S U M M A R Y

Quinolones exhibit both bacteriostatic and bactericidal actions against three strains of Escherichia coli (ATCC 11229/1 NCIB 8114 and 8583). A carboxylic quinolone (N-ethyl-3-carboxy 7-chloro-4-quinolone, "carboxyQ") is characterised by an absolu requirement for growing cells to exert its antibacterial action It does not affect susceptible cells during lag phase when the are not actively dividing. Its effect on growth and viability noticed only after the control enters the logarithmic phase of growth and its action diminishes when the control approaches stationary phase. Moreover, its action is completely antagoniz under conditions where growth is prevented by withdrawal of an essential nutrient. If the essential nutrient is added to cell pre-incubated in deficient medium with carboxyQ, a period of induction is still required before any antibacterial effect of carboxyQ is observed. Inhibition of growth, e.g. by addition o chloramphenicol, reduces the antibacterial action appreciably. Its activity is also proportional to the rate of growth.

A noncarboxylic quinolone (1, 2-dimethyl-6-nitro-4-quino "nitroQ") has been found to be more active against nongrowing c It exerts an immediate action on susceptible cells in the lag p and its activity is reduced when growth occurs. Its activity i not affected by prevention of growth either by withdrawal of ni gen source or by addition of chloramphenicol. Activity of nitr is not proportional to the rate of growth. However, the activi of nitroQ has been found to depend on concurrent energy metabol

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

The antibacterial action of quinolones is accompanied b appearance of long filamentous cells as seen microscopically a by measurement of the increase in average cell size. At bacte cidal concentrations, DNA synthesis is preferentially inhibite compared with RNA and protein synthesis. These results indica "unbalanced growth" analogous to a variety of conditions which affect DNA synthesis. The close inter-relationship of quinolo activity and DNA synthesis is further supported by the observa that the thymine auxotroph, E. coli 8583, becomes independent thymine at the same time as it becomes resistant to carboxyQ. However, DNA synthesis is not the sole process affected by qui lones. Both carboxyQ and nitroQ affect the respiration of E. 11229 at bactericidal concentrations.

The action of quinolones can be completely reversed by removal of drug which suggests that quinolones are not firmly bound to their sensitive site. Partial reversal can also be achieved by addition of precursors of DNA in the thymine auxotroph indicating that inhibition of DNA synthesis is the main if not the sole and primary effect.

Resistance to carboxyQ and nitroQ is developed by different mechanisms which adds weight to the division into carboxylic and noncarboxylic quinolones but does not necessari reflect any fundamental difference in the mode of action.

ANTIBACTERIAL ACTION OF QUINOLONES ON *ESCHERICHIA COLI*

by

Fakhira Mahmood
(née Sabir)

Thesis presented for the
Degree of Doctor of Philosophy
The University of Glasgow

September, 1967.

ACKNOWLEDGEMENTS

I wish to express my thanks and appreciation to Professor J. N. Davidson for providing me with the opportunity to work in the Institute of Biochemistry, University of Glasgow. My heartfelt thanks and gratitude are due to Dr. W. H. Holms for constant help and guidance throughout this work and to Dr. R. Y. Thompson who introduced me to the subject of Biochemistry. I am also deeply grateful to Dr. Holms and Dr. Thompson who both took a great personal interest in my private affairs throughout my stay in Glasgow.

I am also thankful to Dr. R. Eason, Dr. C.A. Fewson, Dr. W.H. Holms and Miss E.J. Millie for the use of their unpublished data.

CONTENTS

Materials and Methods (contd.) Page

LIST OF FIGURES

LIST OF TABLES

List of Tables (contd.) Page

INTRODUCTION

The study of the mechanism of action of antimicrobial drugs has attracted the attention of many biologists since the discovery of penicillin. This area of research has proved to have both fundamental and applied importance. The use of these drugs for treatment of infectious diseases has completely revolutionized medical practice and it is this aspect which is most important. However, these studies have also been useful in uncovering many aspects of cell growth and physiology which would not have been discovered otherwise. The selective action of antimicrobials has led to many advances in the field of comparative biochemistry and physiology. A large number of biologists have been studying the mode of action of antimicrobials. The published literature on the subject is very vast but many excellent reviews have appeared (Eagle & Saz, 1955; Davis & Feingold, 1962; Gale, 1963; Woodruff & Miller, 1963; Newton, 1965 and Goldberg, 1965).

