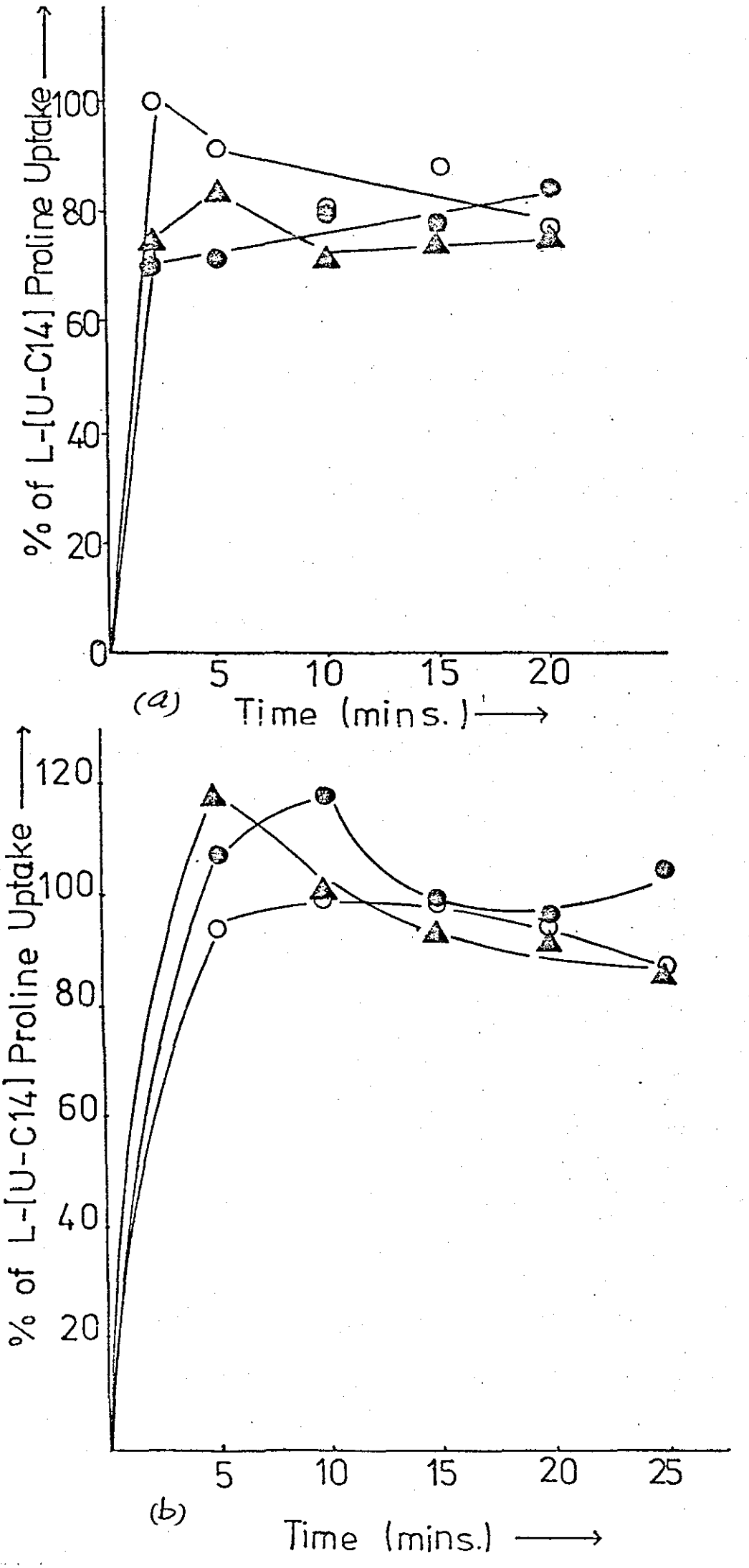
Effect of Phanquon on the uptake of L(U-C14)-proline by membrane vesicles.

a) using ATP (1.0M) as substrate:

●—●: proline only;
○—○: proline plus ATP;
▲—▲: proline plus ATP plus Phanquone (50 µg/ml).

b) using DL-lithium lactate (0.2M) as substrate:

○—○: proline only;
▲—▲: proline plus lactate;
●—●: proline plus lactate plus Phanquone (50 µg/ml).


% of L-[U-C14] Proline Uptake
0
20
40
60
80
100
5
10
15
20
Time (mins.)
(a)
% of L-[U-C14] Proline Uptake
20
40
60
80
100
120
5
10
15
20
25
Time (mins.)
(b)

7. THE EFFECT OF SOME METAL IONS ON THE ANTIBACTERIAL ACTIVITY OF PHANQUONE

7.1 The Formation of Complexes with Some Metal Ions

Fig. (3.45) shows the change in colour observed 15 mins. after mixing Phanquone and some metal ions at 1:2 ratio. The formation and the deposition of precipitates observed for Phanquone - Fe(II), -Cu(II), and -Co(II) mixtures after 24 hrs., is shown in Fig. (3.46). When left for 48 hrs., a precipitate appeared in the Phanquone - Ni(II), and -Zn(II) tubes (Fig. 3.47).

7.2 Job-Plots for Phanquone - Fe(III) Complexes.

While all Phanquone-metal ions complexes precipitated (with the exception of Phanquone - Fe(III) and -Mn(II) complexes, the Phanquone-Mn(II) complex did not produce an appreciable change in colour, and therefore, only Fe(III) was available for the construction of Job-plots. Fig. (3.48) shows the Job-plots for this metal ion when combined with Phanquone at different ratios after 24 hrs. and 48 hrs. As can be noted, the peak in both figures corresponded to a Phanquone-Fe(III) ratio between 3:2 and 1:1.

7.3 The Effect of Metal Ions on the Zone of Inhibition

Table (3.15) shows the effect of some metal ions on the zone of inhibition produced by Phanquone. Here, Co(II), Cu(II), Fe(II) and Fe(III) antagonized its action against both *E.coli* and *S.aureus*; Mn(II) and Ni(II) antagonised its action against *S.aureus* only, while Zn(II) antagonized its action against *E.coli*. Fig. (3.49)

Fig. (3.45)

The changes in colour and/or the formation of precipitates observed for some Phanquone-metal ion mixtures at 1:2 ratio after 15 minutes, indicating the formation of complexes.

All tubes contain Phanquone (5×10^{-4}M) and 10^{-3}M solutions of (from left to right) Co(II), Cu(II), Fe(II), Fe(III), Mn(II), Ni(II) and Zn(II).

Last tube contains Phanquone (5×10^{-4}M) only.

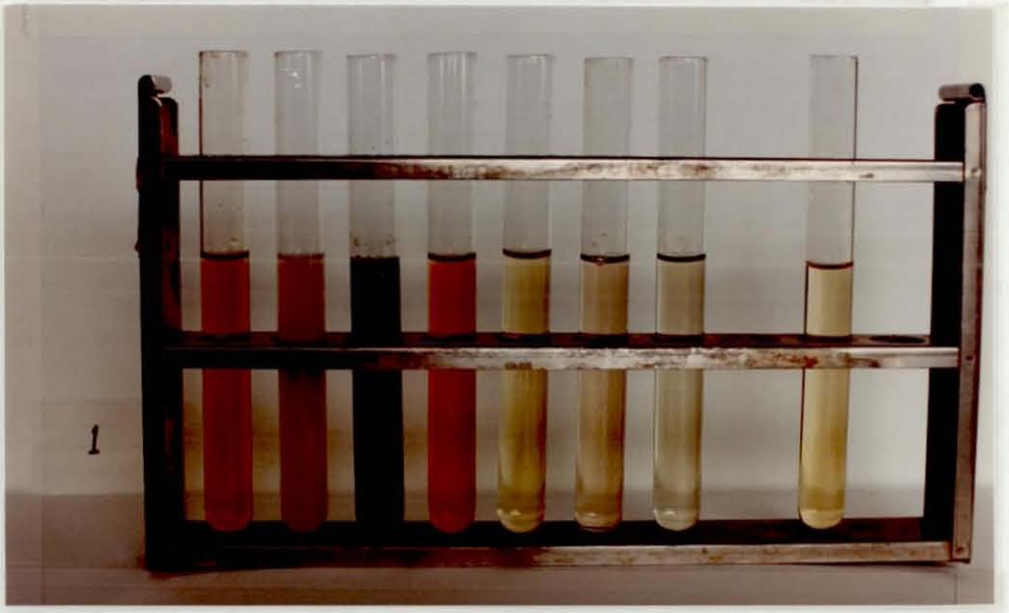
Fig. (3.46)

The changes in colour and/or the formation of precipitate observed for some Phanquone-metal ion mixtures at 1:2 ratio after 24 hours.

Tube contents are similar to those in Fig. (3.45)

Fig. (3.47)

The changes in colour and/or for the formation of precipitates observed for some Phanquone-metal ion mixtures at 1:2 ratio after 48 hours. Tube contents are similar to those in Fig. (3.45).


1


4

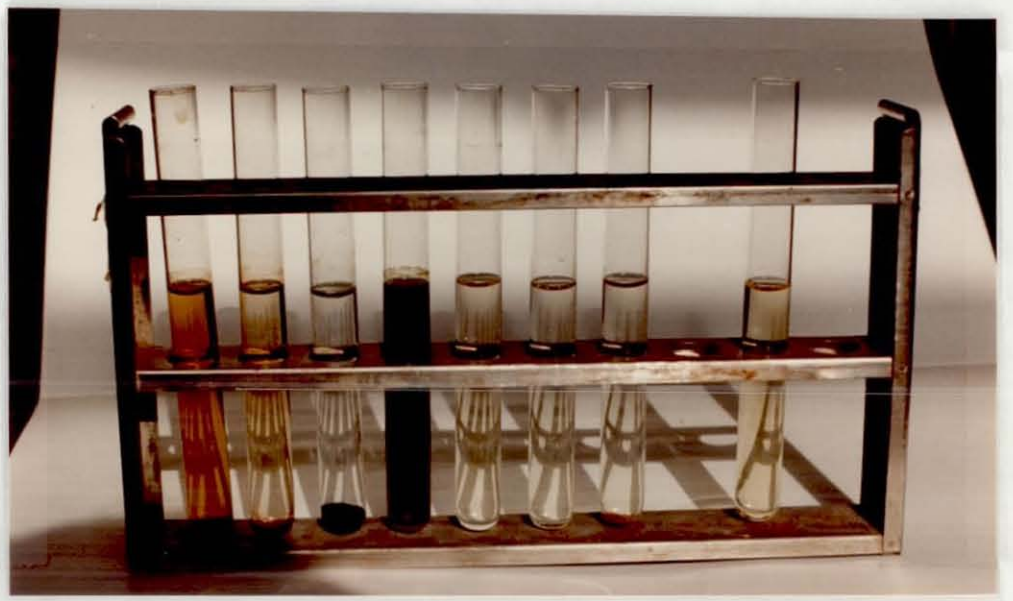


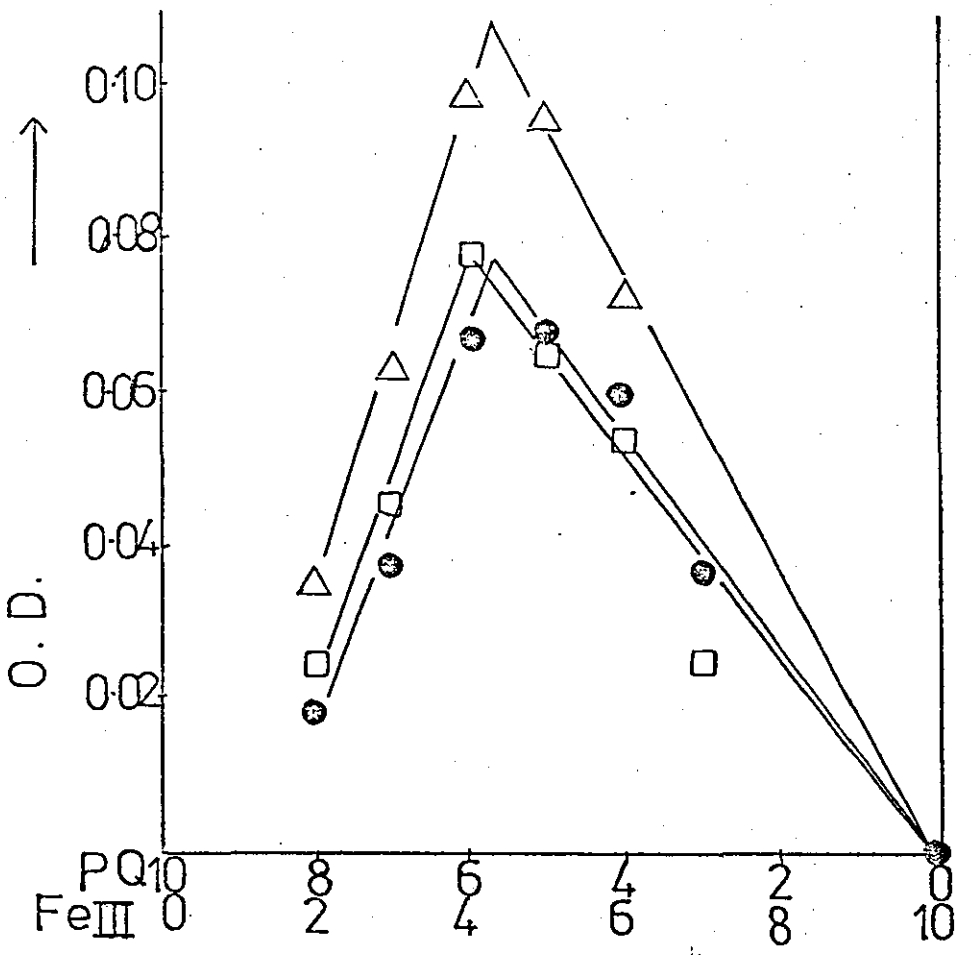
Fig. (3.48)

Job-plots for Phanquone-Fe(III) complexes in 0.1N H_2SO_4 using the method of continuous variation

a) after 24 hours:

●—●: at 500 nm;
□—□: at 600 nm;
△—△: at 650 nm.

b) after 48 hours at 600 nm.


0·10
0·08
0·06
0·04
0·02
O. D.
PQ 10 8 6 4 2 0
Fe III 0 2 4 6 8 10

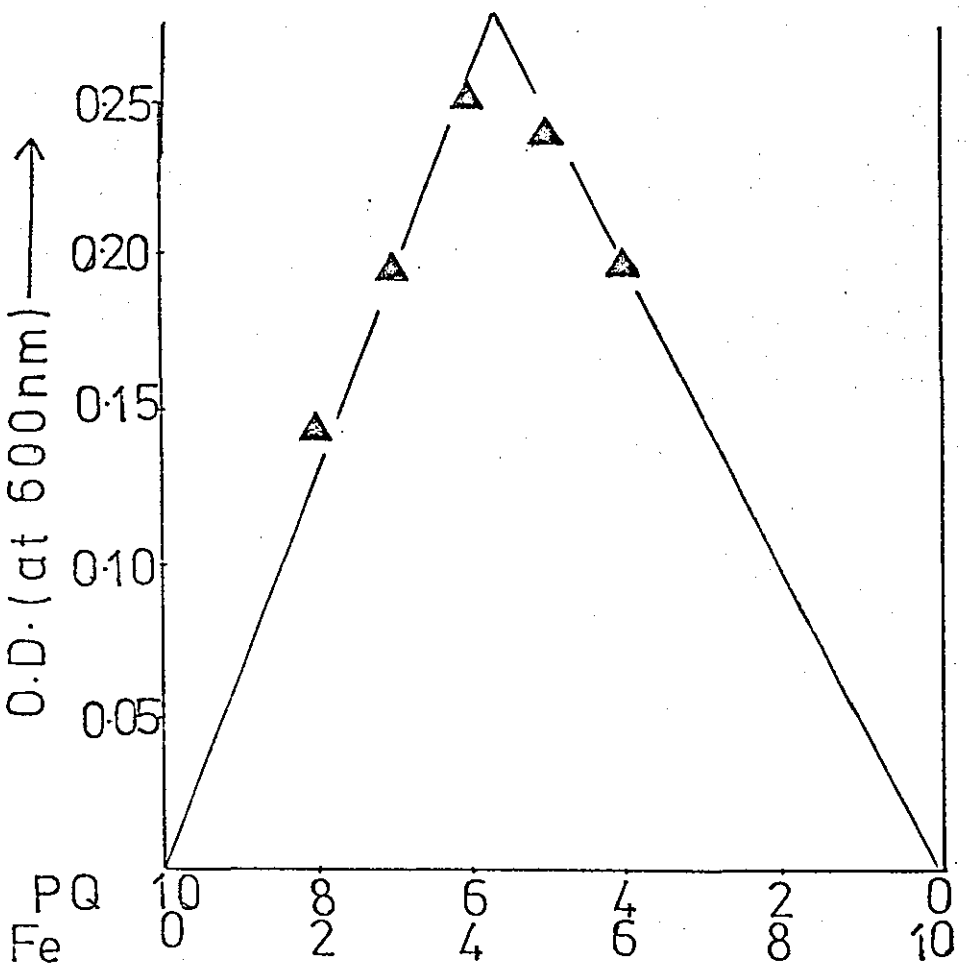
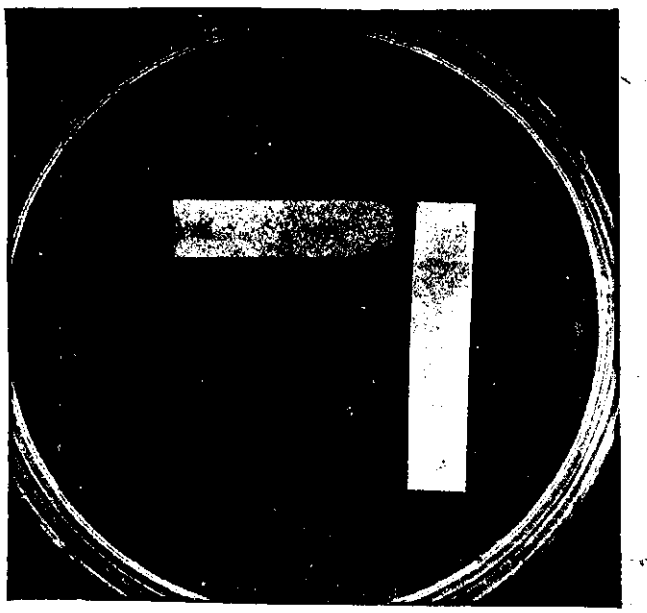

0·25
0·20
0·15
0·10
0·05
O.D. (at 600nm)
PQ 10 8 6 4 2 0
Fe 0 2 4 6 8 10

Table (3.15)

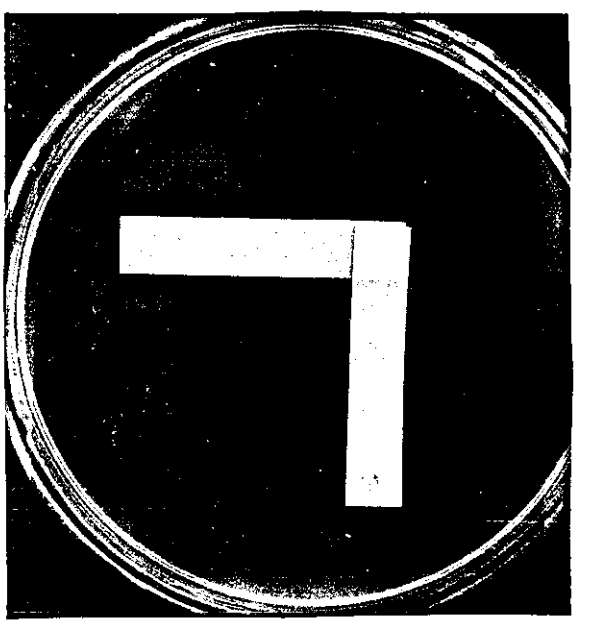
Effect of metal ions on the zones of inhibition produced by Phanquone and related compounds using the right-angled strips method.

-	:	no effect;
+	:	antagonism;
++	:	prominent antagonism;
NZ	:	no zone.

STRIP A soaked in	STRIP B soaked in	Effect on zone of Inhibition	
		E.coli	S.aureus
Phanquone (2000 µg/ml)	$CaCl_2$ (0.1M)	–	–
	$CoSO_4$	+	+
	$CuSO_4$	++	++
	$FeCl_3$	+	++
	$FeSO_4$	+	++
	KCl	–	–
	$MgSO_4$	–	–
	$MnCl_2$	–	+
	NaCl	–	–
	$NiSO_4$	–	+
	$ZnCl_2$	+	–
	$ZnSO_4$	+	–
9,10-phenanthrenequinone (2000 µg/ml)	$FeCl_3$	NZ	–
1,10-phenanthroline (2000 µg/ml)	$FeCl_3$	+	++
Oxine (1000 µg/ml)	$FeCl_3$	++	+
1,10-phenanthroline 5,6quinone (1000 µg/ml)	$FeCl_3$	–	+



(a)



(b)

Fig. (3.49)

Effect of Fe(III) solution on the zone of inhibition produced by Phanquone (strip A, 2000 µg/ml) using the right-angled strips method.

a) *E. coli* : strip B containing 10^{-3}M solution of $FeCl_3$;

b) *S. aureus* : strip B containing 10^{-3}M solution of $FeCl_3$.

shows this antagonistic effect of Fe(III) on *E.coli* and *S.aureus* respectively. However, other metal ions tested did not have any antagonistic effects. By comparison, Fe(III) antagonised the actions of 1,10-phenanthroline and oxine against both *E.coli* and *S.aureus*, while antagonising the action of 1,10-phenanthroline-5,6-quinone against *S.aureus* only. However, Fe(III) had no effect on the activity of 9,10-phenanthrenequinone.

7.4 The Effect of $FeCl_3$ on the MIC Values

The antagonistic effect of Fe(III) on the MIC and MCC values of Phanquone against *E.coli* and *S.aureus*, is shown in Table (3.16). There was a four-fold increase in the MIC and MCC values for both organisms. By comparison, Fe(III) antagonised the activity of 1,10-phenanthroline against both *E.coli* and *S.aureus*.

7.5 The Effect of Metal Ions on the Activity of Phanquone Against Non-Growing Suspensions

The effect of metal ions on the activity of Phanquone against *S.aureus* cells suspended in distilled water is shown in Table (3.17). Here, it is important to note that several metal ions, especially those at molar concentration double that of Phanquone, were toxic themselves (as shown in between brackets), and therefore, this metal ion effect should be taken into consideration when interpreting results obtained from this table, and for that matter, the following one (Table 3.18).

As can be seen from Table (3.17), Phanquone at 10^{-4}M (about 20 µg/ml), did not cause cell death by itself. However, when Phanquone was mixed with the different metal ions at 2:1 and 1:1 ratios, a

Fig. (3.16)

Effect of Fe(III) on the MIC and MCC values of Phanquone and 1,10-phenanthroline for *E. coli* and *S. aureus*.

Antibacterial	$FeCl_3$ added	*E. coli*		*S. aureus*	
		MIC µg/ml	MCC µg/ml	MIC µg/ml	MCC µg/ml
Phanquone	—	25	25	50	50
	2×10^{-3} M	100	100	200	200
1,10-phenanthroline	—	25	50	50	100
	2×10^{-3} M	>200	>200	>200	>200

Table (3.17)

Effect of some metal ions (at different ratios to Phanquone) on the activity of Phanquone against non-growing *S. aureus* cells suspended in distilled water:

+++	:	> 50 colonies;
++	:	20-49 colonies;
+	:	2-19 colonies;
-	:	< 2 colonies;

Signs in brackets denote the effect of the metal ions alone.

	Co^{++}		Cu^{++}		Fe^{++}		Fe^{+++}		Mn^{++}		Ni^{++}		Zn^{++}	
	1 hr	2hrs	1hr	2hrs	1hr	2hrs	1hr	2hrs	1hr	2hrs	1hr	2hrs	1hr	2hrs
PQ only $10^{-4}M$	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
PQ : M^{n+} 2 : 1	− [+++]	− [+++]	++ [+]	− [−]	+ [+++]	− [+++]	− [++]	− [+++]	− [+++]	− [+++]	− [++]	− [++]	− [++]	− [++]
PQ : M^{n+} 1 : 1	− [+++]	− [+++]	++ [−]	− [−]	+ [+]	+ [+]	− [+]	− [+]	++ [+++]	− [+++]	− [++]	− [++]	− [++]	− [++]
PQ : M^{n+} 1 : 2	+ [++]	− [+]	+ [+]	++ [−]	+ [+]	+ [−]	− [++]	− [−]	− [+++]	− [+++]	++ [+]	+ [−]	++ [+++]	− [+++]

Table (3.18)

Effect of some metal ions (at different ratios to Phanquone) on the activity of Phanquone against non-growing *E. coli* cells suspended in distilled water:

+++	:	> 50 colonies;
++	:	20-49 colonies;
+	:	2-19 colonies;
-	:	< 2 colonies;

Signs in brackets denote the effect of the metal ions alone.

	Co^{++}		Cu^{++}		Fe^{++}		Fe^{+++}		Mn^{++}		Ni^{++}		Zn^{++}	
	1hr	2hrs	1hr	2hrs	1hr	2hrs	1hr	2hrs	1hr	2hrs	1hr	2hrs	1hr	2hrs
PQ only 5×10^{-6}M	+	-	+	-	+	-	+	-	+	-	+	-	+	-
PQ:M^{n+} 1 : 1	+ [+++]	- [+++]	++ [+]	++ [-]	+ [+++]	+ [+++]	++ [+++]	++ [+++]	+ [+]	- [+]	- [++]	- [++]	++ [+++]	+ [+++]
PQ: M^{n+} 1 : 2	- [++]	- [++]	++ [-]	++ [-]	++ [+++]	++ [+++]	- [+]	- [-]	- [++]	- [++]	- [+]	- [+]	- [-]	- [-]
PQ:M^{n+} 1 : 4	- [++]	- [++]	+ [-]	- [-]	++ [+++]	++ [+++]	+ [-]	- [-]	+ [++]	- [++]	- [+]	- [+]	- [-]	- [-]

lethal effect was observed. The Phanquone-metal ion mixture at the 1:2 ratio had a lethal effect as well, but some *S.aureus* cells continued to survive when Cu(II), Fe(II), and Ni(II) were used. The rest of the results from this table were not meaningful, due to the toxicity of the metal ions, on their own, to the *S.aureus* cells.