In the following pages an attempt has been made to describe briefly what is known at present about the mode of action of some of the best known antimicrobial drugs. It is not the aim to cover this topic in detail but just to present an idea of how the different types of antimicrobials act and what lines of study have led to the discovery of their mode of action. In doing so I have taken great help from the reviews of Gale (1963) and Newton (1965) and also from the proceedings of the sixteenth symposium of the Society for General Microbiology entitled "Biochemical Studies of Anti-

microbial Drugs" (1966). However, individual references are also cited in the text wherever appropriate.

Antimicrobial substances have been arbitrarily divided into two groups:

1) The "antibiotics" which are produced by a living organism and which inhibit the growth and activity of other organisms.
2) The drugs which have been synthesized in the laboratory and which are also active against some types of living organisms.

No exact line can be drawn between the natural and synthetic groups e.g., phenol is included among the synthetic drugs although it is obtained from coal tar, a natural product, and some antibiotics like chloramphenicol can be synthesized in the laboratory.

Gale (1966) has pointed out the questions to be answered when studying the mode of action of an antimicrobial substance:

1) What is the precise mechanism of the toxic action?
2) What is the site of action within the sensitive cell?
3) Why is the action selective?
4) What is the relationship between the chemical structure of the drug and the chemistry of the sensitive site?
5) By what mechanisms do normally sensitive cells become resistant to the toxic action?

In order to fully understand the mechanism of action of a drug all the above questions must be answered. This was not possible until the present advances in biochemistry, cytology and molecular biology. Even with the sophisticated experimental tools available to research workers it is not always easy to find the exact mode of action of an antimicrobial drug. This is due to the complexity of

the growth processes and various physiological functions of the cell so that unless sufficient knowledge is available about these mechanisms no advances can be made in the study of their inhibitors.

SYNTHETIC ANTIMICROBIAL DRUGS

Salts of heavy metals, phenols, soaps, dyes, sulphonamides and some other antimetabolites are included in this group. Salts of mercury, arsenic, antimony, copper and lead act by combining with sulphydryl (-SH) groups, which are active sites of many enzymes, and thus inactivate the enzyme. These enzymes include alcohol dehydrogenase, succinic dehydrogenase and phosphoglyceraldehyde dehydrogenase. The action of these agents can be reversed by adding an excess of compounds containing -SH group (Fildes, 1940 for mercury salts). The salts of heavy metals stop growth rather than kill the cells. These compounds have been used as antiseptics (mercury salts) and in treatment of protozoal and spirochetal infections (arsenic and antimony salts).

The surface active agents have a lethal action in contrast with growth inhibition by salts of heavy metals. This group includes phenols, soaps, detergents, salts of some organic acids and a small group of basic peptides and amines. They act by lowering the interfacial tension at the surface of the organisms. Some of the antibiotics like polymyxins are also surface active and cause death of cells by leakage of cell materials.

A number of dyes inhibit the growth of bacteria. They include acridines, crystal violet and malachite green. All these dyes are basic dyes and it has been suggested that acidic groups such as phosphates and teichoic acids found on the surface of gram positive bacteria have a strong affinity for these dyes. The basicity of dyes has been found to be directly proportional to

their growth inhibitory properties (Thimann, 1963). The action of proflavin and acriflavin dyes has been attributed to their interaction with nucleic acids. They have been found to be inserted into the DNA molecule between adjacent base pairs (intercalation)(Lerman, 1961). The action of these dyes can be antagonised by addition of nucleotides and nucleic acids (McIlwain, 1941).