Table (3.18) shows the effect of the same metal ions on the activity of Phanquone against *E.coli* cells, suspended in distilled water. Here, Phanquone, at 5 x 10^{-6}M (approximately 1 µg/ml), greatly reduced the viable number after 1 hr. contact, while completely killing the bacterial cells after 2 hrs.. However, when Phanquone was mixed with Cu(II), Fe(II), Fe(III) and Zn(II) at 1:1 ratio, its action was quenched or antagonised. Cu(II) and Fe(II), at 1:2 ratio, and Fe(II) at 1:4 ratio, also quenched the activity of Phanquone. Here also, some of the results obtained were not meaningful, due to the lethal effect produced by the metal ions.

7.6 The Solubility of Phanquone and its Metal Complexes in Octanol

Fig. (3.50) shows the absorbance peak obtained for Phanquone and Phanquone-Cu(II) complexes at 3:1, 2:1, 1:1, 1:2 and 1:3 ratios, in the octanol phase of an octanol-water system. As can be seen, Phanquone alone was quite soluble (as denoted by the size of the peak) in the octanol phase, but as the concentration of the Cu(II) increased, Phanquone became less and less soluble. A similar plot was observed when Fe(III) and Fe(II) were used (Fig. (3.51) and (3.52)).

The same pattern was observed, but to a lesser extent, when Co(II), Zn(II), Ni(II) and Mn(II) were used (Figs. 3.53, 3.54, 3.55, and 3.56 respectively).

Fig. (3.50)

Absorbance peaks for Phanquone and its Cu(II) complexes at 3:1, 2:1, 1:1, 1:2 and 1:3 ratios, using the UV SP800 spectrophotometer.

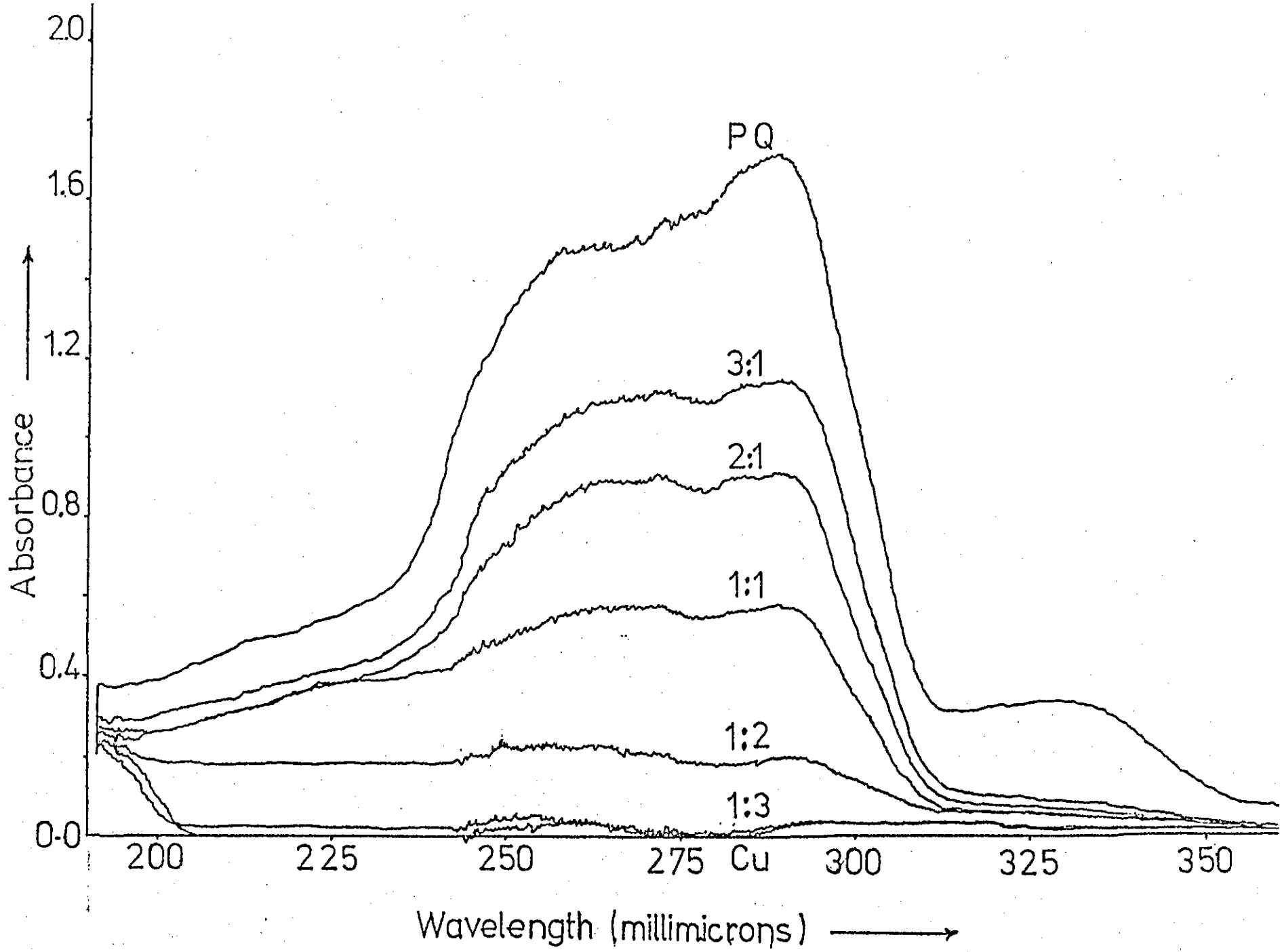
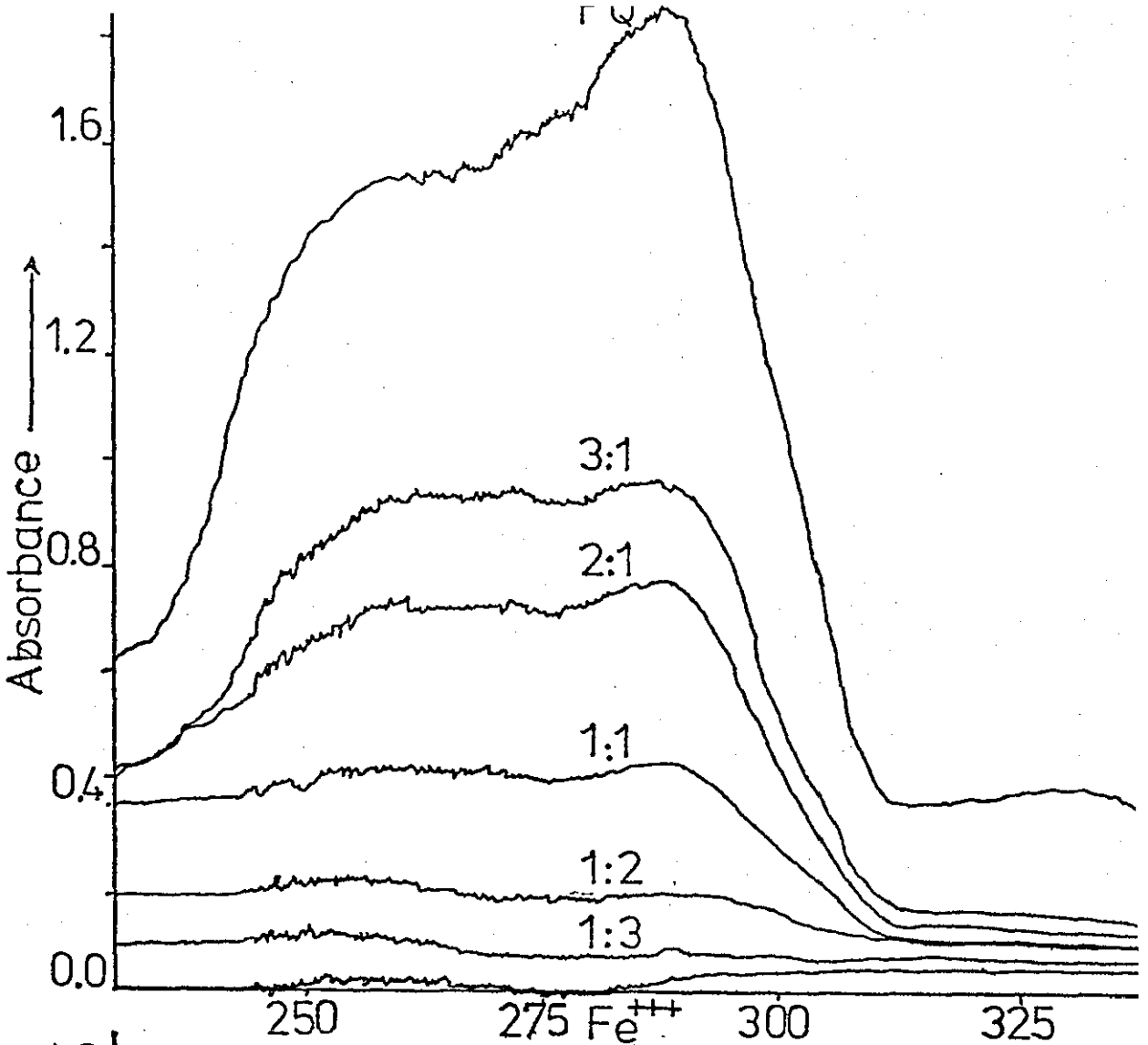

2.0
1.6
1.2
0.8
0.4
0-0
Absorbance
PQ
3:1
2:1
1:1
1:2
1:3
200
225
250
275
Cu
300
325
350
Wavelength (millimicrons)

Fig. (3.51)

Absorbance peaks for Phanquone and its Fe(III) complexes at different ratios.

Fig. (3.52)

Absorbance peaks for Phanquone and its Fe(II) complexes at different ratios.


Absorbance
1.6
1.2
0.8
0.4
0.0
3:1
2:1
1:1
1:2
1:3
250
275
Fe++
300
325

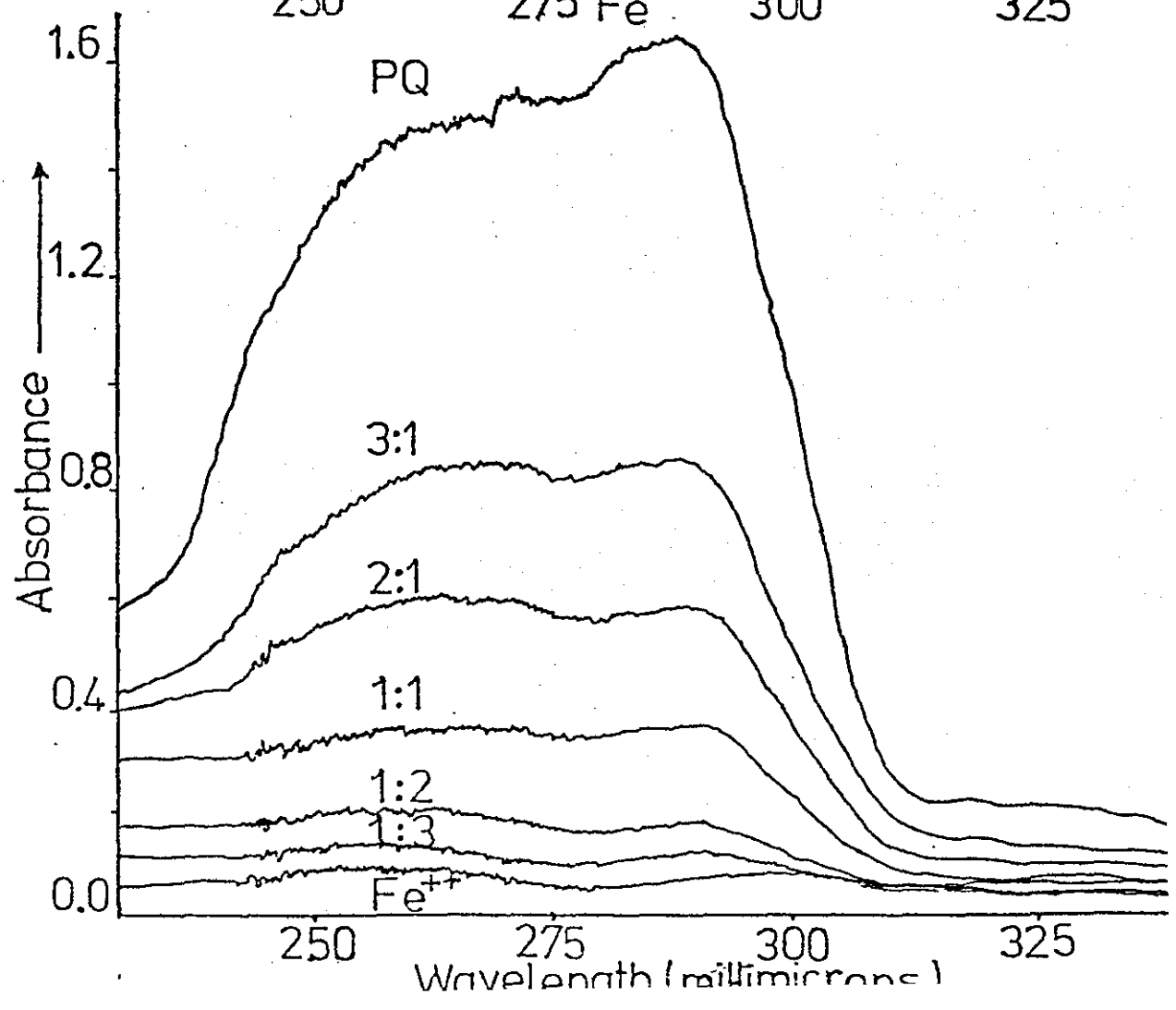

Absorbance
1.6
1.2
0.8
0.4
0.0
PQ
3:1
2:1
1:1
1:2
1:3
Fe++
250
275
300
325
Wavelength (millimicrons)

Fig. (3.53)

Absorbance peaks for Phanquone and its Co(II) complexes at different ratios.

Fig. (3.54)

Absorbance peaks for Phanquone and its Zn(II) complexes at different ratios.

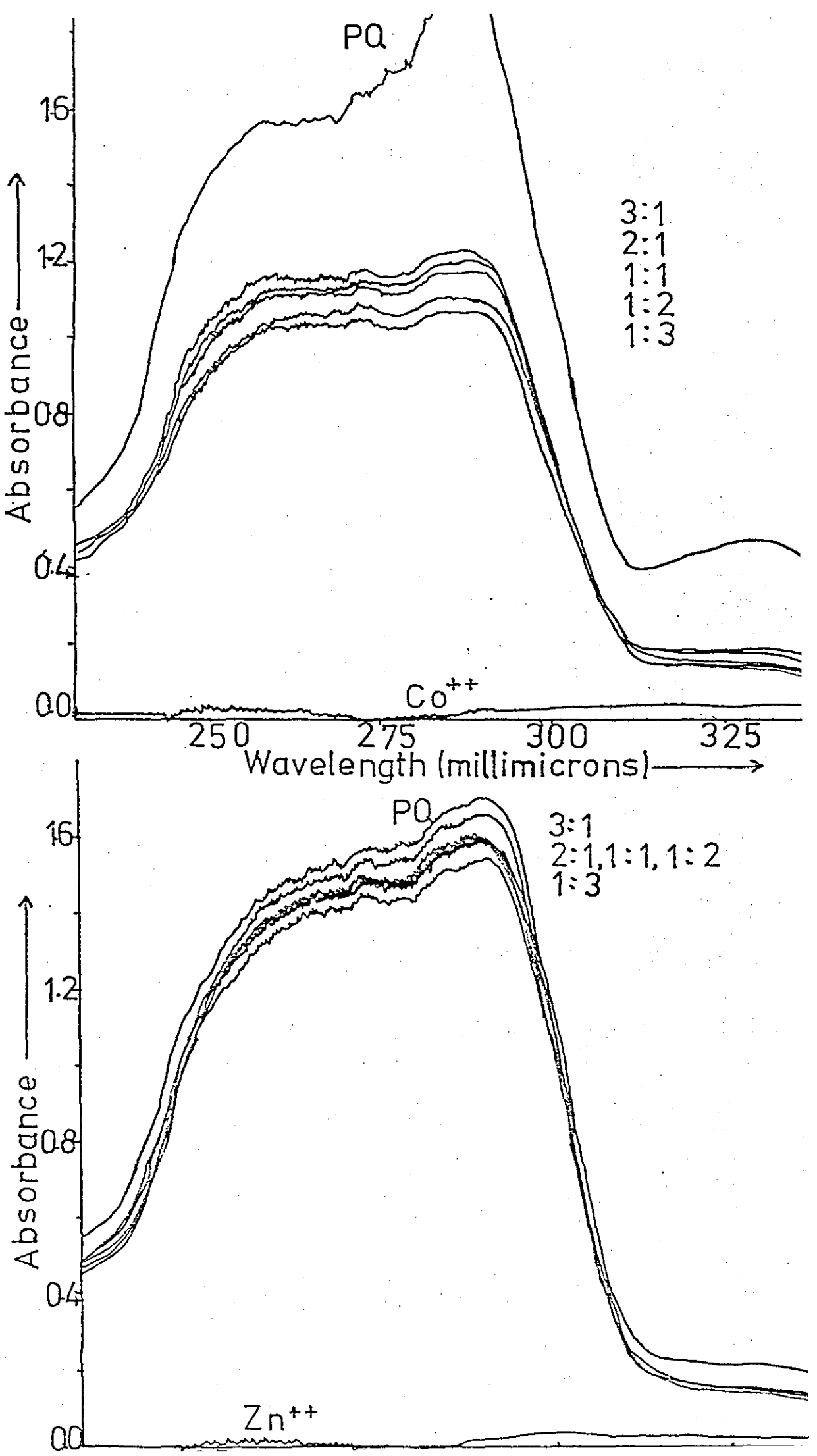
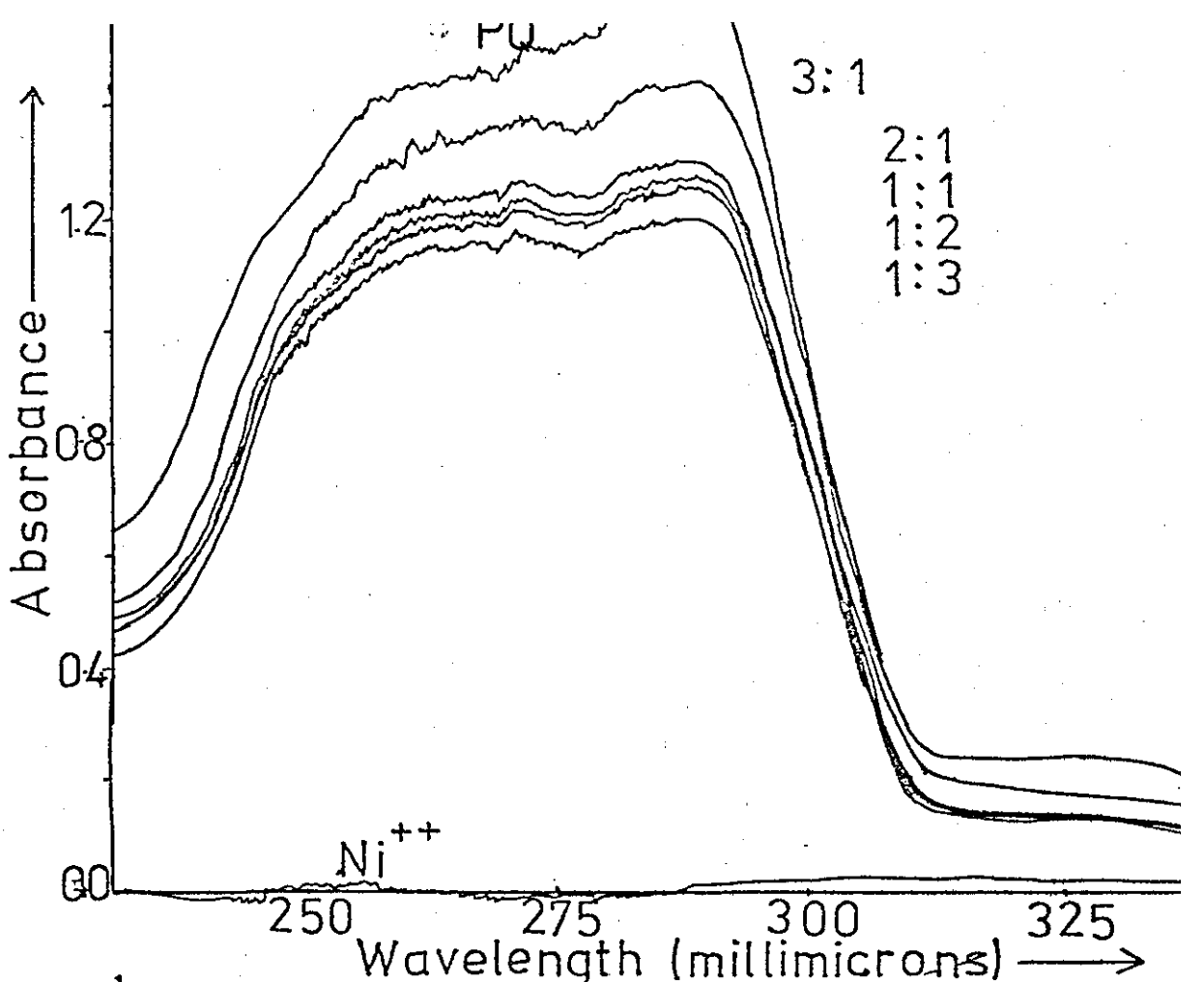

PQ
3:1
2:1
1:1
1:2
1:3
Absorbance
1.6
1.2
0.8
0.4
0.0
Co++
250
275
300
325
Wavelength (millimicrons)
PQ
3:1
2:1, 1:1, 1:2
1:3
Absorbance
1.6
1.2
0.8
0.4
0.0
Zn++

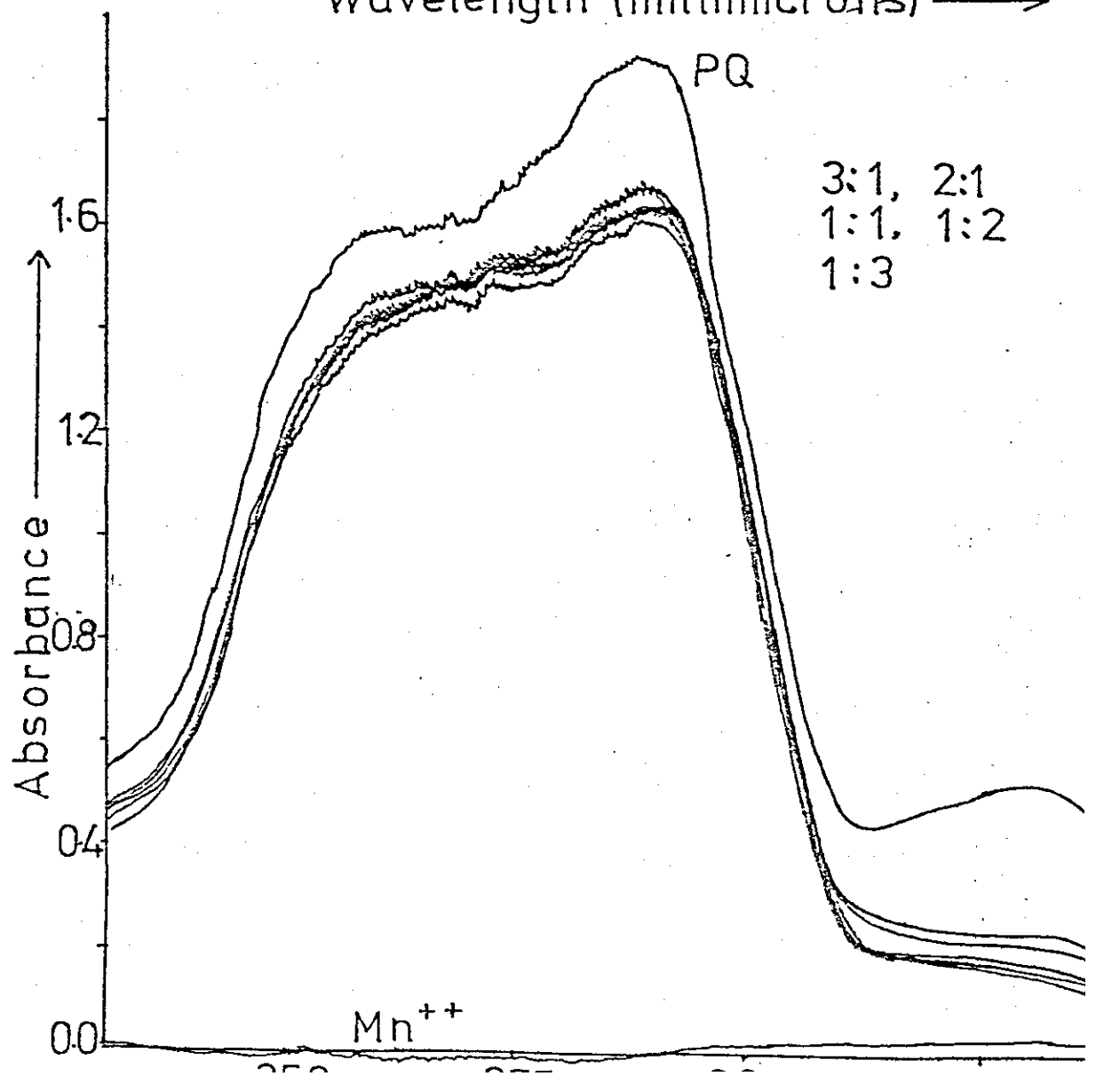
Fig. (3.55)

Absorbance peaks for Phanquone and its Ni(II) complexes at different ratios.

Fig. (3.56)

Absorbance peaks for Phanquone and its Mn(II) complexes at different ratios.