The sulphonamides are the most important of the synthetic drugs not only for chemotherapeutic purposes but also because study of their mode of action drew attention to metabolite analogues as possible chemotherapeutic agents. Fildes (1940) was the first one to suggest the antimetabolite principle as a rational approach to research into chemotherapy. Sulphonamides are active against both gram positive and gram negative bacteria. Woods (1940) first showed that action of sulphonamides can be reversed by p-amino-benzoic acid (PABA), which is an essential metabolite for all susceptible organisms. The first known product of PABA metabolism is folic acid, an essential growth factor for both micro-organisms and higher forms of life. The folic acid molecule contains residues of PABA, glutamic acid and pteridine. Folic acid acts as a coenzyme in reactions involving one-carbon transfers. Such reactions occur at certain steps in the biosynthesis of a number of amino-acids (methionine, serine, etc.), purine ribotides, thymidine and, in some organisms, vitamin B_{12} and pantothenic acid. Sulphona-mides, therefore, attack the synthetic processes of the organism at many points simultaneously. The insensitivity of mammalian cells

is attributed to their absolute requirement for preformed folic acid as they are unable to synthesize it from its precursors. In the sensitive organisms the synthesis of folic acid is inhibited due to antagonism between sulphonamides and PABA. Some of the sensitive parasites can utilize preformed folic acid like their hosts, while others are unable to utilize an external source of folic acid presumably due to permeability barrier. It is only in the latter case that sulphonamides can be successfully used in chemotherapy.

The discovery of the mode of action of sulphonamides led to the study of many compounds as analogues of other essential metabolites but the reversal of their action by addition of the natural metabolites has not been demonstrated in all cases.

ANTIBIOTICS

Antibiotics are defined as compounds which are produced by a living organism and inhibit the growth and activity of another living organism. The antibiotics may be selectively toxic to a variable range of organisms without doing any harm to others. This property makes the antibiotics useful in chemotherapy where they are active against the parasite but not the host cells. The unique structural and metabolic organization of micro-organisms, particularly bacteria, as opposed to higher forms of life makes them susceptible to a number of antibiotics which are not active against the host cells. The selective action reflects fundamental differences in biochemistry and physiology of various forms of life. Antibiotics, with the possible exception of surface active antibiotics, have been found to be active only against growing cells.

The discovery of many antibiotics affecting various sensitive sites in the cells has provided the chemist the basic clues about the drug acceptors in the sensitive cells and the essential reactions one should aim to block. The detailed study of the sensitive processes has also led to the discovery of many reactions involved in biosynthetic pathways which were not known before.

The problem of mode of action of an antimicrobial compound is usually approached in two ways:

1) To find a substance which when added to the sensitive cells in presence of the drug will bring about a reversal of drug action. The most typical example is that of sulphonamides and p-amino-benzoic acid. This approach is not usually successful although

the clue to the action of actinomycin came from the observation that its action can be reversed by addition of DNA to the test system (Kirk, 1960).

2) To study the effect of drug on the various physiological and biochemical processes which occur between the uptake of the nutrients and their eventual elaboration into cell material and the processes which are responsible for maintenance of cell integerity.

The antibiotics have been classified according to their effect on the following processes although this classification is not absolute since an antibiotic can be transferred from one group to another as the knowledge about its mode of action increases.

1) Drugs affecting cell wall synthesis.
2) Drugs affecting synthesis, permeability and integerity of membranes.
3) Drugs affecting energy generating and energy coupling reactions.
4) Drugs affecting protein synthesis.
5) Drugs affecting nucleic acids synthesis and function.

Drugs affecting cell wall synthesis

The bacterial cell wall is the rigid component which protects the cell against osmotic shock. The cell wall can be removed from the rest of the cell, leaving the protoplast, which can perform all the functions of the cell in an osmotically stable medium. If the protoplasts are suspended in a hypotonic solution, they immediately undergo lysis. Thus it is the cell wall that gives osmotic stability to the cell.