PQ
3:1
2:1
1:1
1:2
1:3
Absorbance
1·2
0·8
0·4
0·0
Ni++
250
275
300
325
Wavelength (millimicrons)


PQ
3:1, 2:1
1:1, 1:2
1:3
Absorbance
1·6
1·2
0·8
0·4
0·0
Mn++

Similarly, and as can be seen from Fig. (3.57), the absorbance peak of 1,10-phenanthroline decreased in the presence of Cu(II) at 3:1 and 2:1 ratio, but completely disappeared for 1:1, 1:2, and 1:3 ratios.

The absorbance peaks for oxine and oxine-Cu(II) complexes are shown in Fig. (3.58). In contrast to Phanquone and 1,10-phenanthroline, the absorbance peak at 250 mμ was much larger in the presence of Cu(II) at 3:1 and 1:3 ratio, while the oxine peak at 315 mμ disappeared almost completely, and new peaks appeared at 400 mμ for both 3:1 and 1:3 ratios.

The amount of Phanquone dissolved in octanol, expressed as a percentage of control, was estimated by dividing the height of the Phanquone-metal ion peak at 385 mμ by the height of the Phanquone peak at the same wavelength, and multiplying by 100.

For example, and for Co(II) (Fig. 3.53, p.145), the height of the 1:2 peak was 8.2 cm at 385 mμ, while the height of the control peak was 14.0 cm at the same wavelength.

$$\frac{8.2 \text{ cm}}{14.0 \text{ cm}} \times 100 = 59\%$$

These percentages of the solubility of Phanquone-metal ions at 1:2 ratio, are shown in Table (3.19).

Fig. (3.57)

Absorbance peaks for 1,10-phenanthroline complexes at different ratios.

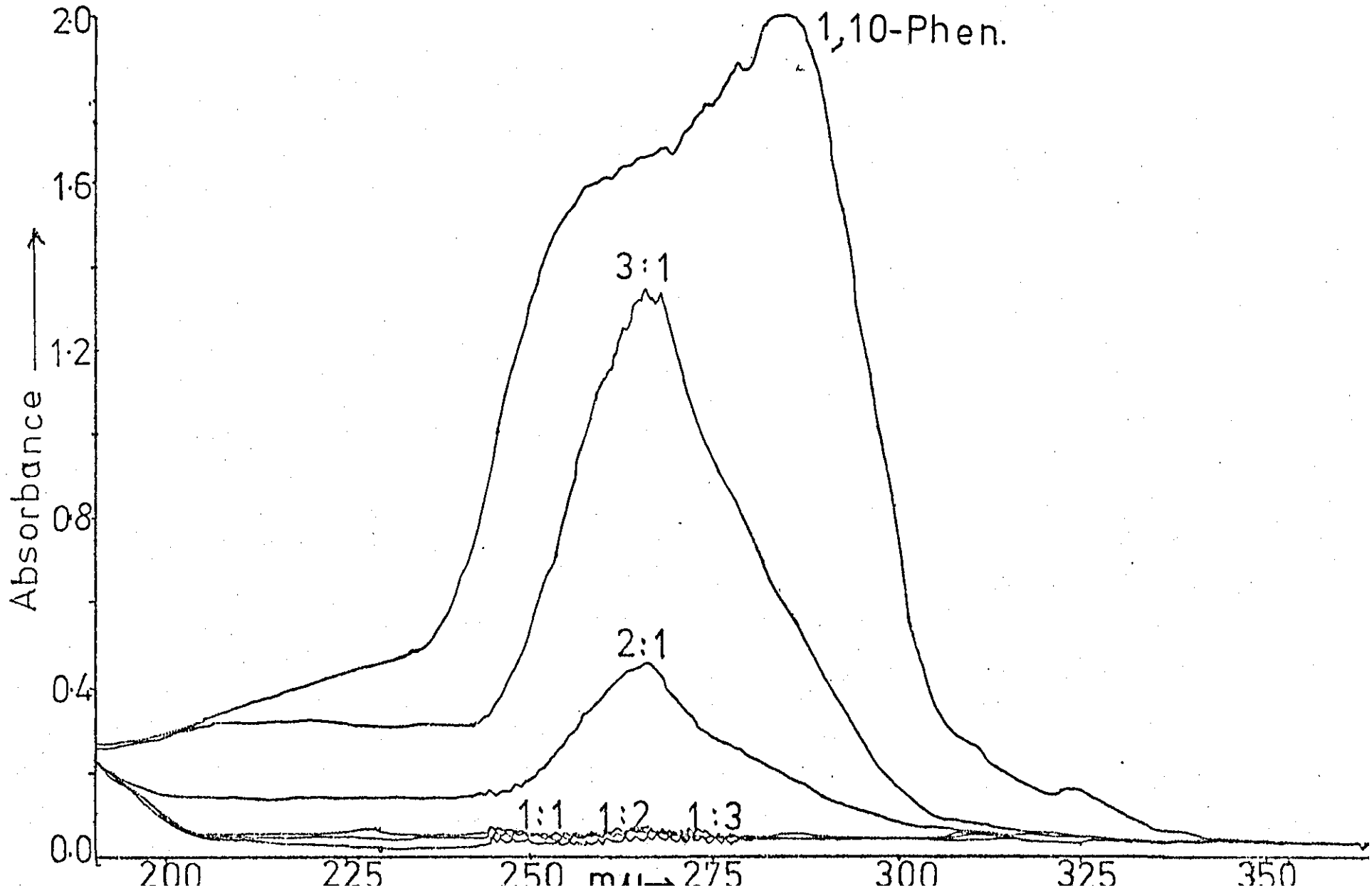

1,10-Phen.
3:1
2:1
1:1 1:2 1:3
Absorbance
2·0
1·6
1·2
0·8
0·4
0·0
200
225
250
275
300
325
350
mμ

Fig (3.58)

Absorbance peaks for oxine and its Cu(II) complexes at 3:1 and 1:3 ratios.

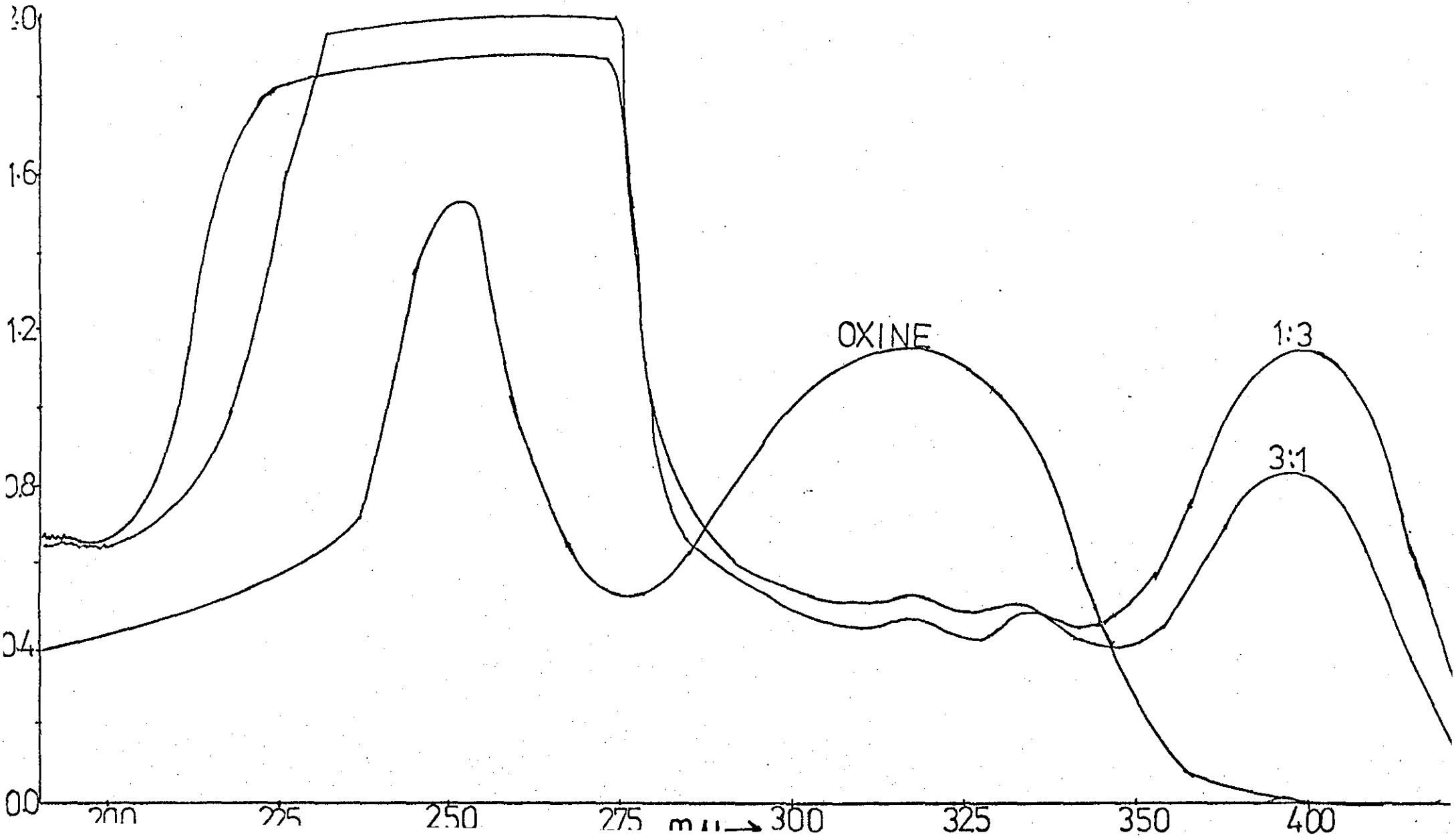

2·0
1·6
1·2
0·8
0·4
0·0
OXINE
1:3
3:1
200
225
250
275
300
325
350
400
mμ

Table 3.19

Percentages of Solubility of Phanquone-Metal Ions at 1:2 Ratio

Metal Ion	% Solubility of Phanquone
Co(II)	59
Cu(II)	11
Fe(II)	11
Fe(III)	10
Mn(II)	85
Ni(II)	77
Zn(II)	94

7.7 The Effect of Metal Ions on the Inhibition of Oxygen Consumption by Phanquone

The effect of Co(II), Cu(II), Fe(II), Ni(II) and Zn(II) on the inhibition of oxygen consumption caused by Phanquone for *E. coli* cells is shown in Figs. (3.59), (3.60), (3.61), (3.62) and (3.63) respectively. As can be noted, all the above mentioned metal ions quenched (or reversed) the inhibition caused by Phanquone on the oxygen consumption. Moreover, in the case of Cu(II), the presence of Phanquone antagonised the inhibitory effect of the metal ion.

Figs. (3.64), (3.65), (3.66) and (3.67) show the effect of Ni(II), Zn(II), Co(II) and Fe(II), respectively, on the inhibition of the oxygen consumption caused by Phanquone. Both Ni(II) and Zn(II) enhanced (or stimulated) the action of Phanquone, while the opposite was true for Co(II) and Fe(II).

7.8 Effect of Some Chelating Agents on the Activity of Phanquone

The effect of EDTA and NTA on the antibacterial activity of Phanquone against *E. coli* and *S. aureus* is shown in Table (3.20). As can be noted, both chelating agents antagonised the activity of Phanquone against *S. aureus* but not against *E. coli*. This antagonism is shown in Fig. (3.68).

Similarly, both EDTA and NTA antagonised the activity of 1,10-phenanthroline against *S. aureus*, but not *E. coli*, while EDTA antagonised the activity of oxine against both organisms. On the other hand, neither of the two chelating agents showed any effect on the activity of 9,10-phenanthrenequinone against *S. aureus*.

Fig. (3.59)

Effect of Co(II) on the inhibition of oxygen consumption by Phanquone for *E. coli* suspended in 0.1M sodium phosphate buffer, pH 7.4, using sodium succinate (0.06M) as substrate.

○—○ : control;
■—■ : plus $CoSO_4$ (2.5×10^{-4}M);
●—● : plus Phanquone at 50 μg/ml (~2.5×10^{-4}M);
△—△ : plus Phanquone (2.5×10^{-4}M) plus $CoSO_4$ (2.5×10^{-4}M).

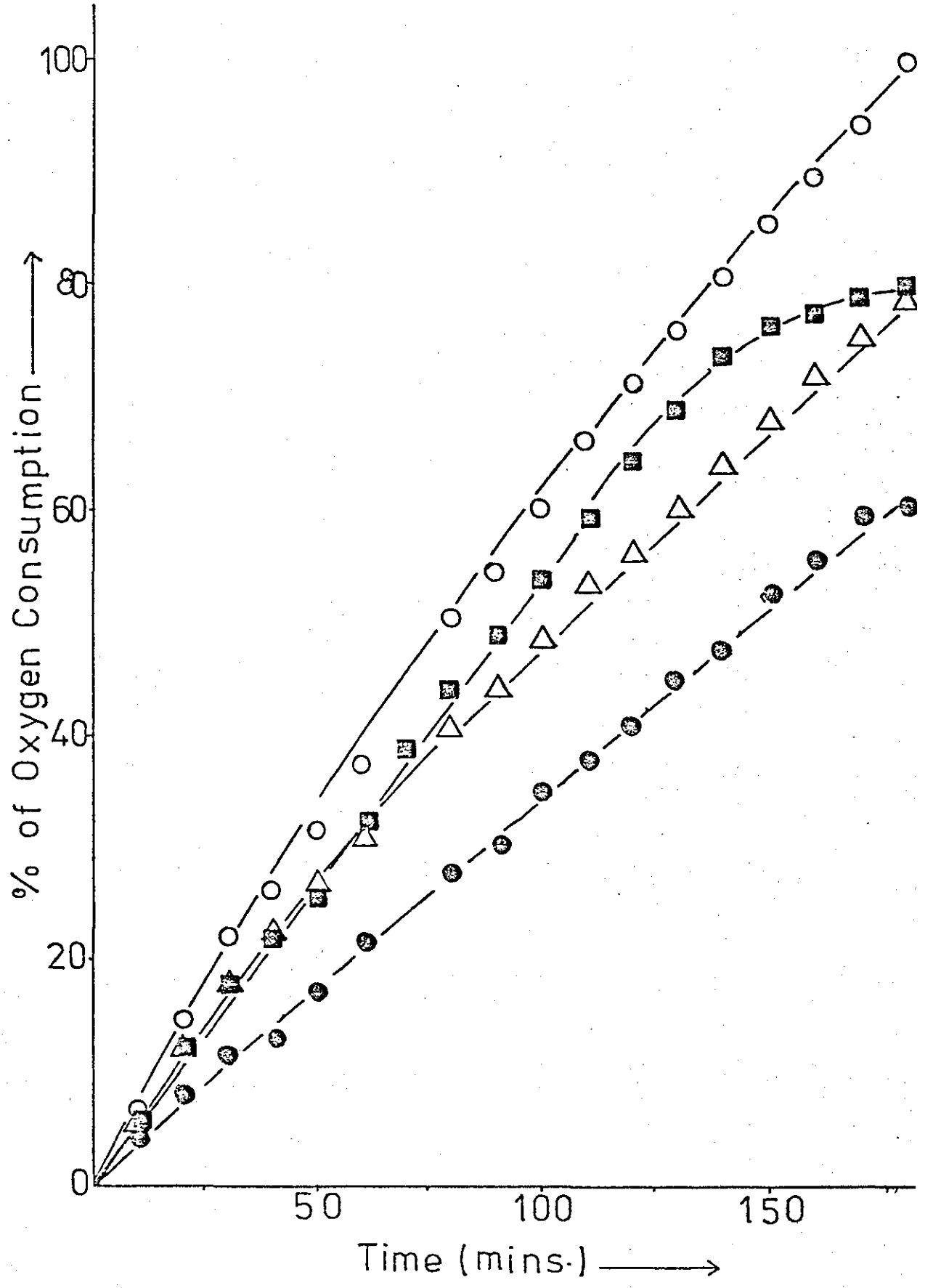

100
80
60
40
20
0
% of Oxygen Consumption
50
100
150
Time (mins.)

Fig. (3.60)

Effect of Cu(II) on the inhibition of oxygen consumption by Phanquone for *E. coli* suspended in 0.1M sodium phosphate buffer, pH 7.4, using D-glucose (0.02M) as substrate:

○—○: control;
■—■: plus $CuSO_4$ (2.5×10^{-4}M);
△—△: plus Phanquone at 50 μg/ml (~2.5×10^{-4}M);
●—●: plus $CuSO_4$ (2.5×10^{-4}M) plus Phanquone (2.5×10^{-4}M).

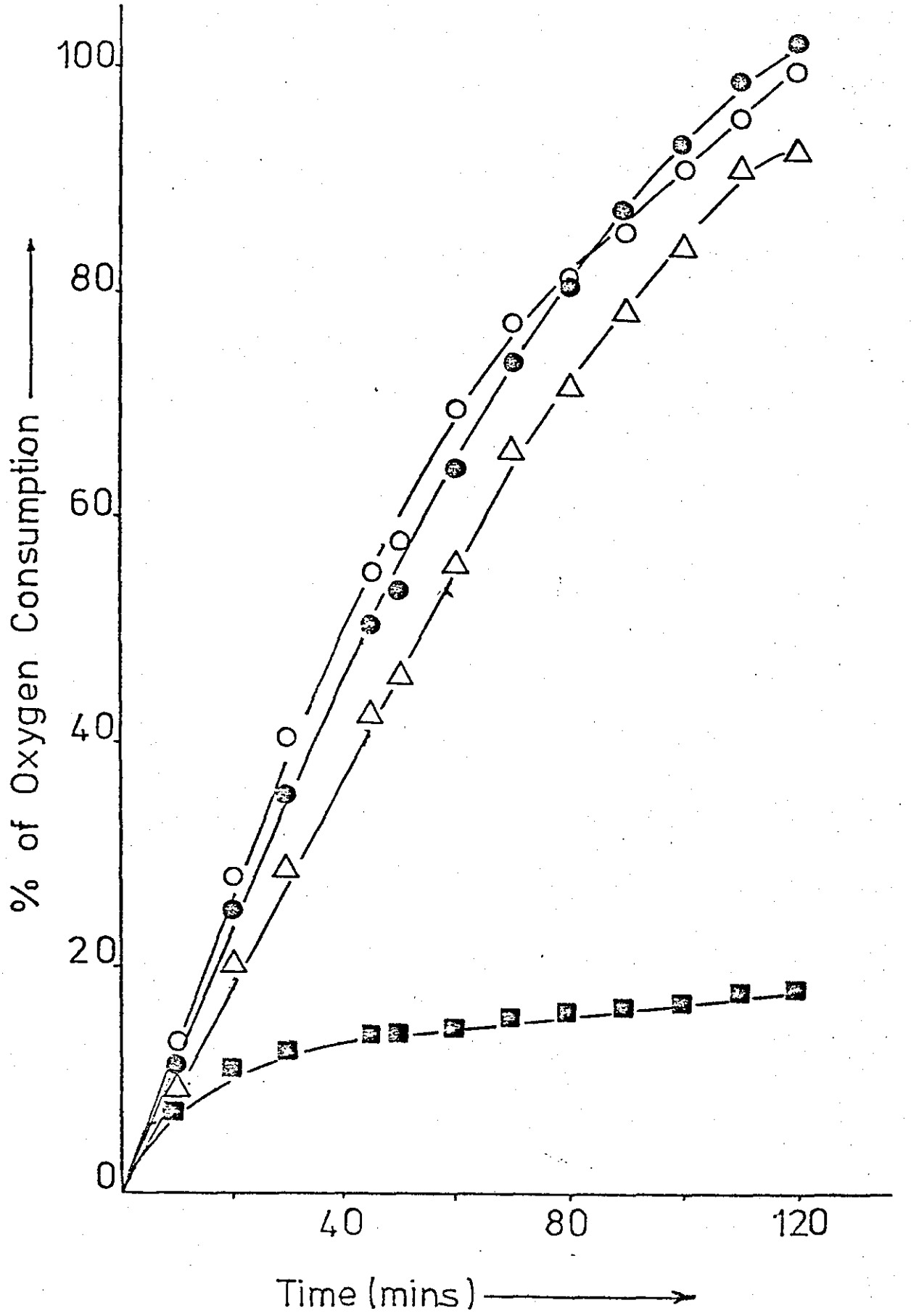

100
80
60
40
20
0
% of Oxygen Consumption
40
80
120
Time (mins)

Fig. (3.61)

Effect of Fe(II) on the inhibition of oxygen consumption by Phanquone for *E. coli* suspended in 0.1M sodium phosphate buffer, pH 7.4, using sodium succinate (0.06M) as substrate.

○—○ : control;
■—■ : plus $FeSO_4$ (2.5×10^{-4}M);
●—● : plus Phanquone at 50 μg/ml (~2.5×10^{-4}M);
△—△ : plus $FeSO_4$ (2.5×10^{-4}M) plus Phanquone (2.5×10^{-4}M).

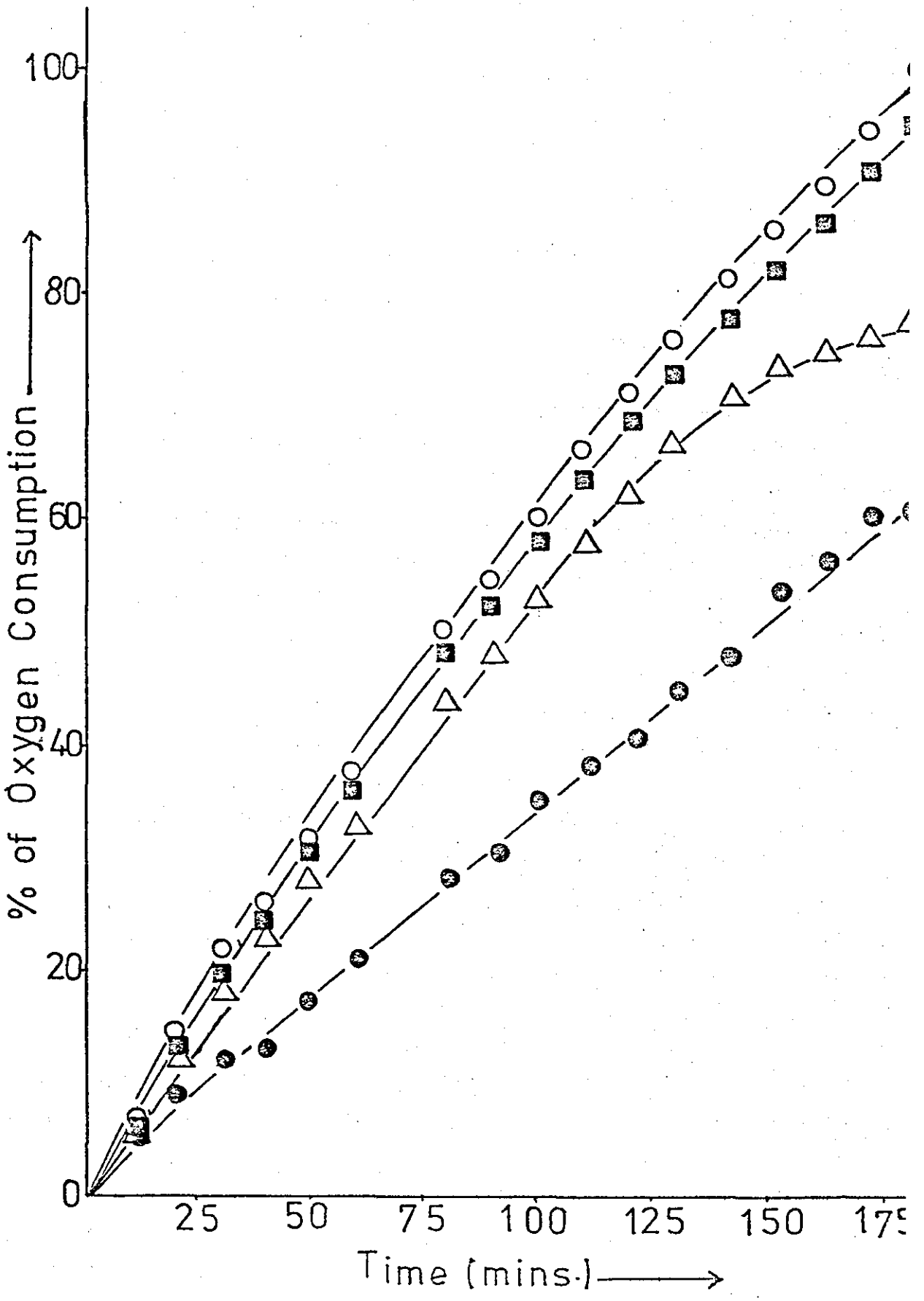

100
80
60
40
20
0
% of Oxygen Consumption
25
50
75
100
125
150
Time (mins.)

Fig. (3.62)

Effect of Ni(II) on the inhibition of oxygen consumption by Phanquone for *E. coli* suspended in 0.1M sodium phosphate buffer, pH 7.4, using D-glucose (0.02M) as substrate.

○——○ : control;
■——■ : plus $NiSO_4$ (2.5 x 10-4M);
△——△: plus Phanquone at 50 µg/ml (~2.5 x 10-4M);
●——● : plus $NiSO_4$ (2.5 x 10-4M) plus Phanquone (2.5 x 10-4M).

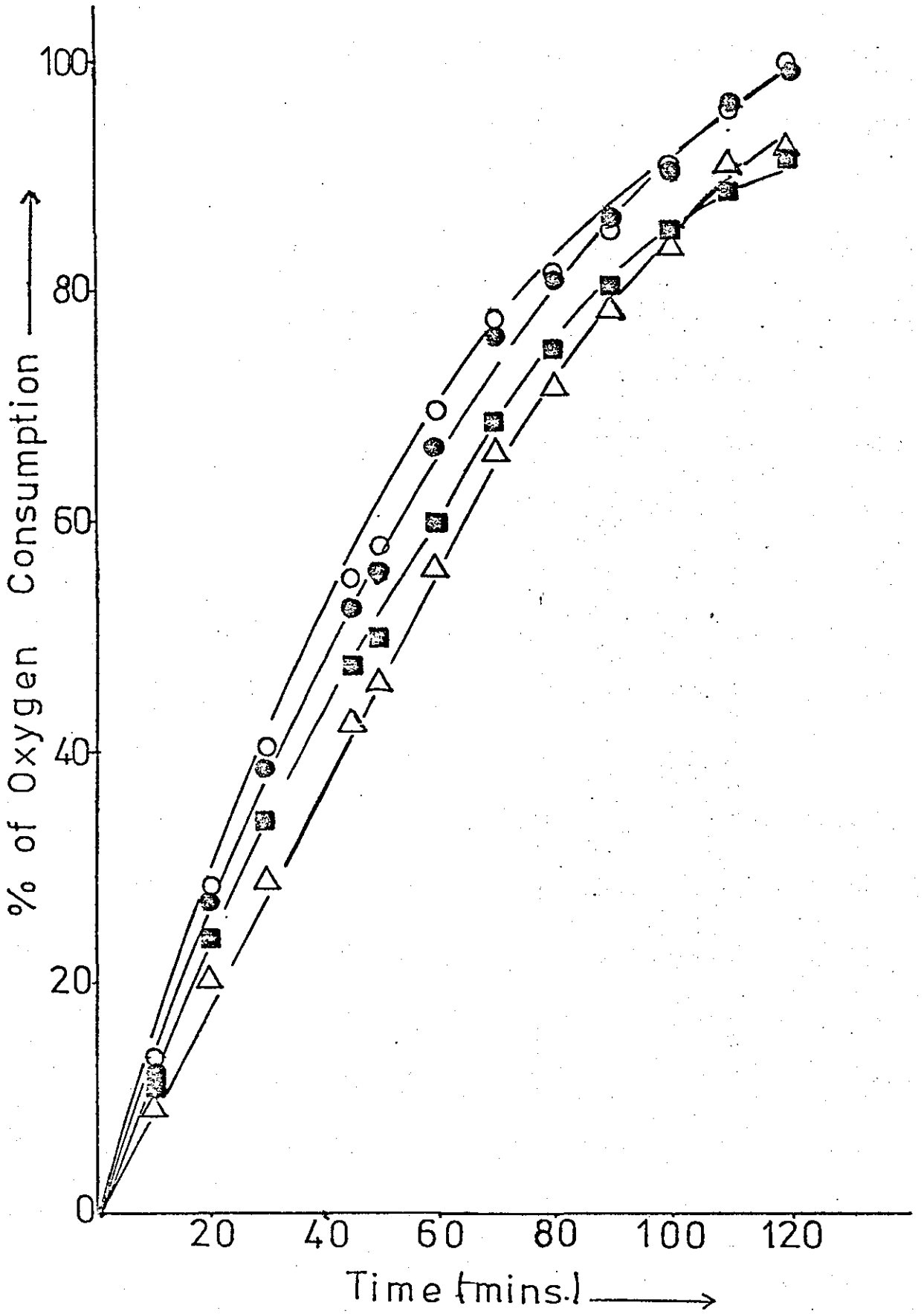

100
80
60
40
20
0
% of Oxygen Consumption
20
40
60
80
100
120
Time (mins.)

Fig. (3.63)

Effect of Zn(II) in the inhibition of oxygen consumption by Phanquone for *E. coli* suspended in 0.1M sodium phosphate buffer, pH 7.4, using sodium succinate as (0.06M) as substrate:

○—○ : control;
▲—▲ : plus $ZnSO_4$ (2.5×10^{-4}M);
●—● : plus Phanquone (~2.5×10^{-4}M);
□—□ : plus $ZnSO_4$ (2.5×10^{-4}M) plus Phanquone (2.5×10^{-4}M).

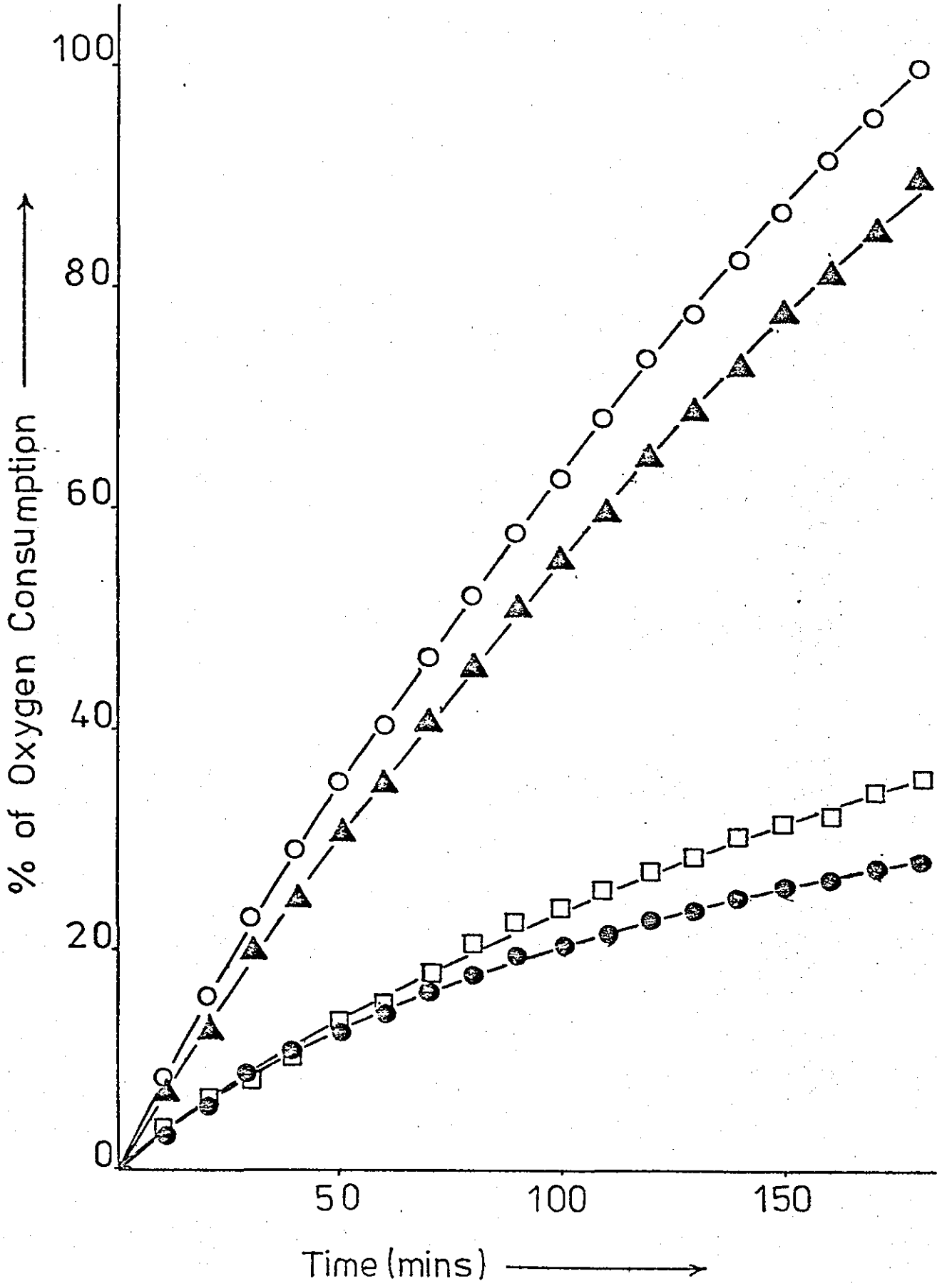

100
80
60
40
20
0
% of Oxygen Consumption
50
100
150
Time (mins)

Fig. (3.64)

Effect of Ni(II) on the inhibition of oxygen consumption by Phanquone for *S. aureus*, using D-glucose (0.02M) as substrate:

○—○ : control;
△—△ : plus $NiSO_4$ (2.5 x 10-4M);
■—■ : plus Phanquone at 50 μg/ml (~2.5 x 10-4M);
●—● : plus $NiSO_4$ (2.5 x 10-4M) plus Phanquone (2.5 x 10-4M).

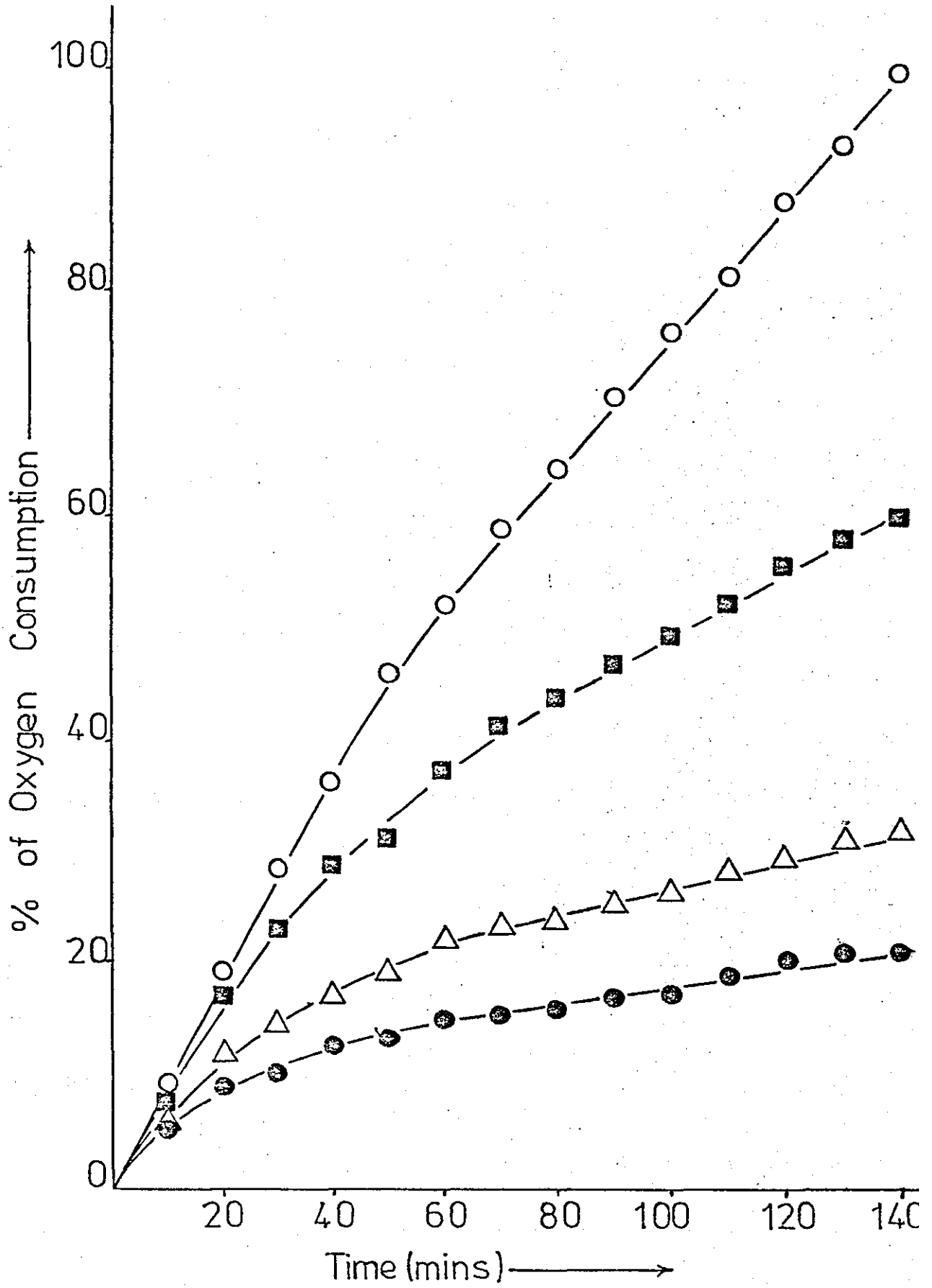

100
80
60
40
20
0
% of Oxygen Consumption
20
40
60
80
100
120
140
Time (mins)

Fig. (3.65)

Effect of Zn(II) on the inhibition of oxygen consumption by Phanquone for *S. aureus* suspended in 0.1M sodium phosphate buffer, pH 7.4, using sodium succinate (0.06M) as substrate:

■—■ : control;
○—○ : plus $ZnSO_4$ (2.5×10^{-4}M);
●—● : plus Phanquone at 50 µg/ml (~2.5×10^{-4}M);
□—□ : plus $ZnSO_4$ (2.5×10^{-4}M) plus Phanquone (2.5×10^{-4}M).
▲—▲ : endogenous respiration.

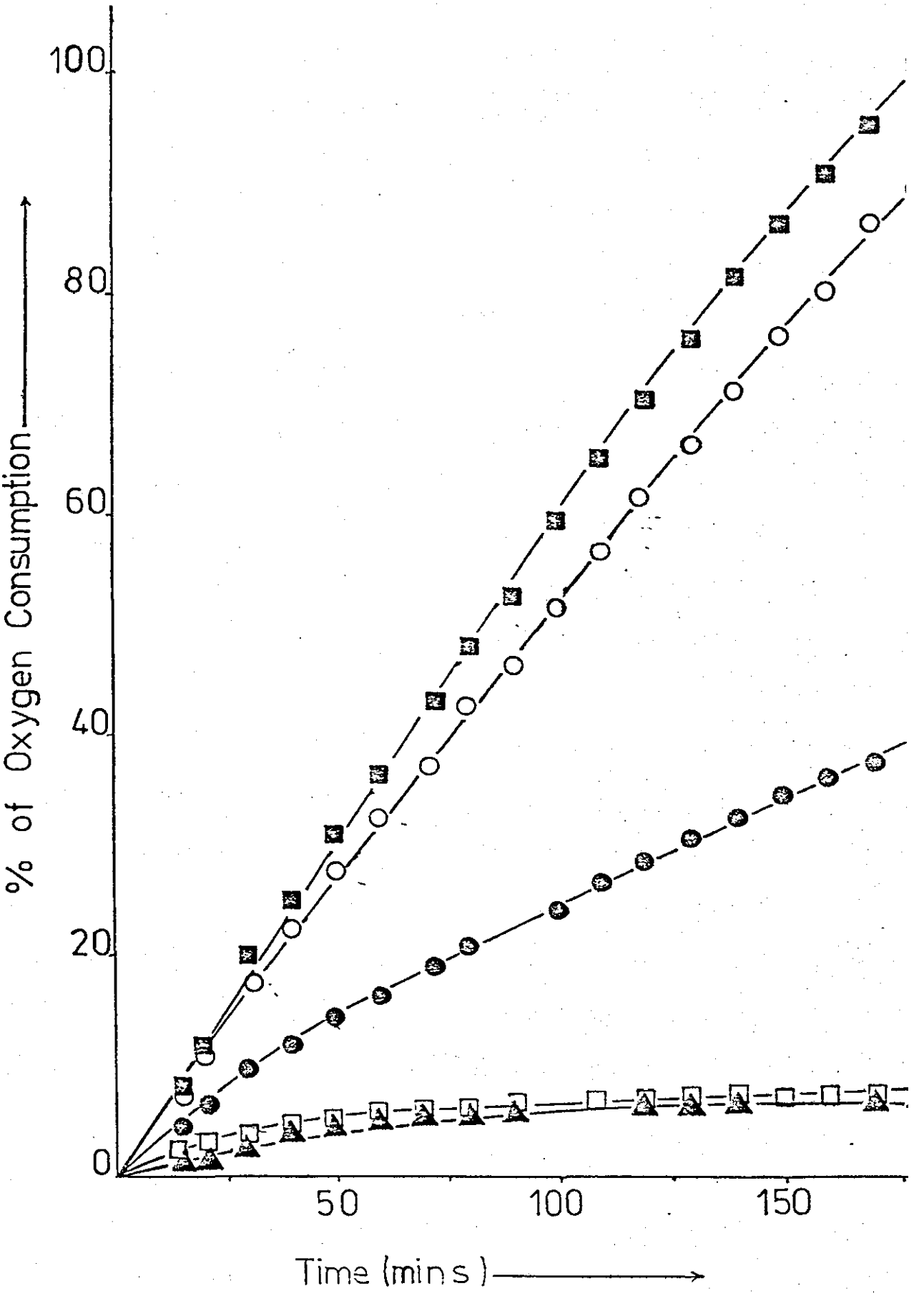

100
80
60
40
20
0
% of Oxygen Consumption
50
100
150
Time (mins)

Fig. (3.66)

Effect of Co(II) on the inhibition of oxygen consumption by Phanquone for *S. aureus* suspended in 0.1M sodium phosphate buffer, pH 7.4, using sodium succinate (0.06M) as substrate:

○—○ : control;
▲—▲ : plus $CoSO_4$ (2.5×10^{-4}M);
●—● : plus Phanquone (~2.5×10^{-4}M);
□—□ : plus $CoSO_4$ (2.5×10^{-4}M) plus Phanquone (2.5×10^{-4}M).

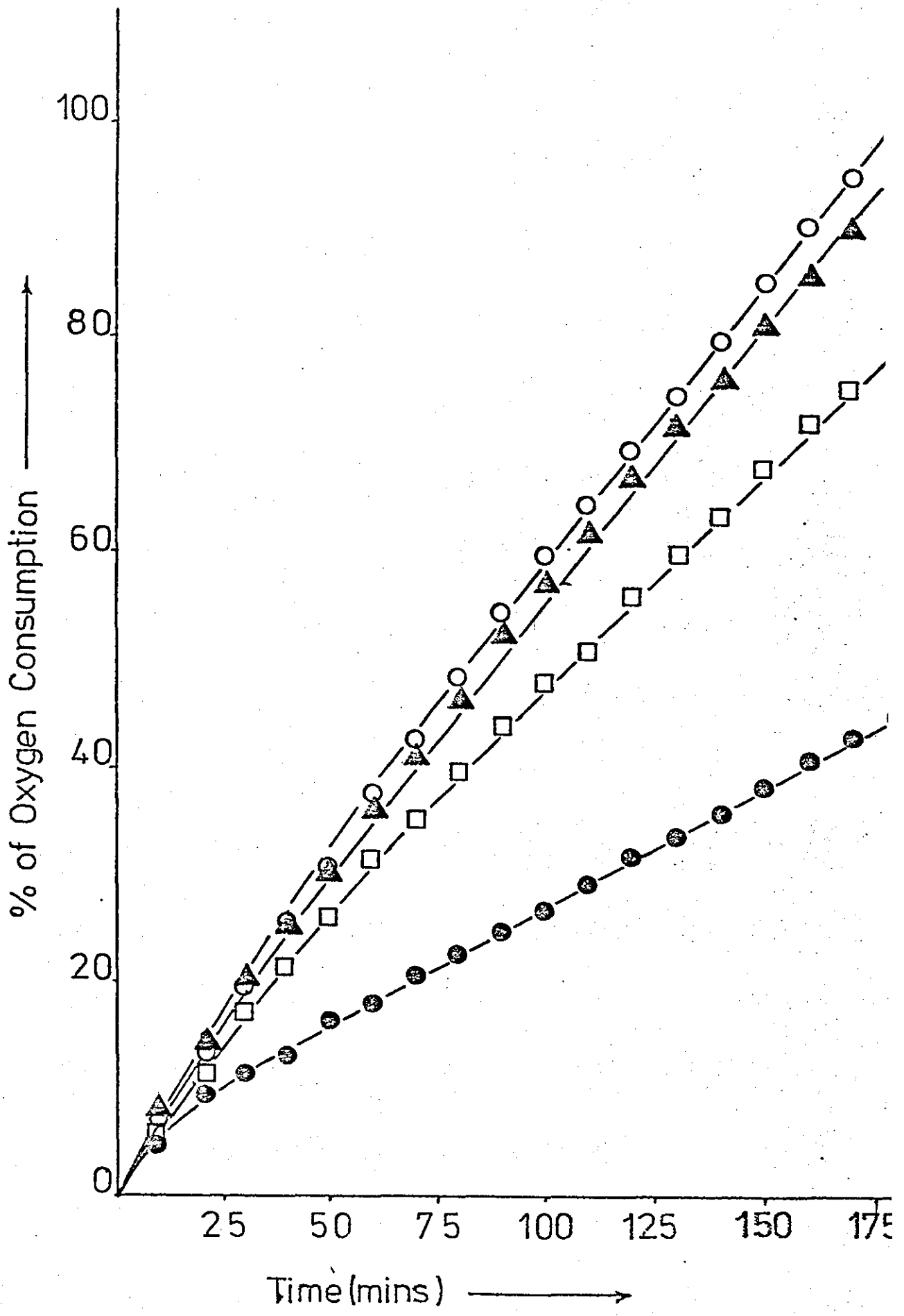

100
80
60
40
20
0
% of Oxygen Consumption
25
50
75
100
125
150
175
Time (mins)

Fig. (3.67)

Effect of Fe(II) on the inhibition of oxygen consumption by Phanquone for *S. aureus* suspended in 0.1M sodium phosphate buffer, pH 7.4, using sodium succinate (0.06M) as substrate.

○—○ : control;
■—■ : plus $FeSO_4$ (2.5×10^{-4}M);
□—□ : plus Phanquone at 50 μg/ml (~2.5×10^{-4}M);
●—● : plus $FeSO_4$ (2.5×10^{-4}M) plus Phanquone (2.5×10^{-4}M).

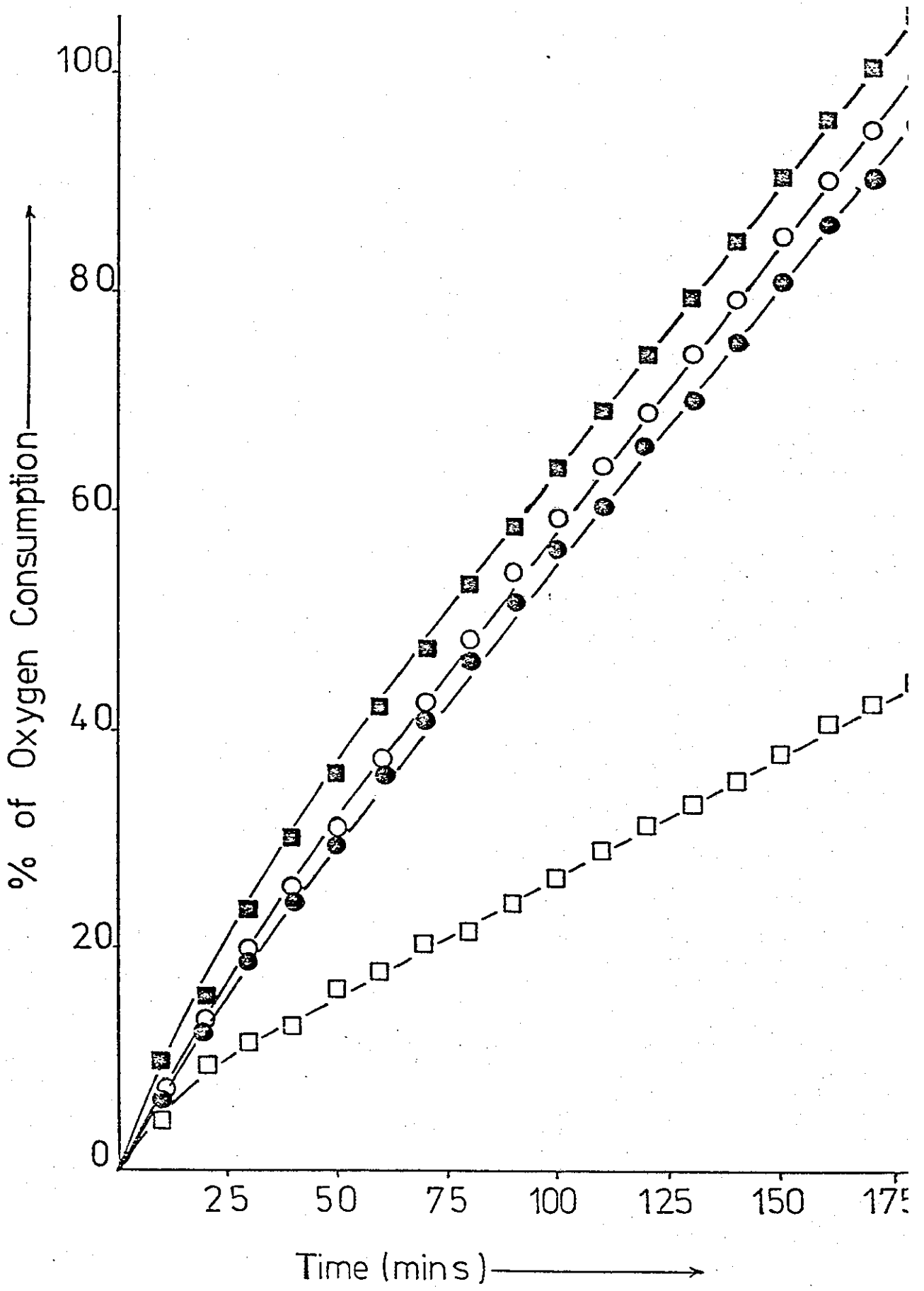

100
80
60
40
20
0
% of Oxygen Consumption
25
50
75
100
125
150
175
Time (mins)

Table (3.20)

Effect of EDTA and NTA on the zone of inhibition produced by Phanquone using the right-angled strips method on nutrient agar

- = no effect;
+ = antagonism;
++ = prominent antagonism.