The composition of isolated cell walls has been extensively studied and has revealed several compounds peculiar to baoterial cell walls. 'Murein sacculus' is a new term introduced by Weidel and Pelzer (1964) to denote the giant molecule of mucopeptide which determines the shape of the bacterial cell. 'Murein' or 'mucopeptide' are names given to the polymer of which the murein sacculus is built. The following chemical constituents appear regularly in bacterial cell wall and are now known to be those of murein (Weidel and Pelzer, 1964):

1) Muramic acid and glucosamine.

   All bacterial cell walls contain these two aminosugars. Muramic acid is a 3-carboxyethyl derivative of glucosamine. Both these sugars are found as their N-acetyl derivatives in the cell wall.

2) 2, 6-Diaminopimelic acid (DAP) and lysine as alternative constituents.

   Bacteria contain either lysine or DAP. Lysine always occurs as L-isomer while DAP can occur as LL-, or meso-, or DD-DAP.

3) D-Glutamic acid.

Glutamic acid in its D-configuration is an invariable murein constituent.

4) L- and D-Alanine

Both isomers occur together in 1:1 ratio.

5) Amino acids not yet to be classified as murein constituents.

Some amino acids like glycine and serine have been found in close association with murein but whether they are indispensible to intact murein sacculus or not is not known.

N-acetyl muramic acid (acMA) and N-acetyl glucosamine (acGN) are linked through β-1, 6-glycosidic linkage. The resulting disaccharide subunits are linked through β-1, 4-glycosydic linkage to form the backbone of the murein. Polypeptides are attached to the acMA part of the backbone. In a typical gram-negative bacterium like Escherichia coli they consist of L-Ala, D-Glu, meso-DAP and D-Ala. The DAP of one chain forms cross-link with its NH_2 group to D-Ala of another chain. In Staphylococcus aureus, a gram positive bacterium, DAP is replaced by L-Lys and the cross links consist of pentaglycine (Park, 1966). However, murein obtained from gram negatives are uniform in composition and structure whereas mureins of gram-positives are more varied in composition and are difficult to take apart down to muropeptide level (Weidel & Pelzer, 1964).

In addition to murein, bacterial cell wall contains other polymers like lipoproteins, lipopolysaccharides, proteins and polysaccharides. The walls of gram positives have been found to contain another class of polymers, the "teichoic acids", which are

composed of ribitol (or glycerol) residues joined by phosphate and substituted by glucosyl or acGN residues and ester linked D-Ala. The lipoproteins and lipopolysaccharides of gram negative bacteria constitute a high proportion of the total weight of the cell wall (Weidel and Pelzer, 1964).

A number of antibiotics affecting cell wall synthesis by impairment of murein synthesis are known at present. Their actions have certain features in common (Gale, 1963):

1) Growing cultures are killed in presence of antibiotic but the drug is without effect on viability of nongrowing cells.
2) Growth of sensitive organisms in the presence of antibiotic and a non-penetrating solute (e.g. sucrose) at a concentration sufficient to balance the internal pressure of the organism, gives rise to the development of osmotically sensitive forms or spheroplasts.
3) Growth of Staphylococcus aureus in presence of the antibiotic leads to accumulation within the cells of "Park Nucleotides" (uridine diphosphate derivatives of N-acetyl-muramyl penta-peptide). Most of the antibiotics have been tested only against Staph. aureus but penicillin has been shown to cause accumulation in certain lactobacilli and streptococci (Strominger, 1957), while vancomycin causes large accumulations in Bacillus megaterium, B. subtilis, B. cereus, Sarcina lutea, Micrococcus lysodeikticus, Streptococcus faecalis and Staph. aureus (Reynolds, 1966).
4) Incorporation of glycine and glutamic acid into mucopeptide fraction is inhibited.

Murein and proteins are synthesized by different mechanisms. Gale and Folkes (1955 a and b) have shown that the overall incorporation of ^{14}C-glutamate of glycine involves steps sensitive to both penicillin or chloramphenicol. The steps leading to murein are inhibited by penicillin while those leading to protein are inhibited by chloramphenicol.

5) Growth of cells in presence of the antibiotic results in progressive damage to the membrane. This damage is reflected in progressive impairment of the mechanisms required for aminoacid accumulation, crypticity (the condition in which an impermeable surface structure prevents intracellular enzymes from acting on external substrates) and ability to retain cell components of small molecular weight e.g. aminoacids, purines and pyrimidines etc. These effects are not observed in non-growing cells. Addition of chloramphenicol which inhibits protein synthesis in growing cells prevents the above effects on the membrane.

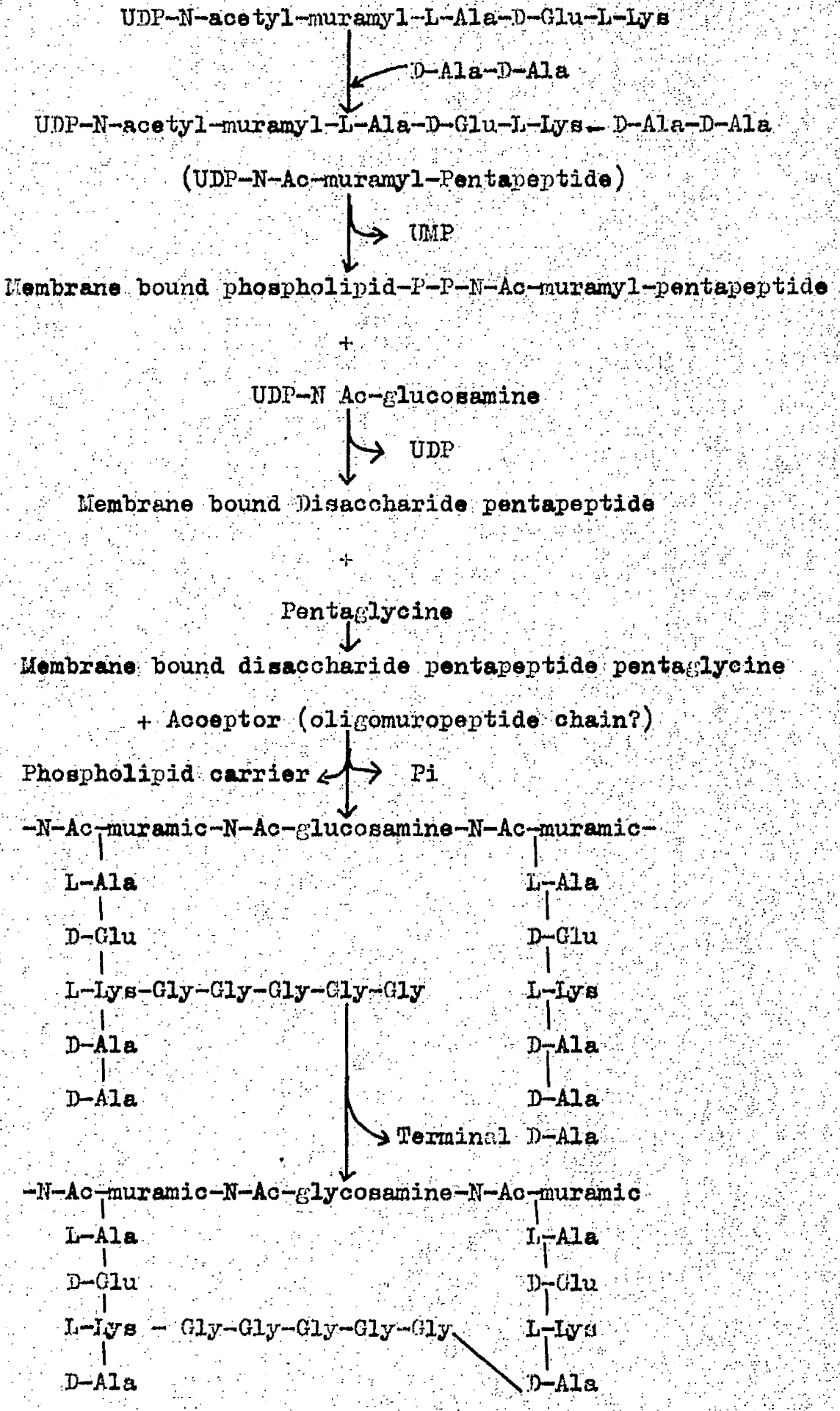


FIGURE 1: Synthesis of murein in Staph. aureus (after Park, 196

Recently Park (1966) has summarised the evidence for the synthesis of murein in Staph. aureus. These reactions are summarised in Figure 1. First a series of reactions within the cell lead to the formation of murein precursors, Park nucleotides, which are uridine-diphosphate-N-acetyl-muramyl pentapeptide. These nucleotides are then attached to the membrane by an unknown phospholipid carrier whose phosphate accepts phospho-NaAC-muramyl pentapeptide (P-P bond) with the release of uridine monophosphate. Next N-AC-glucosamine is transferred from UDP-N-AC-glucosamine to the membrane bound phospholipid-P-P-N-AC-muramyl-pentapeptide with the formation of a disaccharide and release of UDP (transglycosylation reaction). Then a pentaglycine residue is attached to the lysine residue of the disaccharide pentapeptide. This muropeptide (disacoharide-pentapeptide-pentaglycine) is transferred from the lipid carrier to an acceptor (presumably a growing oligomuropeptide chain) with the release of inorganic phosphate and phospholipid carrier which is free to cycle again. Finally in the polymerizing reaction, crosslinking of adjacent chains of oligomuropeptides takes place by a transpeptidation reaction linking the penultimate D-alanine to the free aminogroup of glycine in the neighbouring peptide chain with the release of the terminal alanine.

Reynolds (1966) has suggested that accumulation of the "Park nucleotides" could result from inhibition of a number of processes:

1. Inhibition of the postulated transglycosylation reaction.
2. Inhibition of the transport process or processes.
3. Inhibition of the synthesis or function of the acceptor molecule.

4. Interference with the synthesis or function of the protoplast membrane in general.