Antibacterial	Effect of EDTA		Effect of NTA	
	E. coli	S. aureus	E. coli	S. aureus
Phanquone	–	++	–	++
9,10-phenanthrene-quinone	–	–	–	–
1,10-phenanthroline	–	++	–	++
1,10-phenanthroline-5,6-quinone	–	+	–	+
Oxine	++	++	–	–

Fig. (3.68 (a))

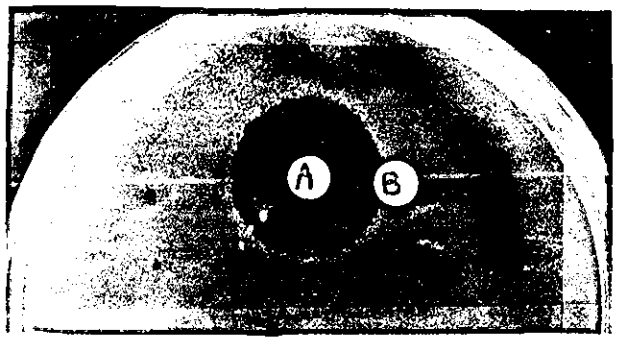
Effect of EDTA on the zone of inhibition produced by Phanquone on nutrient agar. Disc A contained Phanquone (50 μg); while disc B contained EDTA solution (0.05M):

i) *E. coli;*
ii) *S. aureus.*

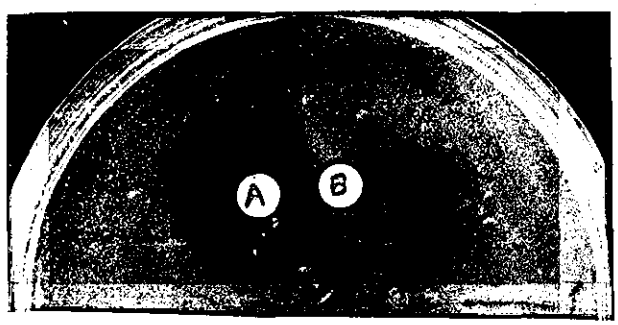
Fig. (3.68 (b))

Effect of EDTA (strip B) on the zone of inhibition produced by Phanquone and 9,10-phanthrenequinone using the right-angled strips method on nutrient agar:

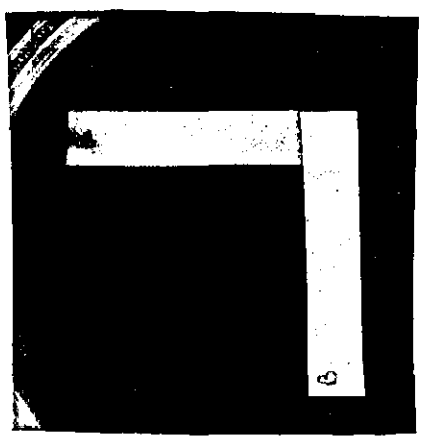
i) for *E. coli* using Phanquone (strip A);
ii) for *S.aureus* using Phanquone (strip A);
iii) for *S.aureus* using 9,10-phenanthrene-quinone (strip A).



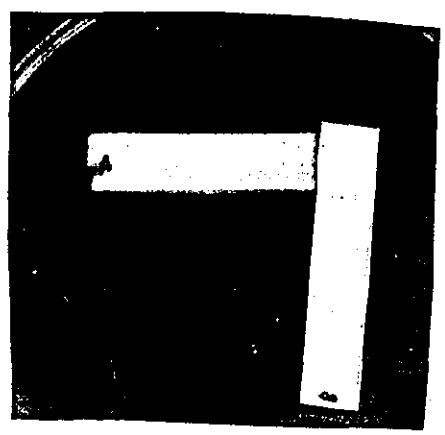
(i)



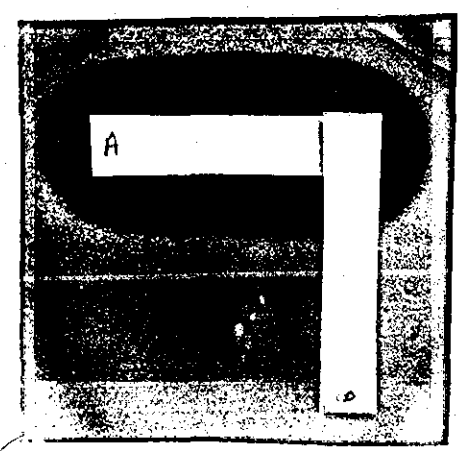
(ii)



(i)



(ii)



(iii)

8. THE EFFECT OF PROLONGING THE PREINCUBATION PERIOD ON THE ZONE OF INHIBITION PRODUCED BY PHANQUONE

Fig. (3.69) shows the effect of prolonging the preincubation period on the zone of inhibition produced by Phanquone for *E. coli* and *S. aureus* respectively. As can be seen, there was a definite zone of growth produced inside the zone of inhibition for *S. aureus*, and this occurred at both pH 7.7 and pH 8.0; while the zone of inhibition produced by Phanquone for *E. coli* contained no zone of growth.

Fig. (3.70) shows the *S. aureus* zone of growth more clearly.

9. THE EFFECT OF PHANQUONE ON THE BACTERIAL CELL MORPHOLOGY USING SCANNING ELECTRON MICROSCOPY

Fig. (3.71) shows *E. coli* cells, coated with gold palladium, and examined under the Scanning Electron Microscopy, in the presence and absence of Phanquone, while Fig. (3.72) shows cells examined under the same microscope, in the presence and absence of Phanquone. In both cases, there were no obvious differences between the control and the test cell morphology.

Fig. (3.69)

Effect of prolonging the preincubation period on the zone of inhibition produced by Phanquone on nutrient agar. Plates were preincubated for 24 hours at $4^{o}C$ (pH 8.0).

a) for *E. coli;*

b) for *S. aureus.*

(a)

(b)

Fig. (3.70)

The zone of growth produced inside the zone of inhibition of Phanquone for *S. aureus* on nutrient agar plates at pH 8.0.

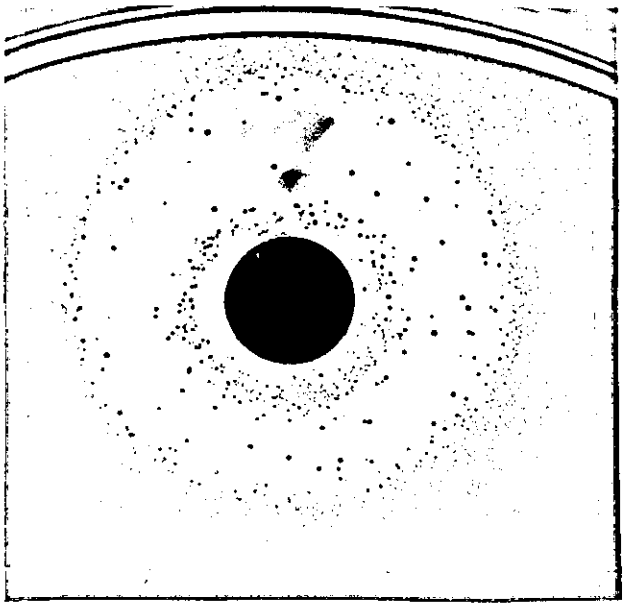


Fig. (3.71)

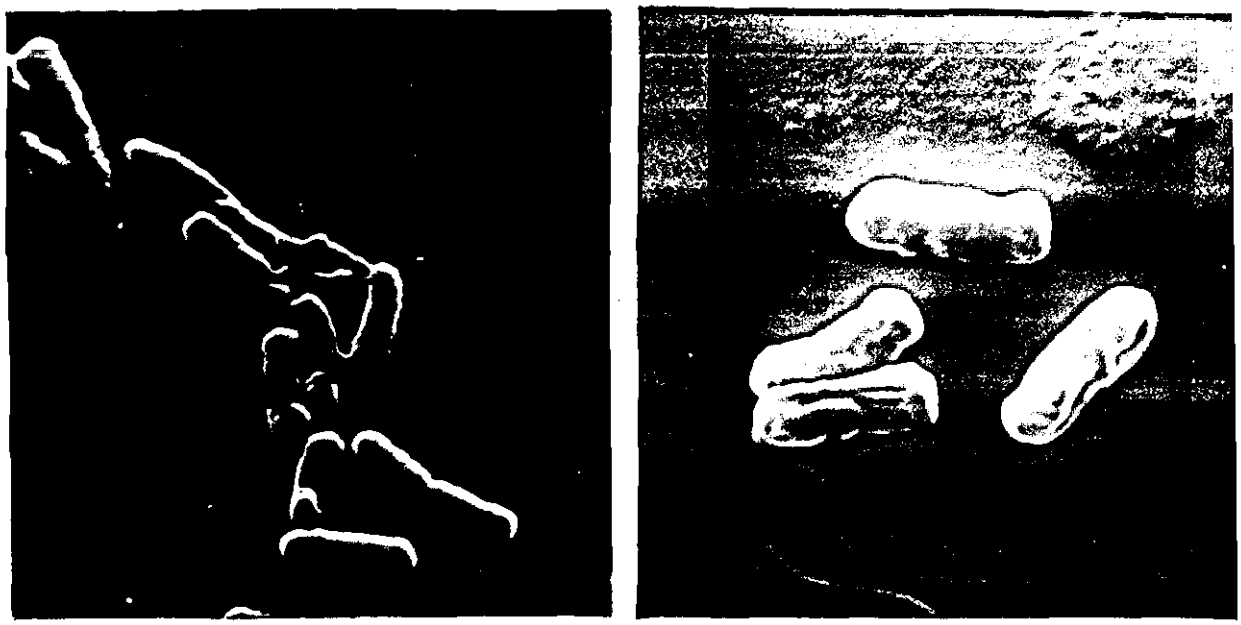
Scanning Electron Microscopy of *E. coli* cells grown in nutrient broth.

a) control cells

i) x10 K;
ii) x22 K.

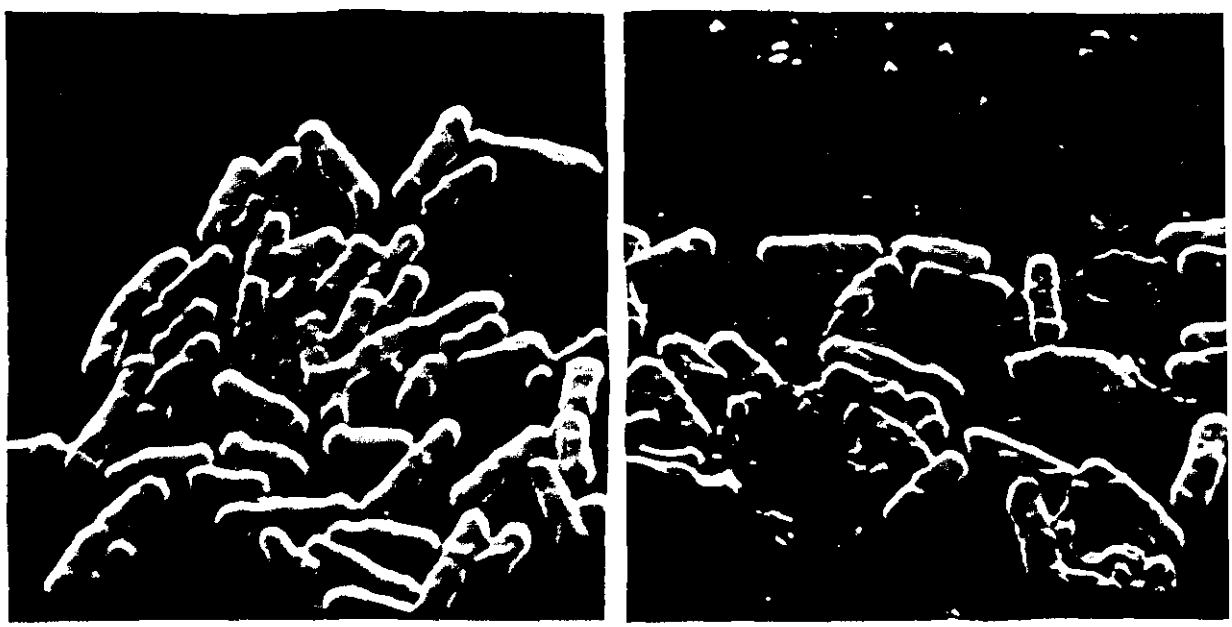
b) cells left in contact with Phanquone for 90 mins.

i) x10 K;
ii) x10 K.



(i) (ii)

(a)



(i) (ii)

(b)

Fig. (3.72)

Scanning Electron Microscopy of *S. aureus* cells grown in nutrient broth.

a) control cells:

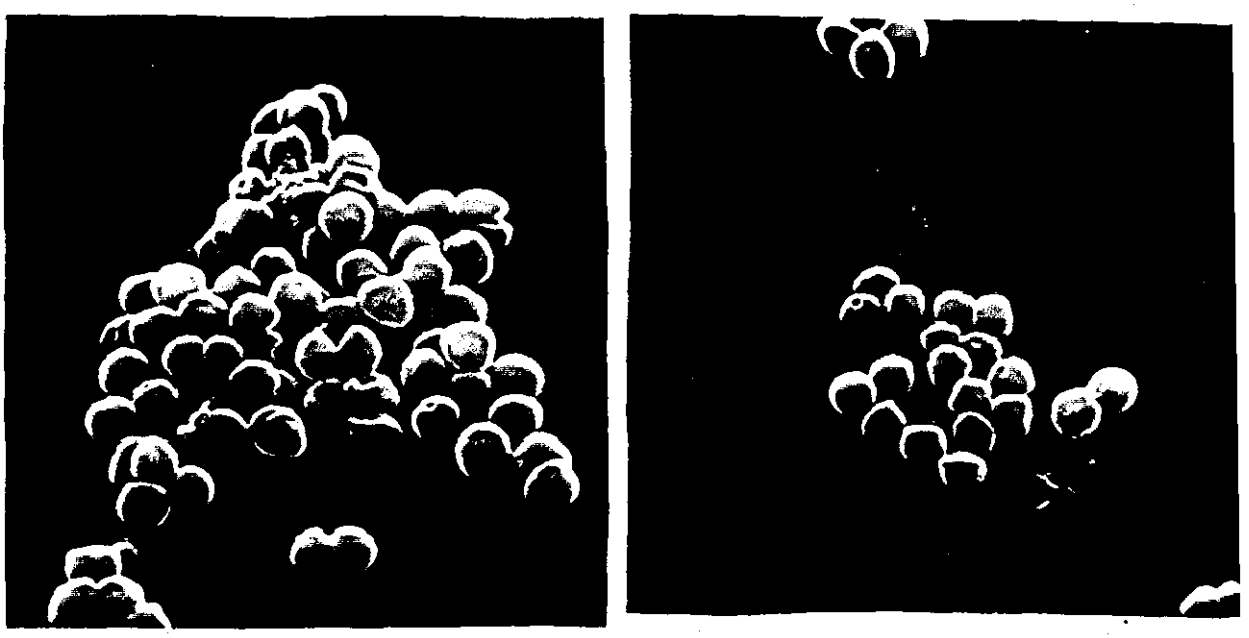
i) x10 K;
ii) x20 K.

b) cells left in contact with Phanquone for 90 mins.

i) x10 K;
ii) x10 K.

(i) (ii)

(a)



(i) (ii)

(b)

10. THE EFFECT OF PHANQUONE ON THE RELEASE OF PHOSPHORUS-(32)-CONTAINING COMPOUNDS BY BACTERIAL CELLS

The effect of Phanquone on the leakage of P-(32)-containing compounds on *E.coli* and *S.aureus* cell suspensions is shown in Figs. (3.73) and (3.74), respectively. For *E.coli*, the leakage in the presence of Phanquone was 10% - 15% less than the leakage from control cells. For *S.aureus*, however, the amount of P-(32)-containing compounds that leaked was more or less the same in the presence and absence of Phanquone.

11. THE EFFECT OF PHANQUONE ON LIVER MONOAMINE OXIDASE

As can be seen from Fig. (3.75), there was some stimulation of oxygen consumption utilised by the liver monoamine oxidase, followed by a drop in consumption. The endogenous respiration, however, was about 85% of the consumption by the control. By comparison, oxine had no effect on the utilization of oxygen by the oxidase.

12. THE MUTAGENIC EFFECT OF PHANQUONE USING THE SALMONELLA/MICROSOME TEST

Table (3.21) shows the effect of different concentrations of Phanquone on the number of reverted colonies produced by the five salmonella mutant strains, compared to the number of spontaneous colonies. The number of colonies increased (at least two-fold) in

Fig. (3.73)

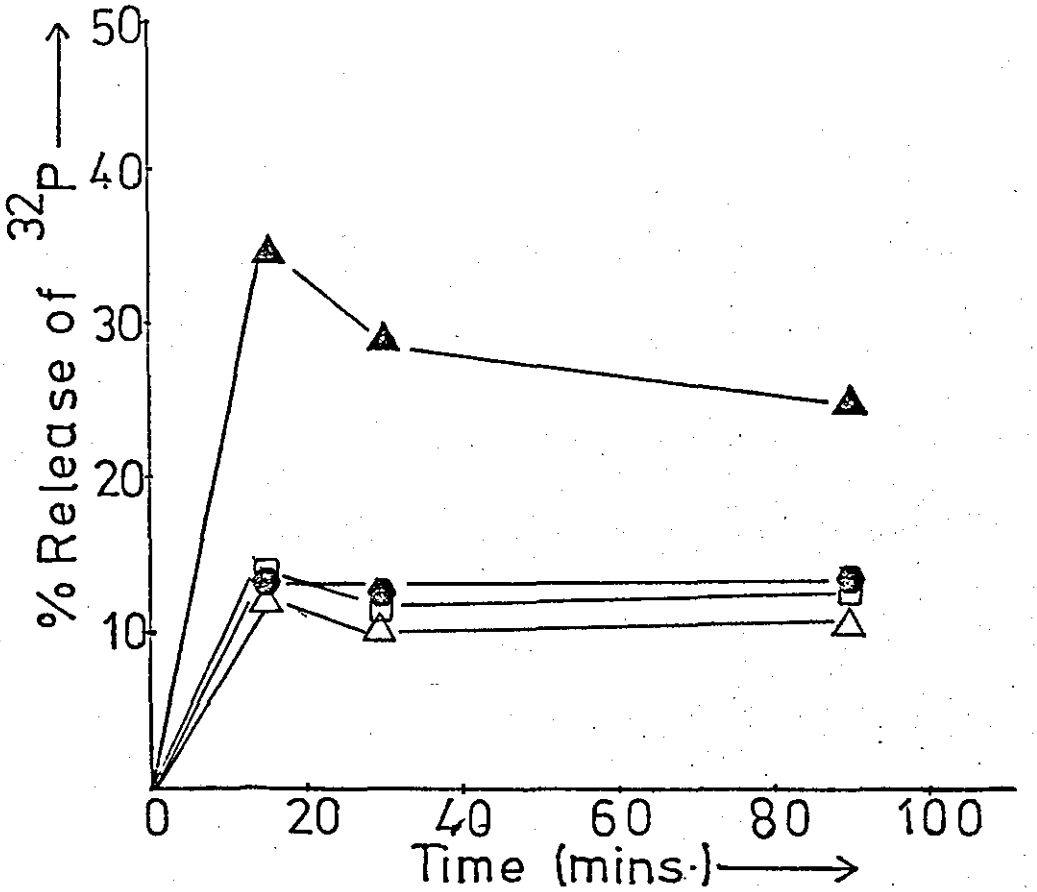
Effect of Phanquone on the release of P(32)-inorganic phosphate by *E. coli* cells suspended in 0.1M sodium phosphate buffer, pH 7.4

▲—▲ : control;
●—● : 100 µg/ml;
□—□ : 200 µg/ml;
△—△ : 300 µg/ml.

Fig. (3.74)

Effect of Phanquone on the release of P(32)-inorganic phosphate by *S. aureus* suspended in 0.1M sodium phosphate buffer, pH 7.4

▲—▲ : control;
●—● : 100 µg/ml;
□—□ : 200 µg/ml;
△—△ : 300 µg/ml.


% Release of ^{32}P
50
40
30
20
10
0
20
40
60
80
100
Time (mins.)

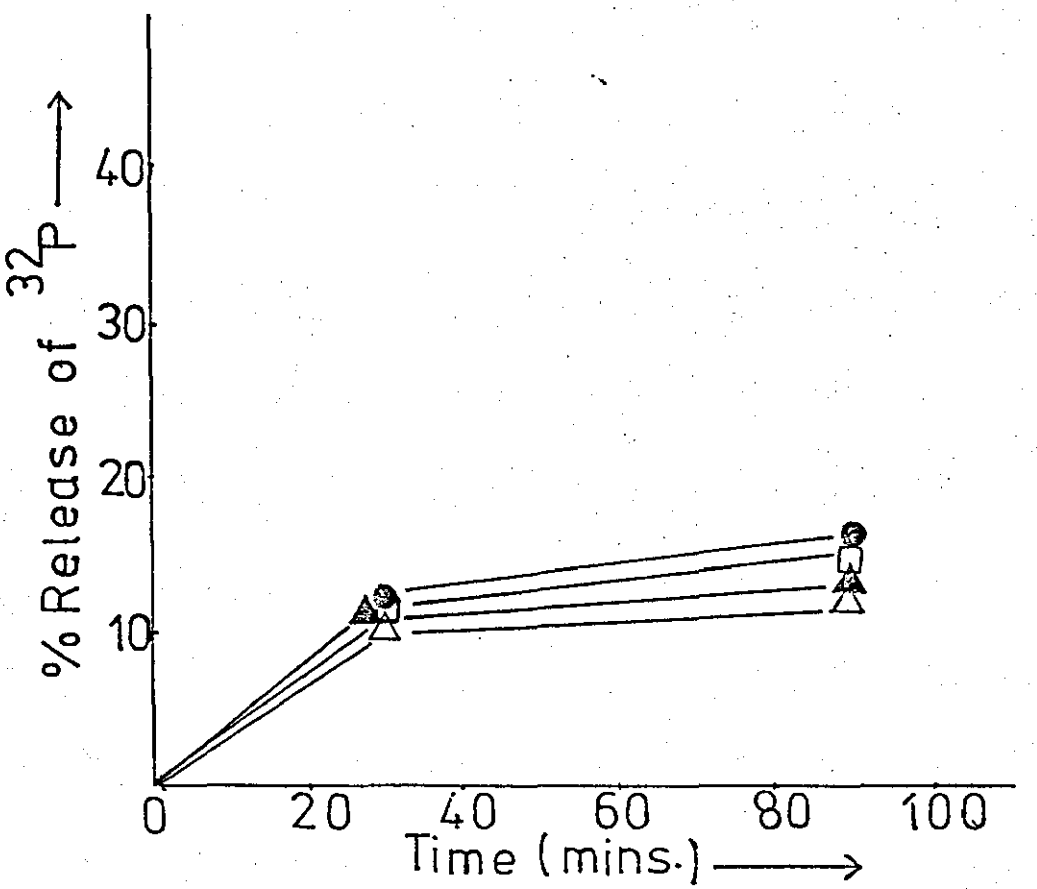

% Release of ^{32}P
40
30
20
10
0
20
40
60
80
100
Time (mins.)

Fig. (3.75)

Effect of Phanquone on the utilization of oxygen by liver monoamine oxidase from guinea-pig

○—○: control (no substrate);
■—■: plus benzylamine (0.04M);
△—△: plus benzylamine (0.04M) plus Phanquone (100 µg/ml).

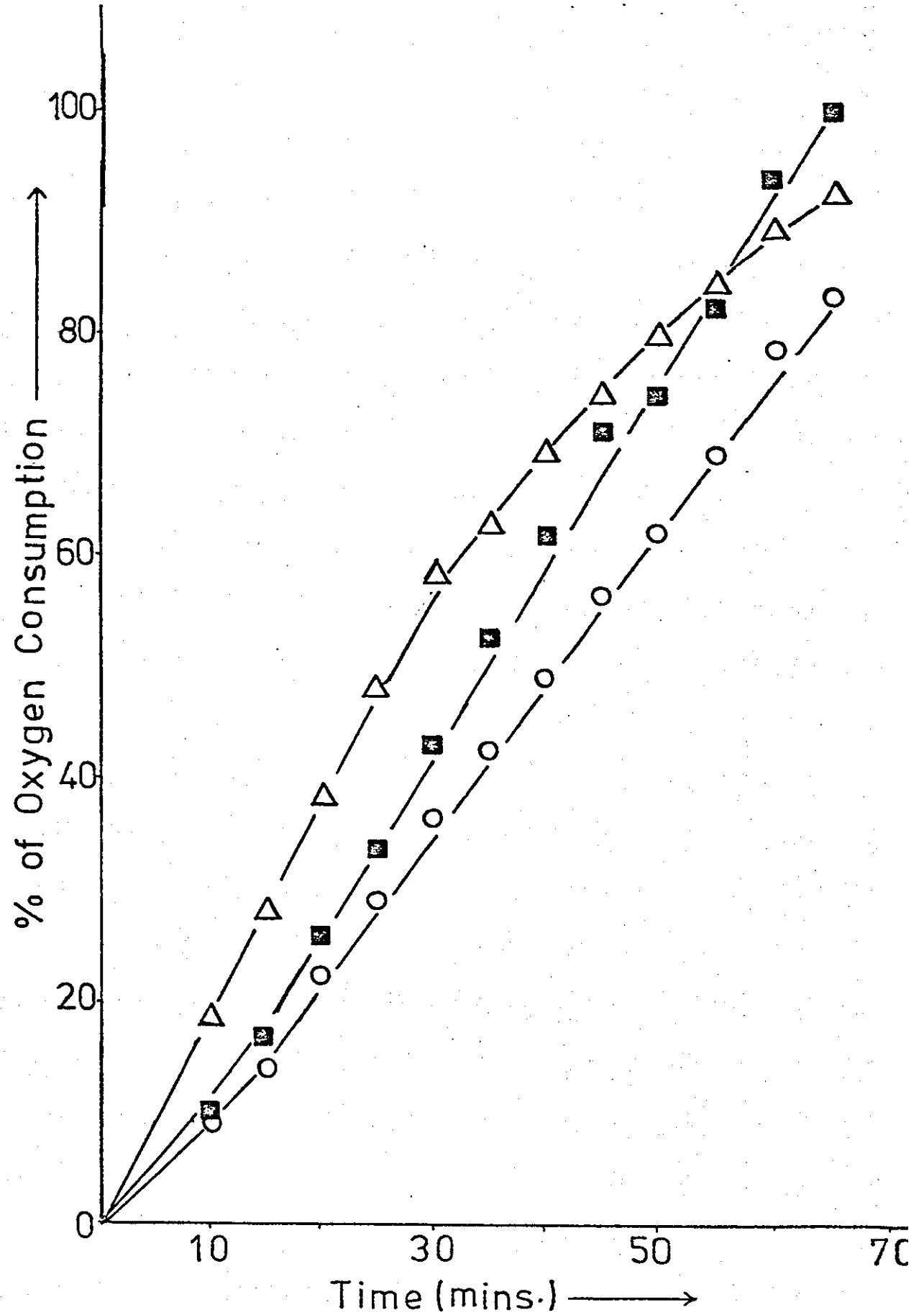

100
80
60
40
20
0
% of Oxygen Consumption
10
30
50
Time (mins.)

Table (3.21)

Effect of Phanquone on the number of revertant colonies produced using the Salmonella/microsome test

±	:	< 400 colonies;
+	:	~400 colonies;
++	:	~800 colonies;
+++	:	>1000 colonies.

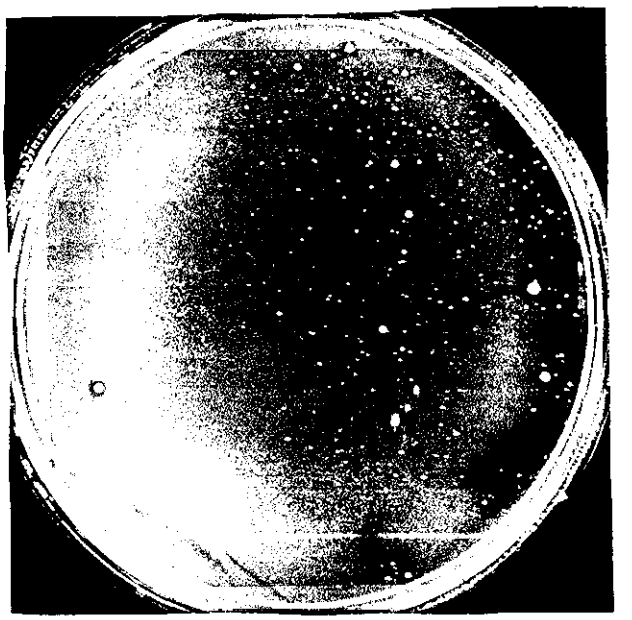
Salmonella typhimurium	Control	Phanquone added		
		10 µg/ml	5 µg/ml	2.5 µg/ml
TA1535	+	+ + +	+ + +	+ +
TA1537	+	+	+ +	+
TA1538	+	±	+	+
TA 98	+	+	+ +	+ + +
TA 100	+	+ +	+ +	+

number, for strains TA 1535, TA 98 and TA 100 (Figs. 3.76 and 3.77) for at least two of the three Phanquone concentrations tested. However, there was no significant increase in the number of colonies for the TA 1537 and TA 1538 strains (Fig. 3.78).

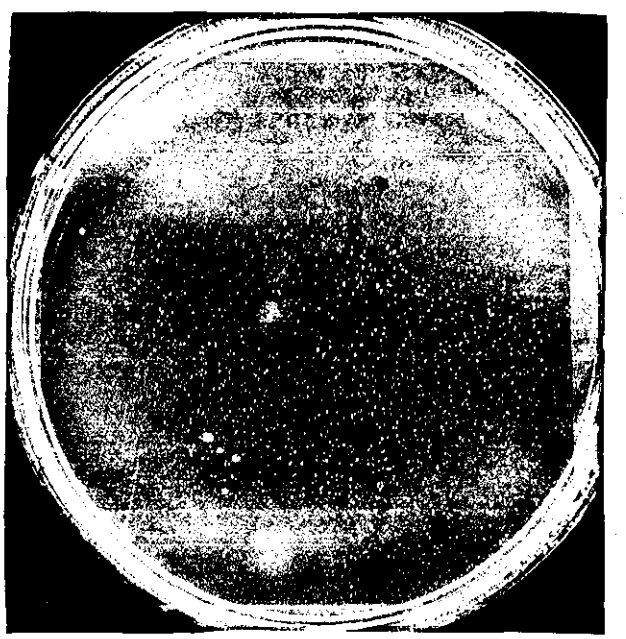
Fig. (3.76)

The increase in the number of revertant colonies produced by Phanquone using *Sal. typhimurium* LT2 TA 1535

a) control;
b) plus 10 µg/ml.



(a)

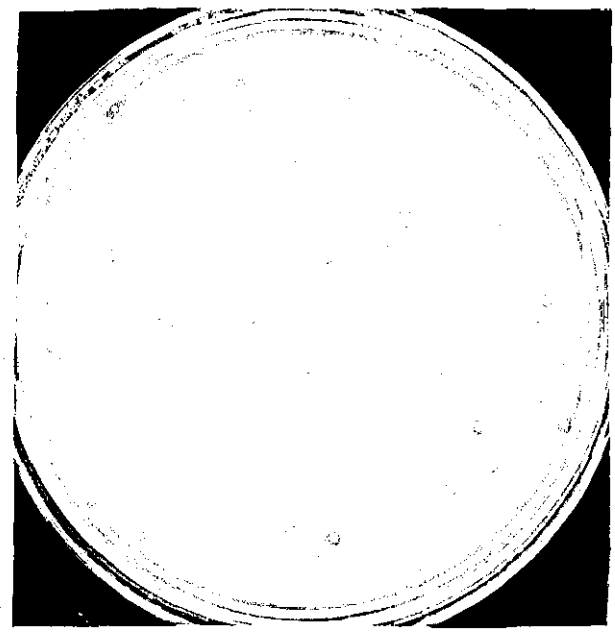


(b)

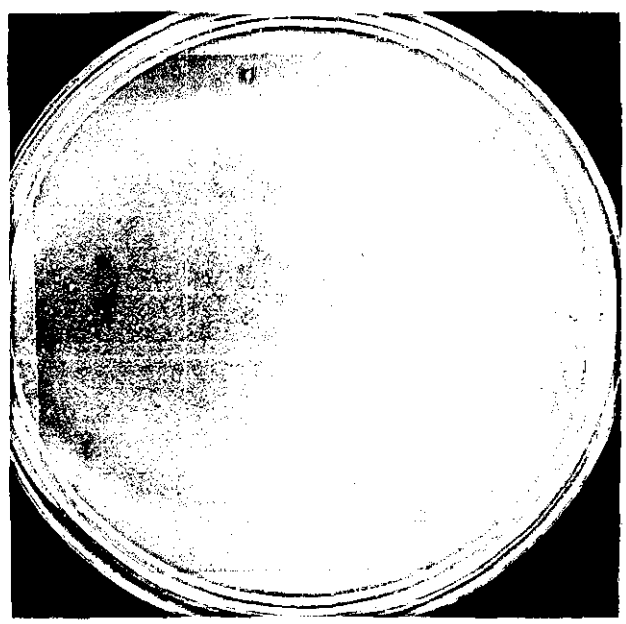
Fig. (3.77)

The increase in the number of revertant colonies produced by Phanquone using *Sal. typhimurium* LT2 TA 100.

a) control;
b) plus 10 µg/ml.



(a)

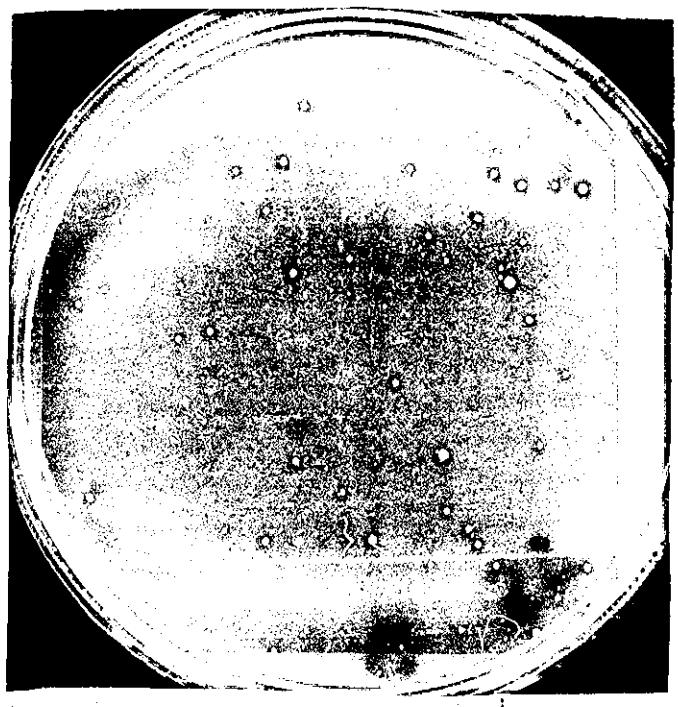


(b)

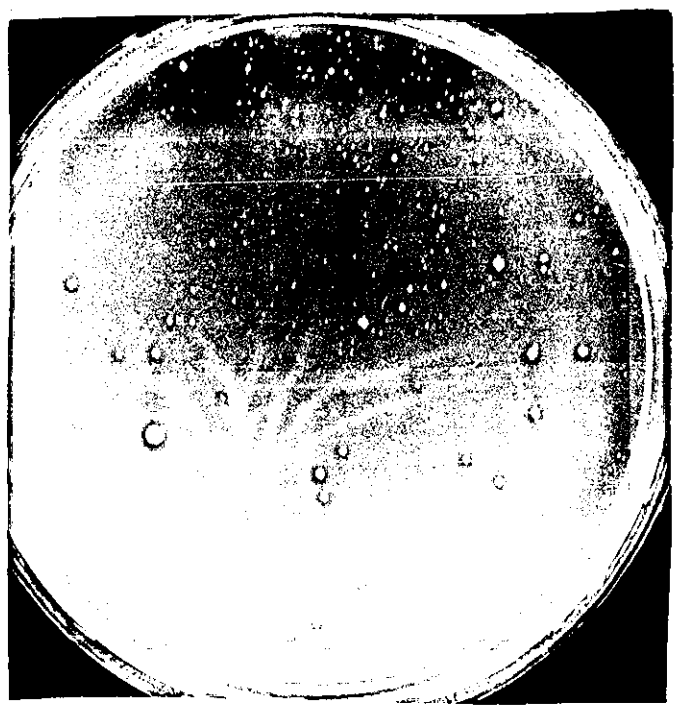
Fig. (3.78)

The no-change in the number of revertant colonies produced by Phanquone using *Sal. typhimurium* LT2 TA 1538.

a) control;
b) plus 25 μg/ml.



(a)



(b)

PART IV

DISCUSSION

1. THE ANTIBACTERIAL ACTIVITY OF PHANQUONE

The MIC and MCC values obtained suggest that Phanquone is a broad spectrum antibacterial agent, affecting the Gram-positive, Gram-negative, as well as Acid-fast bacteria. It is active against a strict aerobe (*Ps. aeruginosa*), a strict anaerobe (*Cl. sporogenes*), and many facultative aerobes such as *E. coli* and *S. aureus*.

This broad spectrum of activity was also reported by Kradolfer and Neipp (1958), and Sackmann and Kradolfer (1961), who found that Phanquone was active against many Gram-positive as well as many Gram-negative bacteria. The most sensitive strain (in terms of MIC) was *Sal. abortus ovis*, while the most resistant was *Strep. faecalis*. The latter was also found to be very resistant by Kradolfer and Neipp (1958), and Sackmann and Kradolfer (1961); this resistance could be significant in understanding the mode of action of Phanquone as *Strep. faecalis* lacks a cytochrome chain (Dolin, 1961).

However, it is important to stress the fact that Phanquone is not only an active antibacterial agent, but also possesses a broad spectrum antimicrobical activity, covering amoeboid protozoans of the class Sarcodina (Kradolfer and Neipp, 1958; Carter, 1961; Kaushiva, 1964; and Krishna Prasad, 1972), protozoan flagellates and ciliates of the classes Mastigophora and Ciliophora, respectively, and helminths of the class Trematoda (Kradolfer and Neipp, 1958; Kradolfer, Sackmann and Bassil, 1960), as well as the fungus *Candida albicans* (Ansel and Thibaut, 1967).

Hence, Phanquone is by no means active against, or specific for, one class of microorganisms only, but is, on the contrary, active against many microorganisms that are quite unrelated.

This, perhaps, suggests that the mode of action of Phanquone might involve a system(s) that is(are) common in, or to, a wide variety of microorganisms; and/or that Phanquone might have more than one mode of action, and therefore, affects different microorganisms in different ways.

The results obtained from the MCC determinations and from the kill curves for growing cultures, indicate that Phanquone is a bactericidal agent. The kill curves also suggest that the action of Phanquone is rapid, as there was an appreciable drop in the viable number of both growing *E. coli* and *S. aureus* cells, almost immediately after its addition.

While the action of Phanquone against growing *E. coli* cultures seemed to be similar to its action against growing *S. aureus* cultures, that is, rapid and bactericidal, its action against non-growing *E. coli* cells seemed to be different from its action against non-growing *S. aureus* cells; in the case of the *E. coli* suspension, the action of Phanquone seemed to be even more rapid than its action on growing cultures, denoting a "direct" action, (Russell, Morris and Allwood, 1973). On the other hand, Phanquone did not seem to be totally lethal (even at 80 µg/ml) to the *S. aureus* suspension. It can be assumed, then, that for *E. coli*, an antagonizing substance(s) existed in nutrient broth, but not in the buffer, whose removal (or absence) caused Phanquone to have a more rapid action. A similar finding was reported for glutaraldehyde, which interacted considerably with broth constituents; therefore, its MIC in broth was

around 50 times higher than was expected (Rubbo, Gardener and Webb, 1967; Munton and Russell, 1970; Russell, Morris and Allwood, 1973).

In the same manner, it can be assumed that for *S. aureus*, a substance(s) which was synergistic to Phanquone, existed in the nutrient broth but not in the buffer or the distilled water; thus, its absence caused Phanquone to be less potent.

Results obtained by the MSST method for the *E. coli* cells confirm the "direct" and bactericidal effect of Phanquone. The MSST method detects the time at which only one single cell is still surviving, and therefore, the times obtained by this method are more accurate than the viable count method. This technique has been utilized by many workers (Berry and Bean, 1954; Hugo and Frier, 1969; Stretton and Manson, 1973) for phenol, dequalinium acetate (1,1-decamethylene-diamine-(4-aminoquinaldinium acetate), and bronopol (2-bromo-2-nitro-propan-1,3-diol), respectively. Compared to both dequalinium acetate and bronopol, Phanquone seemed to be a better bactericide.

Berry and Bean (1954) concluded that the values obtained by this method are comparable, if not better, than those obtained using comparable techniques, such as the Rideal-Walker test (Rideal and Walker, 1903).

As for *S. aureus*, the inability to obtain any meaningful results, confirms the viable count results in that Phanquone is not completely bactericidal in buffer or distilled water, and suggests that probably one (or some) additional component(s) must be present in the medium, in order that Phanquone produces a rapid and complete kill for *S. aureus* cells.

2. ADSORPTION OF PHANQUONE TO BACTERIAL CELLS

A substantial amount of Phanquone was rapidly adsorbed to both *E. coli* and *S. aureus* cells. Although the amount adsorbed to the *S. aureus* cells was nearly twice as much as the amount adsorbed to *E. coli*, yet, Phanquone caused more damage to *E. coli*, which means that Phanquone was adsorbed (and probably taken up) to the *S. aureus* cell without harming it. A related compound, oxine, was found to enter the *S. aureus* cell without harming it (Beckett, Vahora, and Robinson, 1958; Albert, 1973).

The adsorption-time curves (Fig. 3.19, p.89) also indicated that about twice as much Phanquone was adsorbed to *S. aureus* as to *E. coli*. There, maximum adsorption occured after about 5 minutes for *S. aureus*, and 10 minutes for *E. coli*, followed by the release of some of the Phanquone back into the buffer. This probably indicates that the adsorption was not absolutely irreversible, as was the case for Fentichlor (2,2´-thiobis-4-dichlorophenol; Hugo and Bloomfield, 1971(a)).

When plotting the amount of Phanquone adsorbed against the supernatant liquid concentration (Fig. 3.11; p. 88), a curve was obtained which closely corresponded to the Langmuir form (L-form) curve described by Giles, MacEwan, Nakhawa and Smith (1960), where the curvature indicated that as some sites on the bacterial cell wall were filled, it became increasingly difficult for other Phanquone molecules to find a vacant site available. This also implied that either the adsorbed Phanquone molecule was not vertically oriented,

or that there was no strong competition from the solvent.

Moreover, the Phanquone adsorbed probably partitioned between the aqueous phase and the lipid biophase of the cell, as it was soluble in both phases (indicated by the partitioning of Phanquone between the aqueous and octanol phases).

3. THE CHEMISTRY OF CHELATON OF PHANQUONE

Theoretically speaking, Phanquone can be thought of as a quadridentate, and may chelate one, two or three metal ions as shown in Fig. (4.1), giving 1:1, 1:2 or 1:3 Phanquone-metal ion complexes, respectively. Similarly, Phanquone may also form 2:1, 3:1 or even 3:2 Phanquone-metal ion complexes.

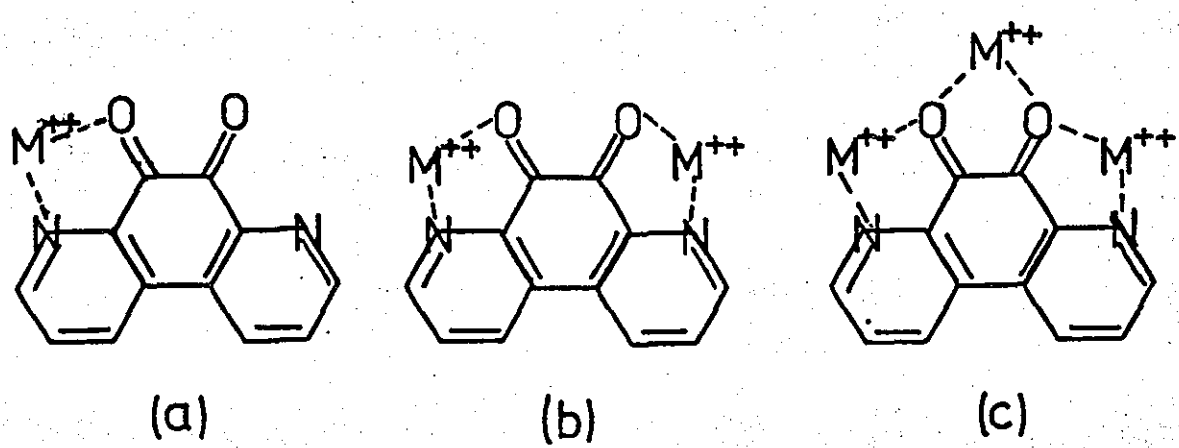


Fig. (4.1)

Some Phanquone-metal Ion Complexes

a) at 1:1 ratio;
b) at 1:2 ratio;
c) at 1:3 ratio.

The change in colour of a solution containing a metal ion and a ligand (such as Phanquone), is probably the most obvious indication of a complex formation. This change in colour may sometimes help to elucidate the stability of the complexes formed, using spectrophotometric methods such as the method of continuous variation for the construction of Job-plots (Job, 1928; Vosburgh and Cooper, 1941; Dagnall and West, 1961; 1964). Moreover, the formation of a precipitate can also be used as an indication of complex formation (Albert and Gledhill, 1947; Albert and Magrath, 1947; Albert, Rubbo, Goldacre and Balfour, 1947).

In the case of Phanquone, the change in colour observed, when mixed with some of the metal ions (e.g. Fe(II), Fe(III), Co(II), Ni(II) and Cu(II)), was a good indication of the formation of metal complexes.

However, the method of continuous variation could not be applied to all Phanquone-metal ion complexes, with the exception of the Fe(III) complex, due to the formation of a precipitate, and especially in the case of Cu(II), Fe(II) and Co(II). Also, Phanquone solutions had a bright yellow colour when dissolved in water, and this colour interfered with the spectrophometric readings. Moreover, when Phanquone was dissolved in 0.1N H_2SO_4, to obtain a colourless solution, the change in colour obtained from the Phanquone-metal ion mixtures, was very small. This was probably due to the hydrogenation of the nitrogen atoms of Phanquone in acidic conditions, as shown in Fig. (4.2), which probably impeded the formation of the Phanquone-metal ion complexes. Therefore, it was quite difficult to measure the stability of the different complexes that might have formed when Phanquone and the metal ions were mixed.

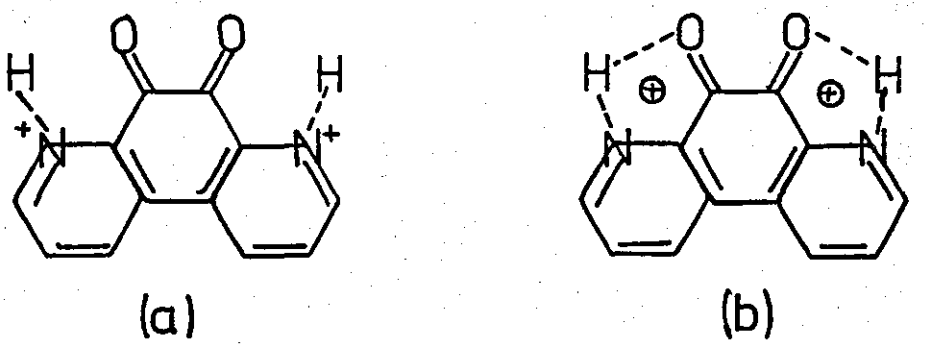


Fig. (4.2)

Hydrogenation of Phanquone in Acidic Media

a) hydrogen bonding with the nitrogen atoms;
b) hydrogen bonding with both nitrogen and oxygen atoms.

An attempt to obtain a Job-plot was made with Phanquone, dissolved in 0.1N H_2SO_4, and Fe(III), where the mixture had to be left for at least 24 hrs. in order to obtain an appreciable change in colour. The Job-plot constructed (p.135) indicated that the most stable complex was either the 3:2 or the 1:1 complex.

The size of the metal ion is an important factor in the determination of the stability of a complex (Albert, 1973); hence the ability of a ligand to form a complex with a metal ion, depends on the metal ion size as the metal ion has to be accommodated between the two "claws" of the chelating agent. As can be noted from Table (4.1), the crystal radii of Co(II), Cu(II), Fe(II), Ni(II) and Zn(II) have almost identical sizes (ranging between 0.72 Å and 0.76 Å),

with Mn(II) slightly larger (0.80 Å). This size range, then, was probably preferred by Phanquone for the formation of a complex. One exception was Fe(III), which had a radius of 0.64 Å, but did

TABLE (4.1)

CRYSTAL RADII OF CATIONS (IN Å)*

Na^{+}	0.95	Mg^{++}	0.65	Fe^{+++}	0.64
K^{+}	1.33	Ca^{++}	0.99		
		Mn^{++}	0.80		
		Fe^{++}	0.76		
		Co^{++}	0.74		
		Ni^{++}	0.72		
		Cu^{++}	0.72		
		Zn^{++}	0.74		

Some Phanquone-Metal Ion Complexes

a) at 1:1 ratio;
b) at 1:2 ratio;
c) at 1:3 ratio.

form, nevertheless, a stable complex with Phanquone (as demonstrated by the colour change). This could be due to the stronger positive charge that Fe(III) carried.

The degree of solubility of Phanquone and Phanquone-metal ions (at different ratios) in the octanol phase suggested that, while Phanquone was quite soluble in octanol, the different Phanquone-metal ions were not. This assumption is based on the fact that as the metal ion concentration was increased in relation to Phanquone, the amount of Phanquone that dissolved into the octanol phase, decreased. Moreover, there was no shift in the Phanquone absorbance

* Pauling. 1960.

peak, to denote the dissolving of a complex in octanol, in contrast to oxine and its Cu(II) complexes (Fig.3.58; p.149), where a peak shift was observed denoting the dissolving of the oxine Cu(II) complexes.

Using this assumption, then, the amount of Phanquone that dissolved in the octanol phase (measured semiquantitatively by the UV Spectrophotometer) was directly proportional to the stability of the Phanquone-metal ion complex. This suggested that if more Phanquone dissolved in octanol, in the presence of one metal ion, in comparison with another, then the former Phanquone-metal ion complex was weaker than the latter, and vice versa.

The percentage of the solubility of Phanquone-metal ions at 1:2 ratio (Table 3.19, p.150), imply that the order of stability of the Phanquone complexes, is probably as follows:

$$Fe(III) > Fe(II),\ Cu(II) > Co(II) > Ni(II) > Mn(II) > Zn(II)$$

This order of stability seems to be consistent with the change in colour observed, as well as with the amount of precipitate formed (with the exception of Fe(III)), when Phanquone was mixed with the different metal ions at a 1:2 ratio. This order of stability is in close agreement with the general order of avidity of metal chelates reported by Albert (1958; 1973), which decreased as follows:

$$Fe(III) > Cu(II) > Ni(II) > Co(II),\ Zn(II) > Fe(II) > Mn(II) > Mg(II),\ Ca(II).$$

However, Phanquone might have promoted Fe(II) up the avidity scale in a similar way to riboflavine and 2,2-dipyridyl, which also promoted Fe(II) up the scale by bringing new orbitals into play (Albert, 1958).

4. A STRUCTURE-ACTIVITY RELATIONSHIP FOR PHANQUONE AND RELATED COMPOUNDS

Before attempting to establish a structure-activity relationship for Phanquone and related compounds, it is imperative to look at their three-dimensional structures. The molecular model of Phanquone (Fig. 4.3) suggested that the Phanquone molecule was not planar but was slightly twisted at the C-C bond in between the two C=O bonds, with each oxygen atom pointing towards a different plane, away from each other. This twist was caused by the longer C-C single bond, joining the two carbon atoms, that hold the two oxygen atoms, in comparison to the adjacent but short C=C double bonds. However, before establishing that this molecule is partially non-planar, the stabilization energies for both the twisted and the planar structures should be taken into consideration, as the planar structure might be stabilized by resonance as shown in Fig. (4.5).