A number of antibiotics besides penicillin are known to affect cell wall synthesis in some way. They are bacitracin, vancomycin, D-cycloserine, cephalosporins and ristocetin. All these antibiotics with the exception of penicillin and cephalosporin are assumed to have different sensitive sites. The mechanism of synthesis of cell wall is so complex that these antibiotics can inhibit anyone of the several steps in biosynthesis of cell wall and thus it is very difficult to exactly define the sensitive site. D-cycloserine is the only antibiotic which is known to act as an analogue of D-alanine. It inhibits two enzymes, namely alanine racemase, which converts L-alanine to D-alanine and D-alanyl-D-alanine synthetase, which catalyses the synthesis of dipeptide. D-cycloserine does not affect the addition of dipeptide to the incomplete uridine nucleotide peptide (Strominger, Ito and Threnn, 1960; Neuhaus and Lynch, 1962). Its action can be reversed by addition of D-alanine to the medium (Strominger, Threnn and Scot, 1959).

Penicillin is one of the most extensively studied antibiotics. Although it was known that penicillin produces all the effects due to inhbition of cell wall synthesis, the exact sensitive site was unknown until recently. Wise and Park (1965) found the presence of excess alanine and a higher content of free amino groups of glycine in penicillin treated cells. They suggest that cross linking of murein is not completed in presence of penicillin. They concluded that penicillin inhibits the transpeptidation reaction which is

essential for cross linking mucopeptide chains. Cephalosporins have been shown to have the same mechanism of action as penicillin. Like penicillin, bacitracin, vancomycin, novobiocin and ristocetin have all been shown to cause accumulation of Park nucleotides. Reynolds (1966) has given a survey of what is known about the action of these antibiotics. Unlike penicillin there are varied reports about the sensitivities of the protoplasts in an osmotically stable medium. Vancomycin has been shown to affect both cell wall synthesis and membrane function. Its action varies with different organisms and it may have more than one sensitive site in the same organism. It binds irreversibly and immediately to the sensitive sites. Whole cells are not protected in hypertonic solutions while protoplasts are not affected at all. Ristocetin seems to have a similar mode of action. Bacitracin appears to have a similar action to vancomycin but differs in some respects. Cells treated with bacitracin in buffer at 0° or in growth medium at 37°, can synthesize β-galactosidase when incubated subsequently in absence of antibiotic whereas vancomycin treated cells cannot. Moreover, bacitracin treated cells recover more rapidly than vancomycin treated cells. These three antibiotics namely, vancomycin, ristocetin and bacitracin have been found to inhibit the transfer of disaccharide pentapeptide from phospholipid carrier to acceptor. The result is incomplete oligomuropeptide (devoid of glycine.)