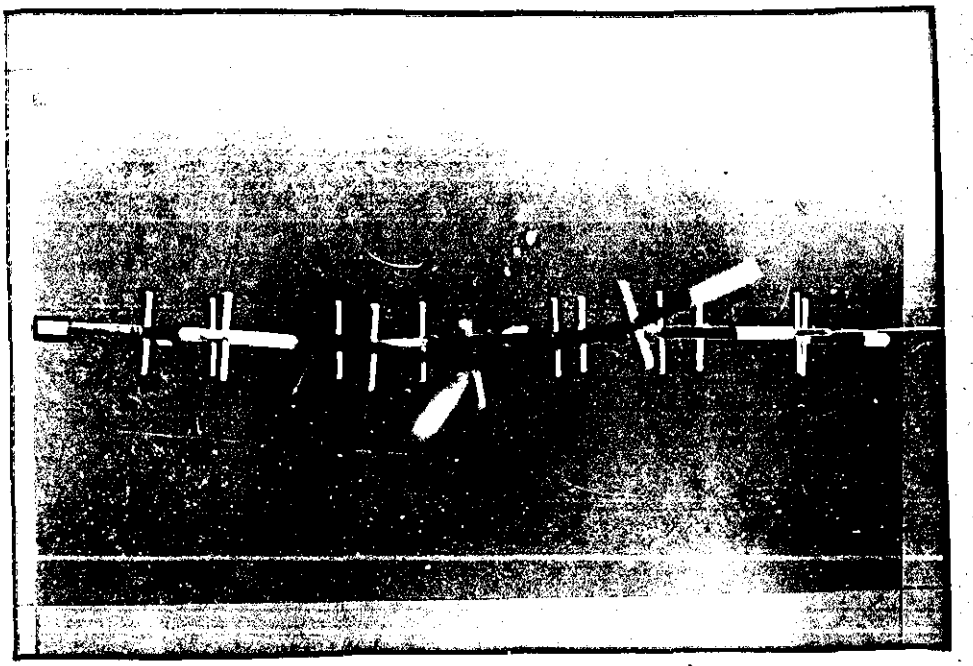
If the Phanquone molecule is flat, then it satisfies the requirement put by Albert, Rubbo and Burvill (1949), in that it possesses a minimal flat area (above 28 square Å) like the acridines, the bezequinolines, the phenanthridines and the benzacridines.

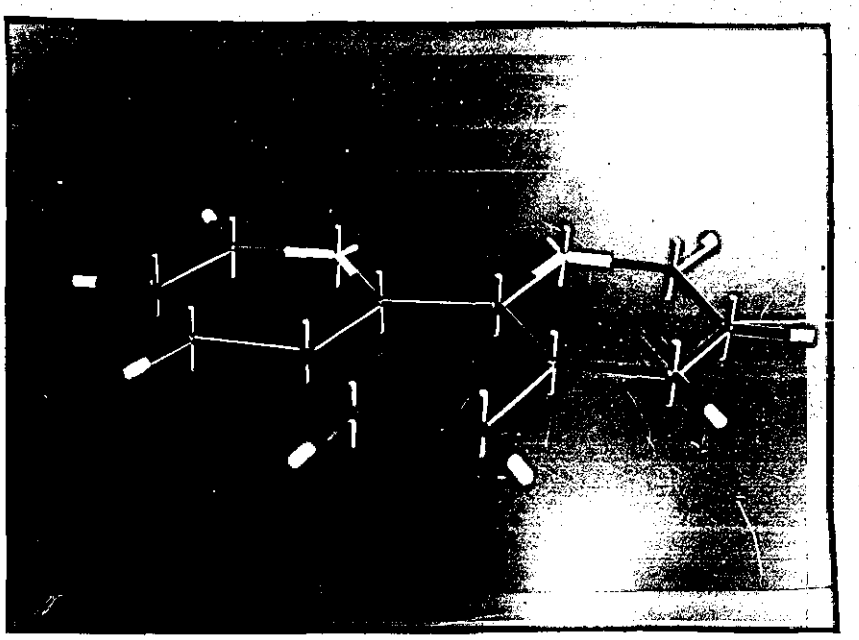
Fig. (4.3)

A molecular model for Phanquone, note the twist of the bond between the oxygen atoms.

Fig. (4.4)

A molecular model for the planar 1,10-phenanthroline.





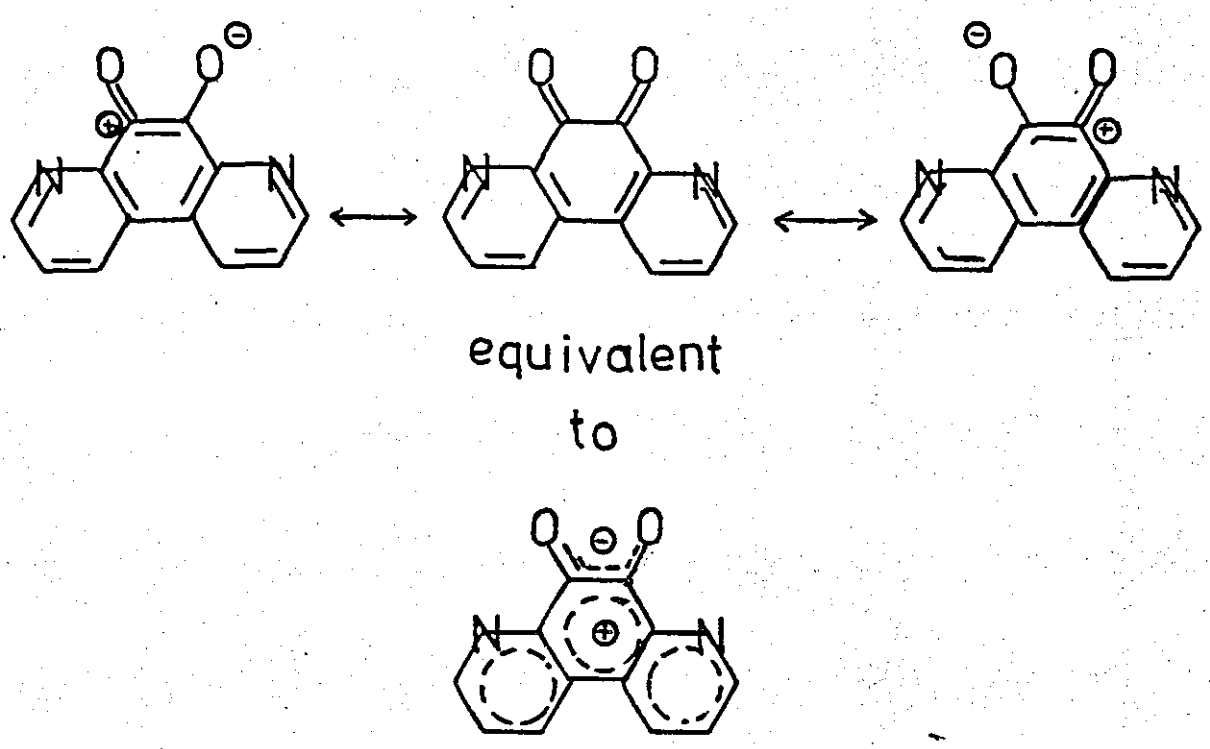


Fig. (4.5)

Some Resonance Structures for Phanquone

The same arguments may be taken into consideration for the prediction of the three-dimensional structures of 9,10-phenanthrene-quinone, and 1,10-phenanthroline-5,6-quinone, while phenanthrene, phenanthridine, oxine, 4,7-phenanthroline and 1,10-phenanthroline (the latter shown in Fig. 4.4) can only be planar molecules.

The MIC and MCC values of Phanquone, when compared to those of the related compounds, place it in the middle of an arbitrary activity scale. At the lower end of the scale is phenanthrene, which seemed to have no practical activity. At the higher end is 1,10-phenanthroline-5,6-quinone, which seemed to be very active against all the strains tested (with the exception of *Strep. faecalis*). 1,10-phenanthroline seemed to have the same range and level of

activity as Phanquone, while phenanthridine and 4,7-phenanthroline were less active.

In general, the antibacterial activity of this series of compounds against Gram-positive bacteria, increased as follows:

phenanthrene < phenanthridine < 4,7-phenanthroline 1,10-phenanthroline, Phanquone < 9,10-phenanthrenequinone, oxine, 1,10-phenanthroline-5,6-quinone;

while the activity against Gram-negative organisms generally increased as follows:

phenanthrene < oxine < phenanthridine, 4,7-phenanthroline < 9,10-phenanthrenequinone < Phanquone, 1,10 phenanthroline < 1,10-phenanthroline-5,6-quinone.

The kill curves for growing *E. coli* and *S. aureus*, show that 1,10-phenanthroline and 1,10-phenanthroline-5,6-quinone were totally bactericidal after less than one hour of their addition, while 9,10-phenanthrenequinone was bacteriostatic against *S. aureus*, whilst not affecting *E. coli*. The inactivity of the latter against *E. coli* did not seem to be caused by the impermeability of the cells, as EDTA-pretreated cells did not become more sensitive to it. Moreover, 9,10-phenanthrenequinone showed no apparent activity against non-growing suspensions of either *E. coli* or *S. aureus*, which confirms its bacteriostatic, rather than bactericidal, effect. Hence, it might be affecting some cellular synthetic process (Russell, Morris, and Allwood, 1973).

The three-ringed structure of the phenanthrene molecule as such did not seem to show any activity (up to 200 µg/ml) on any of the strains tested, although Hass and Applegate (1975) reported its inhibitory effect on *E. coli* strain at 10^{-4}M, and its stimulatory effect at 10^{-6}M. Arnold *et al* (1973) also reported the activity of some phenanthrene methanols against *Plasmodium vivax*.

However, the introduction of nitrogen atom(s) in the phenanthrene rings seemed to increase the activity of this molecule. For example, the introduction of one nitrogen atom at the 9-position, turned the inactive phenanthrene into the slightly active phenanthridine. The introduction of two nitrogen atoms at the 4 and 7 (or at the 1 and 10) positions, yield the active 4,7-phenanthroline and 1,10-phenanthroline, respectively.

The mode of action of 1,10-phenanthroline was mainly attributed to its chelating ability as discussed earlier; the mode of action of phenanthridine was attributed to its ability to intercalate with the DNA of the microbial cell (Newton, 1957; Tomchick and Mandel, 1964; Waring, 1965; Urbanke, Romer and Maass, 1973), while the mode of action of 4,7-phenanthroline has not been investigated.

The introduction of two double-bonded oxygen atoms, at the 9 and 10, or the 5 and 6 positions, seemed to increase the activity of the phenanthrene or phenanthroline molecules respectively. For example, when the two oxygen atoms were introduced on phenanthrene, the resultant 9,10-phenanthrenequinone was quite active, 1,10-phenanthroline became 1,10-phenanthroline-5,6-quinone which was very active; while 4,7-phenanthroline became the more active

4,7-phenanthroline-5,6-quinone (Phanquone).

This increased activity, due to the introduction of the two double-bonded oxygens, has not been extensively investigated. McNew and Burchfield (1951) attributed the activity of 9,10-phenanthrenequinone to one or more of three possible modes of action: binding to enzymes by substitution or addition at the double bond, oxidation of sulfhydryl enzymes, or changing the redox potential in some Gram-positive bacteria; while Hayashi, Tanoue, Ujiaka and Yano (1955), attributed its activity against Gram-positive organisms to the interference with the RNA in the cell wall. The increased activity of 1,10-phenanthroline-5,6-quinone in comparison to 1,10-phenanthroline has not been investigated.

Some of the compounds related to Phanquone are strong metal chelators, and their antimicrobial activity was attributed to their chelating properties.

As mentioned earlier, 1,10-phenanthroline was found to be active against bacteria (McNaught and Owen, 1949; Turian, 1951; Feeney, Peterson and Sahinkaya, 1957; Dwyer *et al*, 1969; Butler, Hurse, Thursky and Shulman, 1969; Nikolaeva, 1972), fungi (Blank, 1951; Cade, Cohen and Shulman, 1970; Shulman, Cade, Dumble and Laycock, 1972; Shulman and White, 1973), as well as helminths (Baldwin, 1948). Recently, it was found to inhibit lymphoblast cell cycle by chelating metals involved in processes essential for those cell cycle events (Falchuk and Kirsham, 1977) while its Ru(II) metal chelates were found to be lethal to P 388 mouse lymphocytic leukaemic cells (Shulman and Laycock, 1977). These findings should

stimulate interest in investigating the activity of other phenanthrolines (including Phanquone) against tumor cells.

The ability of oxine and its related compounds to chelate metals is the essence of its mode of action (Zentmyer, 1944; Albert, Rubbo, Goldacre and Balfour, 1947; Albert, Gibson and Rubbo (1953; Albert, Rees and Tomlinson, 1956; Albert, 1961; 1973).

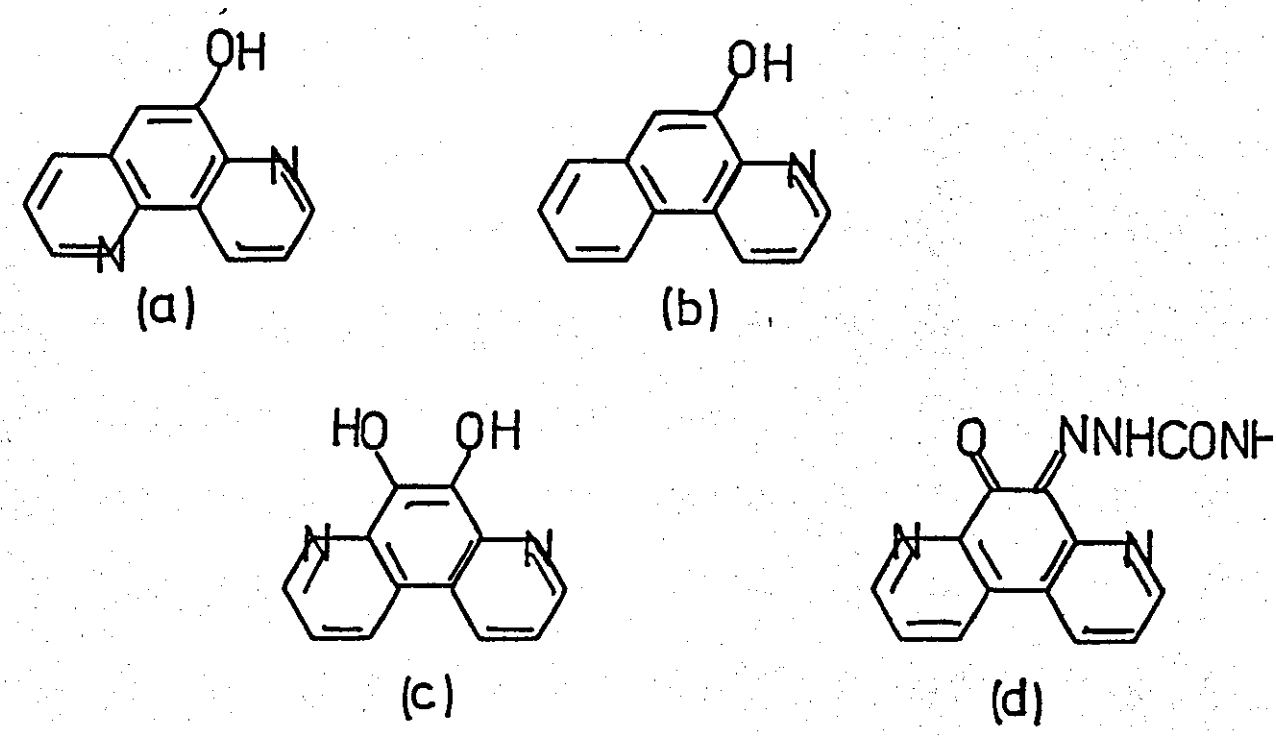


Fig. (4.6)

Structures of Compounds Related to Phanquone

a) 5-hydroxy-1,7-phenanthroline;
b) 5,6-benzoxine;
c) 5,6-dihydroxy-4,7-phenanthroline;
d) semicarbazone of Phanquone.

Some other compounds, whose structure was related to Phanquone, were studied and their antimicrobial activities were also attributed to their chelating properties, such as 6-hydroxy-1,7-phenanthroline, 5,6-benzoxine (Albert, Rubbo, Goldacre and Balfour, 1947), 5,6-dihydroxy-4,7-phenanthroline (Schmidt and Druey, 1957), and the semicarbazone of Phanquone (Kradolfer and Neipp, 1958; Kradolfer, Sackmann and Bassil, 1960). The structures of these compounds are shown in Fig. 4.6.

Therefore, it seems very reasonable, at this stage, to assume that the antibacterial activity of Phanquone can be attributed to its chelating ability.

5. THE INTERACTION OF PHANQUONE WITH OTHER ANTIBACTERIAL AGENTS

In medicine, Phanquone is used either by itself or in conjunction with iodochlorohydroxyquinoline for the treatment of diarrhoeas of different etiology (as discussed earlier on p. 35); therefore, the assessment of the interaction of Phanquone with other antibacterial agents, is of importance, not only from the mechanistic, but from the chemotherapeutic point of view as well.

In practical terms, the synergistic interaction of Phanquone with another antibacterial agent, means a larger spectrum of activity for both, a lower frequency of selection of resistant colonies, and a reduction of the total dose of both antibacterial agents administered (Maccacaro, 1961). On the other hand, an antagonistic interaction would certainly mean the avoidance of the simultaneous administration of the two agents. From the mechanistic

point of view, a synergistic or antagonistic interaction between Phanquone and another antibacterial agent, can help to elucidate many problems of bacterial physiology, such as synthetic pathways and their cross-linkages, selective permeability and physiological or genetical adaptation (Lacey, 1958; Maccacaro, 1961).

As can be seen from Table 3.10 (p. 105), Phanquone did not interact either synergystically or antagonistically with any of the antibacterial agents tested, with the exception of ethidium bromide, and 9,10-phenanthrenequinone, where the synergistic interaction was only slight.

The interaction between Phanquone and 9,10-phenanthrenequinone is interesting, because of the destruction of the resistant *B. subtilis* colonies around the zone of inhibition of Phanquone, only on the side near the 9,10-phenanthrenequinone disc, although the distance between the two discs was greater than the sum of the radii of the zones of inhibition (Fig.3.23, p.108).

In an attempt to explain such a phenomenon, Lacey (1958) assumed that two drugs could delay the outgrowth of resistant mutants by one drug (Phanquone in this case) inhibiting the wild type, while the other (9,10-phenanthrenequinone), principally or exclusively inhibiting mutants resistant to the first, thus, exploiting a collateral sensitivity.

This phenomenon, together with the fact that the MIC and MCC values of Phanquone, for *B. subtilis* and *B. cereus*, dropped when subinhibitory concentrations of 9,10-phenanthrenequinone were added, implies that there was a definite synergistic interaction between the two compounds.

6. THE ACTIVITY OF PHANQUONE ON THE METABOLIC ACTIVITIES OF THE BACTERIAL CELL

The larger aerobic zones of inhibition produced by Phanquone against a number of facultative bacteria, in comparison with the smaller anaerobic zones, probably imply that Phanquone is more active under aerobic conditions, and therefore might be affecting an aerobic metabolic pathway rather than an anaerobic one.

Taking into consideration the fact that both the aerobic and metabolic respiratory pathways contain a large amount of bound metal ions, mainly in the form of non-haem iron (Green, 1961; Hatefi, Haavik and Griffiths, 1962; Ziegler and Doeg, 1962; Rieske and Zaugg, 1962; Green and Wharton, 1963; Cox *et al*, 1970; Hall, Cammak and Rao, 1974; Sun, Phelps and Crane, 1975), it is not surprising for a chelating agent to inhibit some of the aerobic metabolic pathways; hence producing a larger zone of inhibition when the bacteria were grown under aerobic conditions.

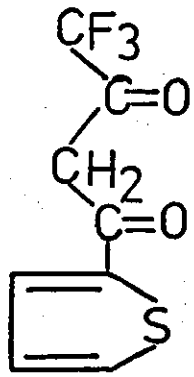
The discrepancy between the levels of bactericidal concentrations obtained by the MCC method on one hand, and by the kill curves (for growing cultures) on the other, being lower in the latter case, can be attributed to what is known as "oxygen poisoning", in which the aeration of the bacterial culture by shaking - when determining the kill curves - brought some of the cells anaerobic metabolic pathways to a halt (Baldwin, 1967; Morris, 1975). This probably forced the bacterial cells to follow the more sensitive aerobic pathway(s) and, hence, were affected by smaller concentrations of Phanquone. On the other hand, some anaerobiasis probably prevailed at the bottom of the test tubes used for the MIC determinations and, therefore, some of the cells were allowed to follow the more

resistant anaerobic metabolic pathway(s), thus needing higher concentrations of Phanquone to bring about the bactericidal effect.

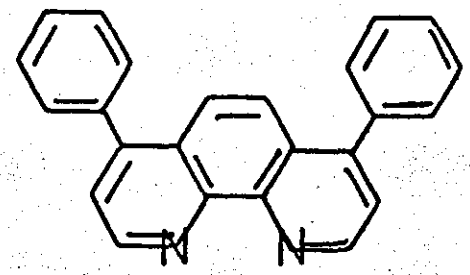
The effect of Phanquone on the oxygen consumption by *E. coli* and *S. aureus*, was more prominent, when succinate, rather than D-glucose, was used as a substrate. Although Phanquone slightly, but notably, inhibited the oxygen consumption, when glucose was used as a substrate, it suppressed succinate-driven oxygen consumption appreciably. This suggests that the aerobic succinate oxidation pathway(s) was probably more susceptible to Phanquone, than the glucose oxidation pathway. This is confirmed by the fact that the former pathway was found to be sensitive to many chelating agents. 1,10-phenanthroline, as well as oxine, inhibited succinate oxidation and coupled phosphorylation in *M. phlei* by inhibiting the succinate oxidase activity, while having little or no effect on the oxidation of another substrate, β-hydroxybutarate, the reason probably being that the NAD^+-linked chain of *M. phlei* either did not contain a metal, or did not have the same type of metal-protein combination as present in the succinate chain (Kurup and Brodie, 1967).

Another metal chelator, thenoyltrifluroacetone (4,4,4-trifluro-(2-thienyl)-1,3-butandione; Fig. 4.7) was shown to be a potent inhibitor of the succinate oxidase system (Toppel, 1960; Ziegler, 1961).

4,7-diphenyl-1,10-phenanthroline (Bathophenanthroline; Fig. 4.7) a lipophilic metal chelator, caused a marked and immediate inhibition on the rate of oxygen uptake, when succinate was used as the energy source, by fresh mitochondria;



(a)



(b)

Fig. (4.7)

a) thenoyltrifluroacetone;
b) 4,7-diphenyl-1,10-phenanthroline

1,10-phenanthroline behaved similarly (Palmer, 1970). It also inhibited the electron transport in all the complexes of the electron transport chain, in which the site of inhibition was related to non-haem iron (Phelps, Harmon and Crane, 1974). Crane, Sun and Crane (1975) concluded that the Bathophenanthroline inhibition of both lactate and NADH oxidase activities in *E. coli* membranes, was also consistent with a non-haem iron site between cytochrome b and oxygen in the cytochrome chain; while Trumpower and Katki (1975) assumed that the site of its inhibition in bovine heart mitochandria and succinate-cytochrome reductase complex was probably the non-haem iron of the complex.

Hence, it is not at all surprising for a strong metal chelator, such as Phanquone, to cause a reduction in the total amount of oxygen consumed by the bacterial cell, and to cause a general

retardation in the process of respiration as a whole, for - as mentioned earlier on p.18 - the bacterial respiratory chain is known to be extensively bound to metal ions. However, the greater sensitivity of the succinate oxidation system towards Phanquone, can be probably attributed, as was the case with other chelating agents, to the fact that this system contains large amounts of bound metal ions, mainly iron (Massey, 1958; Ziegler and Doeg, 1959; Rieske, Hanson and Zaugg, 1962; Asano and Brodie, 1964; Beinert, Palmer, Cremona and Singer, 1965; Redfearn, Whittaker and Burgos, 1965). Succinate dehydrogenase, a metalloenzyme with iron atoms closely bound to it, was capable of forming complexes with chelating agents, leading to the eventual inhibition of the electron transport activity (Massey, 1958; Kurup and Brodie, 1967).

The succinate-driven oxygen consumption for *E. coli* was greatly induced in the presence of low concentrations of Phanquone. A similar stimulating effect on the oxygen consumption was observed by Hugo and Street (1952) with phenol and phenoxetol, and by Hugo and Bloomfield (1971 (b)) with Fentichlor (2,2'-thiobis-4-dichlorophenol), where they suggested that this increase in the total oxygen consumption was typical of the action of energy uncouplers. Kurup and Brodie (1967) showed that oxine also acted as an uncoupler, by completely uncoupling the phosphorylation associated with the oxidation of β-hydroxybutarate in *M. phlei*. Whether Phanquone has the ability to act as an uncoupler or not, requires further investigation.

The fact that Phanquone (at low concentrations), as well as 5-iodo-7-chloro-8-hydroxyquinoline, were found to stimulate the growth of coliform bacteria in the intestines of rats, although the bacterial counts were reduced to start with (Kradolfer and Neipp, 1958; Eisman, Weerts, Jaconia and Barkulis, 1960; Sackmann and Kradolfer, 1961), agrees well with the stimulation of the oxygen consumption by *E. coli*. Some of these workers, however, have attributed this increase in the faecal count, to the destruction of sensitive bacteria, and the subsequent development of strains resistant to the action of Phanquone (and iodochlorohydroxy-quinoline).

Many Pseudomonads have the ability to degrade and metabolize many aromatic compounds (Dagley, 1971), and even using some of them as sole carbon sources. Phenanthrene was found to be easily oxidized by *Ps. putida* (Jeffrey *et al*, 1975) and by some other soil microorganisms (Groenewegen and Stolp, 1975). Moreover, Gibson (1971) found that the enzyme catalyzing the initial oxidation of naphthalene, isolated from a *Pseudomanas* species, rapidly oxidised phenanthrene as well, of which 1,10-phenanthroline was a potent inhibitor.

The results obtained from Fig.3.31(p.118) did not show that Phanquone was used as a carbon source by *Ps. aeruginosa*, but it rather inhibited the endogenous respiration.

Phanquone, at a concentration much lower than that inhibiting the oxygen consumption, inhibited succinate, malate and lactate dehydrogenases, to varying, but appreciable, extents, using the reduction of triphenyltetrazolium chloride (TTC) as an indicator of the enzyme activity.

These three dehydrogenases are metalloenzymes. As pointed out earlier, succinate dehydrogenase, isolated from different sources (including bacteria), was found to contain non-haem iron (Warringa, Smith, Giuditta and Singer, 1958; Warringa and Giuditta, 1958; Singer, 1965). Malate dehydrogenase isolated from pig heart contained zinc (Vallee, Hoch, Adelstein and Wacker, 1956; Kun, 1963), and was found to be in close association with the lactate dehydrogenase (Baldwin, 1967); while lactate dehydrogenase, isolated from different sources, contained zinc as well as iron (Vallee and Wacker, 1956; Boeri and Tosi, 1956; Vestling, Hsieh, Teramaya and Baptist, 1963; Everse and Kaplan, 1973). It is highly probable then, that Phanquone acted on these three dehydrogenases by complexing with their metal components, thus altering their structures and/or their specificities, and causing the retardation of their activities. This is substantiated by the fact that the activity of succinate dehydrogenase, assayed using TTC, was inhibited by 1,10-phenanthroline (Kurup and Brodie, 1967). Moreover, Massey (1958) and Kurup and Brodie (1967) concluded that the non-haem iron of the succinate dehydrogenase was capable of forming complexes with metal-chelating agents, which inhibited the electron transport activity.

When the reduction of TTC was monitored in the presence of Phanquone, using pyruvate or D-glucose as substrates, an increase in the red colour was observed, implying that these two dehydrogenases

were stimulated. This probably means either that both glyceraldehyde-3-phosphate dehydrogenase (which is the first dehydrogenase to be encountered during the oxidation of glucose by the Embden-Meyrhof pathway, the major catabolic pathway in *E. coli* (Sokatch, 1969), and pyruvate dehydrogenase, were not metalloenzymes, and/or that these two dehydrogenases were inhibited, at some stage, by a metal ion; in the presence of a metal chelator, such as Phanquone, this inhibitory metal was inactivated by chelation, thus causing the indirect stimulation observed for the activities of these dehydrogenases.

This is substantiated by the fact that these two enzymes were never directly associated with metal ions. Indirect evidence also comes from the findings by Sutherland (1949) and Milstein (1961), that phosphoglucomutase, the enzyme converting glucose-1-phosphate to glucose-6-phosphate, was inhibited by zinc ions, and that oxine, as well as 1,10-phenanthroline, stimulated its activity by binding the inhibitory zinc ions. This might mean that more glucose-6-phosphate was formed in the presence of Phanquone, eventually leading to the formation and dehydrogenation of more glyceraldehyde-3-phosphate.

There are many reports describing the stimulation of many enzymes and enzyme systems by chelating agents. The activation of creatine phosphotransferase by oxine was thought to be due to the chelation by oxine of some inhibitory metal ions, such as Cd(II), Vo(II) and Zn(II) (O'Sullivan and Morrison, 1963). Glucose dehydrogenase of *Aspergillus oryzae* was specifically induced by hydroquinone and p-benzoquinone; this inductive synthesis of the enzyme was further stimulated by 1,10-phenanthroline and oxine (Bak

and Sato, 1967). This stimulation by 1,10-phenanthroline was due to the binding of the chelating agent to metal ions which interacted with the regulatory substance in such a way that more enzyme was produced (Sinohera, 1968). Moreover, Kamataki, Ozawa, Kitade and Kitagawa (1977) found that 1,10-phenanthroline stimulated rat liver peroxidation, while Tochikuto (1974) reported the stimulation of NADH oxidase of *B. subtilis* by the same chelator.

The TTC method did not seem to be a good method for the assaying of dehydrogenases activities of *S. aureus*, as no meaningful results were obtained when succinate, malate lactate or pyruvate were used. Nevertheless, the stimulation obtained, when glucose was used, can be explained on the same basis as that for *E. coli*.

The induction in the uptake of phosphate by *E. coli* in the presence of low concentrations of Phanquone, can be perhaps related to the induction of the dehydrogenase, as well as the induction of the oxygen consumption observed earlier, as glucose was the energy source in the three inductions.

However, the inhibition of the phosphate uptake by higher concentrations of Phanquone, is consitent with the rest of the results discussed so far. For, if Phanquone inhibited certain aspects in the process of aerobic metabolism and respiration, then the process of the phosphate uptake, which is an energy dependent process (Hotchkiss, 1944; Harold, Harold and Abrams, 1965, Harold and Baarda, 1966; 1969; Hamilton, 1968; Harold, Baarda, Baron and Abrams, 1969), would be retarded or inhibited. So would be the synthesis of ATP.

The chelator Bathophenanthroline caused an immediate and nearly complete inhibition of the ATPase activity of purified beef-heart F_1 (Phelps, Nordenbrand, Nelson and Ernsten, 1975). It also inhibited *E. coli* K12 membrane-bound ATPase activity, and the inhibition was assumed to be due to the chelation of a non-haem iron (Sun, Phelps and Crane, 1975). Therefore, Phanquone may be specifically affecting the ATPase activity in a similar fashion.

The inhibition of the uptake of phosphate by *S. aureus* in the presence of Phanquone, was much greater than in the case of *E.coli* probably signifying a greater susceptibility of the phosphate uptake system in the former organism.

The uptake of L-glutamic acid by *S. aureus* was found to be an energy-dependent process (Gale, 1949; Hugo and Bloomfield, 1971(b)). The results obtained confirm this active transport process, in that at least a substantial amount of this amino acid was actively transported. It is a possibility, however, that the whole glutamic acid transport process was energy-dependent, and in the absence of the energy source (glucose), the preformed ATP provided the energy for transporting the amino acid. It is also a possibility that the glutamic acid was partially passively transported, and the uptake was only enhanced in the presence of the energy source. This is substantiated by the findings of Gale and Folkes (1967), that lysine, in *S. aureus*, was accumulated by two processes, one energy-dependent and the other energy-independent.

Results obtained for the uptake of L-proline by *S. aureus* showed that this amino acid was also, at least partially, actively transported.

Phanquone seemed to inhibit the actively transported fraction of the glutamic acid. This inhibition was probably due to either the direct interference of Phanquone with the active transport by affecting the ATPase activity (as in the case of Bathophenanthroline), and/or by causing the energy uncoupling of the active transport (as in the case of oxine and 1,10-phanthroline), or indirectly by interfering with the respiratory process, resulting in the retardation of ATP production, and eventually slowing the process of active transport.

Gale (1949) found that oxine, as well, retarded the uptake and assimilation of glutamic acid in *S. aureus*, while Pashev (1967) reported the inhibition of glutamate dehydrogenase by 1,10-phenanthroline.

On the other hand, Phanquone inhibited the uptake of proline only slightly. This probably indicates that, for some reason, the glutamate transport system was more vulnerable.

Many *E. coli* strains (such as W, H, K12 and B) have been shown to transport glutamate with difficulty (Halpern and Umbarger, 1961; Halpern and Lupo, 1965; Frank and Hopkins, 1969). Glutamate permeation in *E. coli* was assumed to be due to active transport mediated by a specific cellular component, whose formation was enhanced during the growth on glutamate (Halpern and Even-Shoshan, 1967).

The results obtained showed that the uptake of glutamate by *E. coli* NCTC 9001 was greater in the absence of glucose than in its presence. This probably indicates that this amino acid was not

actively transported by this strain, and that in the presence of the amino acid only, the bacterial cells probably used it as an energy source, as well as an essential amino acid; while in the presence of glucose, the cells only used it as an essential amino acid only, thus taking greater amounts in the former case. The presence of glucose, then, seemed to control the uptake of the glutamate.

Halpern and Even-Shoshan (1967) found that the glutamate transport system was induced when *E. coli* cells were grown in the presence of glutamate alone. On the same lines and for some reason, the glutamate transport system in this *E. coli* strain was either very quickly, or has been already, induced in the presence of glutamate alone, thus more of the amino acid was taken up by the cells.

The uptake of proline by *E. coli* followed a similar pattern to that of glutamic acid. Although many workers reported its uptake as an energy-dependent process (Hotchkiss, 1944; Hamilton, 1968; Hugo and Bloomfield, 1971(b)), yet there was more proline taken up in the absence, rather than in the presence, of glucose. Here as well, the presence of glucose seemed to limit the amino acid uptake.

The inhibition of the proline uptake by Phanquone was more prominent for *E. coli*, while the inhibition of the glutamate uptake was more prominent for *S. aureus*.

The inhibition curves for both amino acids by *S. aureus* and *E. coli*, showed a distinct pattern, consisting of a fast rate of uptake at the begining, followed by a slower rate, then by a faster rate once again.

One possible, and very probable, explanation takes into consideration the fact that Phanquone was added at the same time with the labelled amino acids; hence, the rate of uptake was not affected to start with, because Phanquone had not reached its site of action. The slow rate following probably indicated that Phanquone had reached its site of action, and was exerting its inhibitory effect. The faster rate following indicated that the cell "absorbed the shock" produced by Phanquone, modified its properties, and somehow counteracted the action exerted by Phanquone.

Another possible explanation for this fast-slow-fast pattern of uptake takes into consideration that the Phanquone molecules might be blocking the cell wall and/or the membrane temporarily. Thus, at the beginning, the molecules of both amino acids were taken up at a fast rate, then as the Phanquone molecules got to the cell wall and/or membrane, they block it (them), thus inhibiting the uptake. However, after Phanquone got into the cell, and equilibrium was achieved so that no more Phanquone molecules were taken up, the uptake of the amino acids was resumed at quite a normal rate.

The antagonistic effect exerted by some thiol-containing compounds on the activity of Phanquone against *E. coli* is probably of significance, as there are many reports associating the activity of some metal chelators with thiol groups. Bernheim and Bernheim (1939) reported the oxidation of some thiol-containing compounds such as thioglycollic acid and cysteine by oxine. Colwell and McCall (1945) assumed that the antagonism of the antibacterial action of 2-methyl-1,4-naphthoquinone on *E. coli* by neutralized thioglycollic acid, and sodium thioglycollate, was due to either blocking essential

enzymes through combination with sulphydryl groups, or through combination with sulphydryl groups of essential bacterial metobolites. Albert (1961) concluded that chelators could act on essential thiol groups, while Hugo (1967) assumed that the mode of action of oxine was by forming a 1:1 oxine-Fe(II) complex, which oxidized essential sulphydryl groups inside the bacterial cell.

Moreover, quinones were found to have an oxidative effect on sulphydryl enzymes (McNew and Burchfield, 1951). Thus it seems very possible that Phanquone (being a quinone as well) oxidized some essential sulphydryl groups in *E. coli* cells, and that oxidation was competitively antagonized by the addition of thiol-containing compounds.

Thiol-containing compounds, however, did not seem to affect the activity of Phanquone against *S. aureus*. This probably indicates that Phanquone has not reached and/or interacted with essential sulphydryl groups in this Gram-positive cell, and implies that its mode of action is probably different from that in *E. coli*. A similar finding was reported by Geiger (1946), who observed that sodium thioglycollate and cysteine prevented the inhibition of growth of Gram-negative bacteria by quinones, while their action against the Gram-positive organisms was not prevented by those sulphydryl compounds.

Monoamine oxidase (MAO) has been found to be a metalloenzyme with copper being its metal constituent (Frieden, McDermott and Osaki, 1964; Baldwin, 1967). It catalyzes the following reaction:

$$R\text{-}CH_2\text{-}NH_2 + O_2 \overset{MAO}{\rightleftharpoons} R\text{-}CH = NH + H_2O_2$$

where R-CH = NH is then oxidized by H_2O to give R-CHO and NH_3 (Baldwin, 1967).

Phanquone did not have an inhibitory effect on the enzyme, but rather a slight inductive effect, probably because the liver homogenate was loaded with iron, which is more preferably bound by Phanquone.

7. THE INTERACTION OF THE METAL CHELATES OF PHANQUONE WITH THE BACTERIAL CELL

The antagonism observed by many metal ions such as Co(II), Cu(II), Fe(II) and Fe(III) for both *E. coli* and *S. aureus* when the strip method was employed, clearly indicates that the activity of Phanquone was retarded in the presence of excess metal ions. Although this method is both crude and qualitative and must only be regarded as a first step, it was, nevertheless, indicative of the interaction and complexing between Phanquone and some metal ions.

The activities of two other chelators, oxine and 1,10-phenanthroline, were also antagonized by Fe(III), for both bacterial strains. The activity of 9,10-phenanthrenequinone against *S. aureus* was not antagonized by Fe(III), indicating that the mode of action of this compound was probably independent of the metal ion.

The antagonistic effect of Fe(III) ions on the MIC and MCC values of Phanquone for *E. coli* and *S. aureus*, is consistent with the results obtained by the strip method. This suggests that Fe(III) and probably other metal ions - rendered Phanquone inactive either by filling its complexing sites, hence becoming inable to complex

with metals in the bacterial metal-containing systems, and/or by rendering Phanquone impermeable through the bacterial cell wall or membrane, thus becoming inable to reach its site of action. The ability of Fe(II) ions not only to inhibit the action of Phanquone, but also to stimulate the succinate dehydrogenase of *E. coli* may be explained accordingly.

In a similar fashion, the addition of Fe(II) to 1,10-phenanthroline-treated particles antagonized the activity of the chelator, and restored completely both oxidative and phosphorylative activities, when succinate was used as the electron donor in *M. phlei* (Kurup and Brodie, 1967).

The inability of Phanquone at five times the MCC (500 µg/ml) to cause a complete kill for *S. aureus*, is consistent with the phenomenon of "concentration quenching" observed for oxine (Albert, Gibson and Rubbo, 1953), where the activity of oxine decreased as its concentration was increased. Albert (1973) suggested that the toxic substance was the 1:1 or 2:1 oxine-metal ion complex; in the presence of excess oxine, the 3:1 complex dominated and was unable to penetrate the cell, and was therefore not lethal (see Introduction, p.25). Therefore, in the presence of excess Phanquone the same phenomenon might occur.

The fact that Phanquone - on its own - was not completely lethal to *S. aureus* cells suspended in buffer or distilled water, together with the fact that the Phanquone-metal ion complexes at 2:1 and 1:1 ratios (especially those of Co(II), Fe(II), Fe(III),

Mn(II), Ni(II), and Zn(II) caused a complete kill within two hours, indicates that there was a "cooperative interaction" between Phanquone and those metal ions (at these ratios), similar to that found for oxine (p. 25).

The reversal of the toxic action of the Cu(II), Fe(II) and Ni(II) by Phanquone at the 1:2 ratio, indicates that these Phanquone-metal ion complexes were probably less toxic than the metal ion and, in that case, Phanquone seemed to protect the bacterial cell rather than destroy it.

However, the fact that Phanquone, on its own and at a very low concentration (1 µg/ml), was capable of causing a complete kill for *E. coli* suspended in distilled water within two hours, together with the fact that the presence of Cu(II), Fe(II), Fe(III) and Zn(II) ions, at 1:1 ratio, tended to antagonise the lethal action of Phanquone, indicates that there was an antagonistic interaction, in contrast to the cooperative interaction observed against *S. aureus*. This is similar to the findings of Rubbo, Albert and Gibson (1950) in that although the activity of oxine was enhanced in the presence of some metal ions such as Fe(II) and Cu(II) for *S. aureus*, its activity against *E. coli* was antagonized by Zn(II), Fe(II), Cu(II), Ni(II) and Mn(II). They concluded that the mode of action of oxine did not depend on the presence of metal for *E. coli*. The same conclusion can be drawn for the action of Phanquone.

The antagonism produced EDTA and NTA on the activity of Phanquone for *S. aureus* but not for *E. coli*, strengthens the hypothesis that metal ions cooperated with Phanquone against the former organism,

while antagonizing or having no effect on the latter, in that those chelating agents might have complexed with some of the metal ions in the medium, that otherwise cooperated synergistically with Phanquone against *S. aureus*, and possibly antagonistically against *E. coli*. Adler and Snoke (1962) found that the activity of bacitracin towards *S. aureus* was completely suppressed in the presence of EDTA and concluded that bacitracin required metal ions for its action.

While 1,10-phenanthroline and 1,10-phenanthroline-5,6-quinone behaved similar to Phanquone, against *E. coli* and *S. aureus*, in the presence of these chelating agents, the activity of 9,10-phenanthrene-quinone was not affected. Strangely enough, the activity of oxine was antagonized in the presence of EDTA, but not NTA, against both *E. coli* and *S. aureus*.

The antogonism of the action of Phanquone on the oxygen consumption by *E. coli*, observed in the presence of Co(II), Cu(II), Fe(II) and Zn(II), further supports the "antagonistic effect" of these metal ions on Phanquone for this organism. The enhancement of its activity against *S. aureus* by Zn(II) and Ni(II) also supports the "cooperative effect" described earlier. However, and contrary to what was expected, the antagonistic effect by Co(II) and Fe(II), on the inhibition of the oxygen consumption by Phanquone, may be probably related to the fast precipitation of both Co(II) and Fe(II) complexes, while this was not the case for Ni(II) and Zn(II).

The production of a zone of growth inside the zone of inhibition of Phanquone for *S. aureus*, but not for *E. coli*, renders further support to the hypothesis that Phanquone might be acting differently against the two organisms.

Rings of growth and inhibition were earlier obtained by Feeney, Petersen and Sehinkaya (1957) with oxine and 1,10-phenanthroline, using *Micrococcus pyogenes*. The conditions for obtaining such zones were the utilization of a simple growth medium (P-2; Feeney and Nagi, 1952) consisting of 0.8% nutrient broth, 0.5% Na_2PO_4, 0.03% citric acid, 1.5% Oxoid agar, and conalbumin (added in amounts equivalent to the iron content), adjusting the pH to 8.0, and placing the inoculated plates at $2^{o}C$ for 24 - 48 hrs. to allow for diffusion. The same conditions were followed except that nutrient agar was utilized, to which no iron (or conalbumin) was added, as the peptone in the nutrient agar contained a large amount of metal ions, including iron (Albert, 1958).

Feeney, Petersen and Sehinkaya (1957) suggested that the chelating agents, placed on discs, diffused through the medium, and complexed with the metal ions to varying degrees, depending on the dissociation constants and the relative concentrations, and the ring of inhibition was obtained in the areas where the particular mixture was inhibitory, while a ring of growth represented areas where a different particular mixture was not inhibitory. They concluded that the mode of action of such chelating agents was related to the relative concentrations of a variety of metal ions, the chelating agents, and the metal-chelator complexes.

The same conclusions can therefore be drawn for Phanquone with regard to the formation of the zones of growth and inhibition around its disc, when using *S. aureus*.

8. ASSESSING THE EFFECT OF PHANQUONE ON THE LEAKAGE OF PHOSPHOROUS-CONTAINING COMPOUNDS FROM THE BACTERIAL CELL

The amount of Phosphorous-(32) released by *E. coli* cells in the presence of Phanquone was, surprisingly, less than the amount released by the control cells. One possible explanation is that Phanquone might have either precipitated some P-(32)-containing cellular constituents and cytoplasmic matter inside the cell, so that it could not leak out; or that Phanquone somehow blocked the cytoplasmic membrane, which retarded the release of the P-(32)-containing compounds.

Moreover, it seems reasonable to assume, provided that Phanquone did not cause any leakage, that if Phanquone inhibited the uptake of phosphate by the bacterial cell, then the cell might have released smaller amounts of phosphate-containing compounds than it normally did.

For *S. aureus*, however, Phanquone did not have any effect on the leakage of P-(32)-containing compounds.

9. THE MUTAGENICITY OF PHANQUONE

The results obtained from the Salmonella/Microsome mutagenicity test described by Ames and Co-workers (Ames, Durston, Yamasaki and Lee, 1973; Ames, Lee and Durston, 1973; Ames, McCann and Yamasaki, 1975; McCan, Choi, Yamasaki and Ames, 1975; McCann and Ames, 1977) indicated that Phanquone - at low concentrations - was mutagenic to *Sal. typhimurium* LT2 TA 1535, and to a lesser extent, to TA 98 and TA 100.

The TA 1535 mutant contains the histidine mutation his G 46, which is a missence mutation, and is well reverted by a wide variety of mutagens that caused base-pair substitutions (Ames, McCann and Yamasaki, 1975). The TA 100 mutant is derived from the TA 1535 mutant, and hence contains the missence mutation G 46 as well (Ames, McCann and Yamasaki, 1975). It was found to be extremely effective in detecting carcinogens like nitroquinoline-N-oxide and 7,12-dimethylbenzanthracene (McCann, Spingarn, Kobori, and Ames, 1975).

Since Phanquone was most mutagenic to the TA 1535 and the TA 100 strains, among the five strains tested, then there is a possibility that Phanquone caused base-pair substitutions in the DNA molecule of these two Salmonella mutants. Nevertheless, Phanquone showed a mutagenic effect on the TA 98 strain, which is a mutant derived from the TA 1538, and contains the histidine frameshift mutation his D 3052, but was found to be more sensitive than the latter mutant in the detection of all mutagens tested (Ames, McCann and Yamasaki, 1975). Therefore, one cannot exclude the possibility that Phanquone may be able to bring about frameshift mutations, for even the TA 1537 strain, which contains the histidine frameshift mutation his C 3076, seemed to be reverted at one of the three Phanquone concentrations tested.

The mutagenicity of Phanquone has been reported by Bignam *et al* (1974), who found that this compound was 15% mutagenic, using a system that rapidly tested the non-disjunction and crossing-over induction of diploids of *Aspergillus nidulans*.

The structure of Phanquone resembles many of the compounds that are known to interact with the DNA by intercalation. Some of these compounds are shown in Fig. (4.8). Craig and Isenberg (1970),

studying the binding of various polycyclic aromatic hydrocarbons to DNA, concluded that the structure of the intercalated complexes was almost entirely determined by the size criterion. The intercalation of the DNA by Phanquone, then, is neither impossible nor improbable.

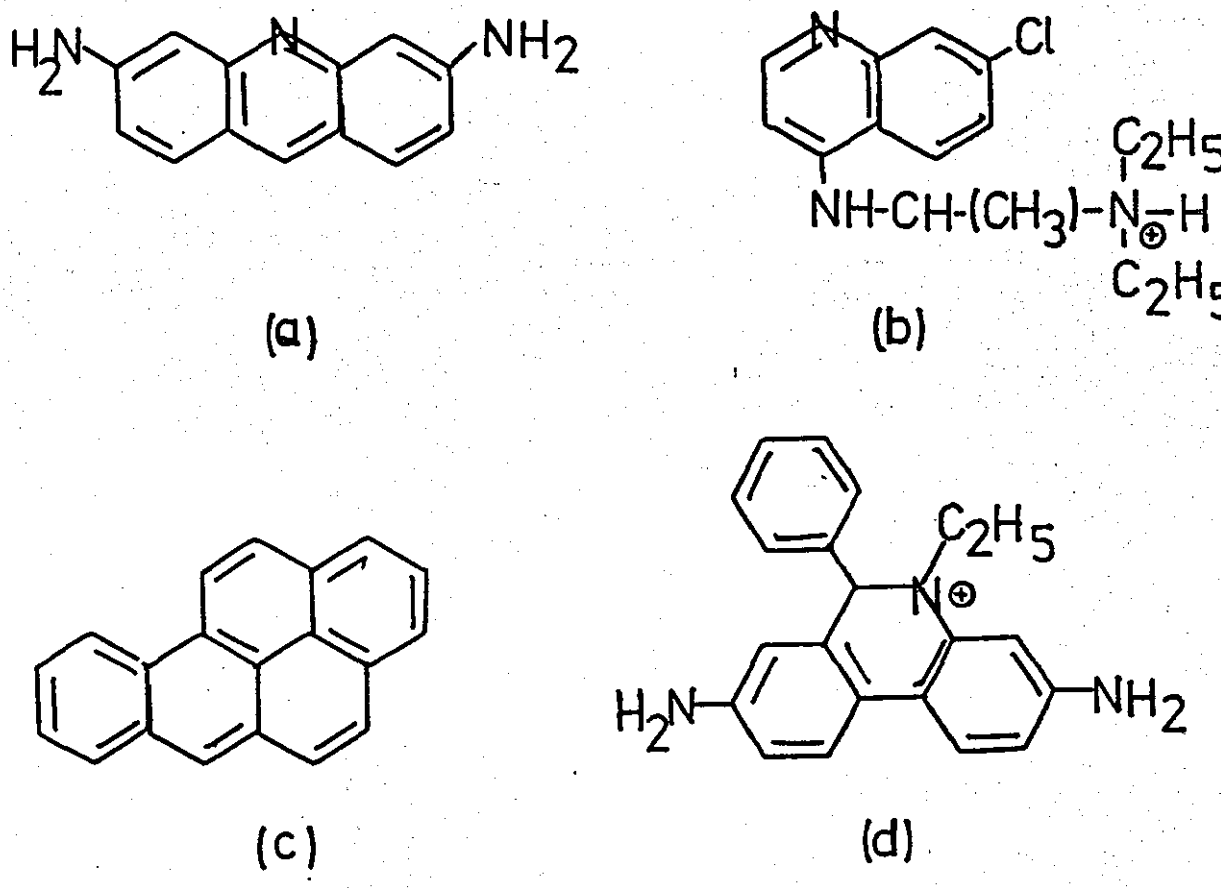


Fig. (4.8)

Structures of Some Intercalating Compounds

a) proflavine (Lerman, 1961; 1964 (a));
b) chloroquine (O'Brien, Allison and Hahn, 1966);
c) 3,4-benzpyrene (Lerman, 1964 (b); Lesko, Hoffmann, Tso and Mahler, 1971);
d) ethidium (Fuller and Waring, 1964).

10. CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

It appears that Phanquone has two different modes of action against *E. coli* and *S. aureus*. In the case of *E. coli*, Phanquone probably interacted with some metal-containing systems, rendering them inactive, and leading to the bacterial cell death. The addition of metal ions to the medium either antagonized, or had no effect on, its effect.

For *S. aureus*, however, Phanquone was not lethal. Yet in the presence of metal ions at a certain ratio, activity was greatly enhanced; this "co-operative effect" caused rapid cell death.

These two different modes of action of Phanquone are very similar to the modes of action of oxine suggested by Albert and co-workers against Gram-negative and Gram-positive bacteria in general.

Moreover, Phanquone seemed to be mutagenic to some *Sal. typhimurium* strains, probably by intercalating with their DNA.

Further work may utilize the use of radiolabelled Phanquone and/or radiolabelled metal ions, on isolated cell components, in order to pinpoint its exact site of action as well as its exact toxic complex (in the case of *S. aureus*).

The mutagenic effect of Phanquone should be further investigated, as this antibacterial agent is prescribed for internal use in the treatment of non-specific enteritis.

The antiviral and antitumor effects of Phanquone and its related compounds (especially 1,10-phenanthroline-5,6-quinone) should be studied on tissue culture systems, (such as Hela and KB cells) in order to attempt to broaden the practical applications of Phanquone.

B I B L I O G R A P H Y

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