Drugs affecting membrane synthesis

Vancomycin, bacitracin and novobiocin not only affect cell wall synthesis but are also active in other sites. There have been conflicting reports about the effect of vancomycin, bacitracin and novobiocin on the membrane. Hancock and Fitz-James (1964) reported that vancomycin and bacitracin inhibit the growth of protoplasts of Bacillus megaterium. Reynolds (1966), however, reported that growth and uptake of amino acids by whole cells was inhibited while protoplasts were relatively unaffected. Jordan (1965) has found that in protoplasts of Staphylococcus aureus treated with vancomycin, incorporation of P^{32} into membrane fractions is inhibited 80% after 30 minutes whereas 100% inhibition of incorporation of glycine into mucopeptide of whole cells is observed within 54 seconds of addition of antibiotic. This suggests that both cell wall and membrane synthesis are affected. The former is inhibited more rapidly and completely.

Ristocetin, like vancomycin, inhibits the growth of Bacillus megaterium (Shockman and Lampen, 1962). Bacitracin's effects on protoplasts are as controversial as with vancomycin. Reynolds (1966) has reported the same effects on whole cells and protoplasts of B.megaterium as reported for Staphylococcus aureus and vancomycin i.e., amino acid incorporation in whole cells was immediately and strongly inhibited while in protoplasts no effect was observed up to two hours.

Drugs affecting membrane function

The membrane is an important structure of the microbial cell and various functions are attributed to it. The first and the most important is the protection afforded to the cell by maintenance of an osmotic barrier which prevents the intracellular metabolites leaking out into hypotonic media. Besides this it is also thought to be the site of specific proteins (permeases) which control the active transport of amino acids and sugars into the cell (Cohen and Monod, 1957). It may also be the site of electron transport (oxidative phosphorylation)(Mitchell and Moyle, 1956) and cell wall synthesis in bacteria and may be involved in protein synthesis (Butler, Godson and Hunter, 1961). The membrane is mainly composed of phospholipid, protein and some carbohydrate. Due to the importance of the membrane to the integerity of the cell, any antibiotic affecting the membrane function may be lethal to the cell.

These antibiotics can be divided into two groups:

1) Cyclic peptide antibiotics

This group includes tyrocidin, gramicidin S, and the polymyxins. Tyrocidin acts as a surface active agent and destroys the osmotic barrier of the membrane, releasing amino acids, phosphates, purines, pyrimidines, phosphate esters etc. into the surrounding medium (Hotckiss, 1944; Gale and Taylor, 1947; Newton, 1953). Gramicidin S is similar to tyrocidin but in addition acts as an uncoupler of oxidative phosphorylation (Brodie and Gray, 1956). Polymyxins also affect the cell membrane as shown by leakage of small molecular weight compounds (Hotckiss, 1946).

2) Polyenes

This group of antibiotics is characterised by their highly selective action on fungal membranes. Lampen (1966) has reviewed the effect of polyene antibiotics on functions of fungal membrane. These antibiotics cause leakage of small molecular weight substances like carboxylic acids, phosphate esters, inorganic phosphates and K^+ ion from sensitive cells. The specific site of polyene action is attributed to their interaction with sterols of the fungal membranes. This accounts for their selective action towards fungi. It has been suggested that polyenes cause a reorientation of the sterol in the membrane and thus alter cell permeability sufficiently to kill the cells (Demel, Van Deenen and Kinsky, 1965).

Besides these antibiotics, novobiocin also affects the integerity of the bacterial membrane as shown by the release of materials absorbing at 260 mμ and changes caused in a mutant cryptic for β-galactosidase (Brock and Brock, 1959). This is one of the many effects produced by this antibiotic and may not be the main lethal event. Streptomycin also causes a change in permeability of the membrane as revealed by an efflux of potassium ions in presence of streptomycin (Dubin and Davis, 1961; Dubin, Hancock and Davis, 1963).

Drugs affecting energy metabolism

There are very few agents known which selectively interfere with energy metabolism of different organisms. Antimycin A is an antifungal agent which inhibits the transport of electrons across the respiratory chain (Chance and Williams, 1956). However, it is toxic for mammalian cells also so that it can be used as a research tool only. Gramicidin acts as an uncoupler of oxidative phosphorylation (Brodie and Gray, 1956). Moore and Pressman (1964) have obtained similar results for valinomycin.

Grant (1966) has suggested that although there are basic similarities between energy yielding reactions of the host and parasite, the biochemical diversity may be expressed in several ways. There may be differences in rates of enzyme action. Enzyme variants (isoenzymes) may exhibit different affinities for allosteric and other inhibitors. There are also variations in the structural organization and enzyme distribution which affect selective penetration and absorption of drug. These differences might provide some possibility of selective inhibition of energy yielding reactions.

Drugs affecting protein synthesis

Protein synthesis is a highly complex phenomenon and extensive research has been carried out to determine the sequence of events occurring during its operation. The mechanism of protein synthesis, as known today (Ingram, 1965) is briefly as follows. Proteins are built from amino acids units linked by peptide bonds. The DNA of the cell carries the genetic information for the sequence of amino acids in a protein chain. It does so in the form of a linear sequence of bases (adenine, thymine, guanine and cytosine). Each amino acid is coded by three bases (triplets) in the DNA. The base sequence coded in DNA is copied in a complementary fashion by messenger RNA (mRNA). This process is called the transcription process. (The base thymine is replaced by uracil in RNA). The mRNA is then carried to the site of protein synthesis, the ribosomes. Ribosomes are ribonucleoprotein particles of variable size depending on Mg^{++} ion concentration. At .01M Mg^{++} concentration, most of ribosomes sediment at 80 or 100 Svedberg units (100S and 80S particles). As Mg^{++} ion concentration decreases 100S particles dissociate into 70S and 70S are further dissociated into 50S and 30S particles. Protein synthesis takes place in 70S or larger particles but not in smaller particles. The ribosomes take part in protein synthesis in the form of chains called polysomes. The amino acids synthesized in the cell are first activated by conversion to a reactive amino acid adenylate in the presence of ATP and a specific enzyme with the release of inorganic pyrophosphate. The activated amino acids are then attached to another species of RNA, the soluble or transfer RNA (sRNA or tRNA).

The sRNA has a chain length of about 100-200 nucleotides with one specific site for attachment to activated amino acid and another specific recognition site for triplet code in mRNA. The product of reaction between activated amino acid and sRNA is called amino-acyl--sRNA. The amino acyl-sRNA then passes to the ribosomes where under the control of a specific mRNA, the amino acid residues are assembled in specific sequence and peptide bonds are formed. The sRNA is then released and is free to cycle again.

The mechanism of protein synthesis as outlined above is a very complex process and is liable to interference by antimicrobials at a number of points. Antibiotics affecting protein synthesis have been extensively studied because their mode of action illuminates the mechanism of protein synthesis. The possible sites of inhibition of protein synthesis are summarised in Figure 2.

FIG. 2 : Mechanism of protein synthesis and possible sites of interference by antibiotics (After Gale, 1963).

Dark arrows indicate the sites of inhibition.

Dashed lines separate the ribosomal stage of protein synthesis.

$\alpha_1-\alpha_2-\alpha_3-$ sRNA^3 : Growing peptide chain

α^* and sRNA* : Amino acid and corresponding sRNA to be added next.

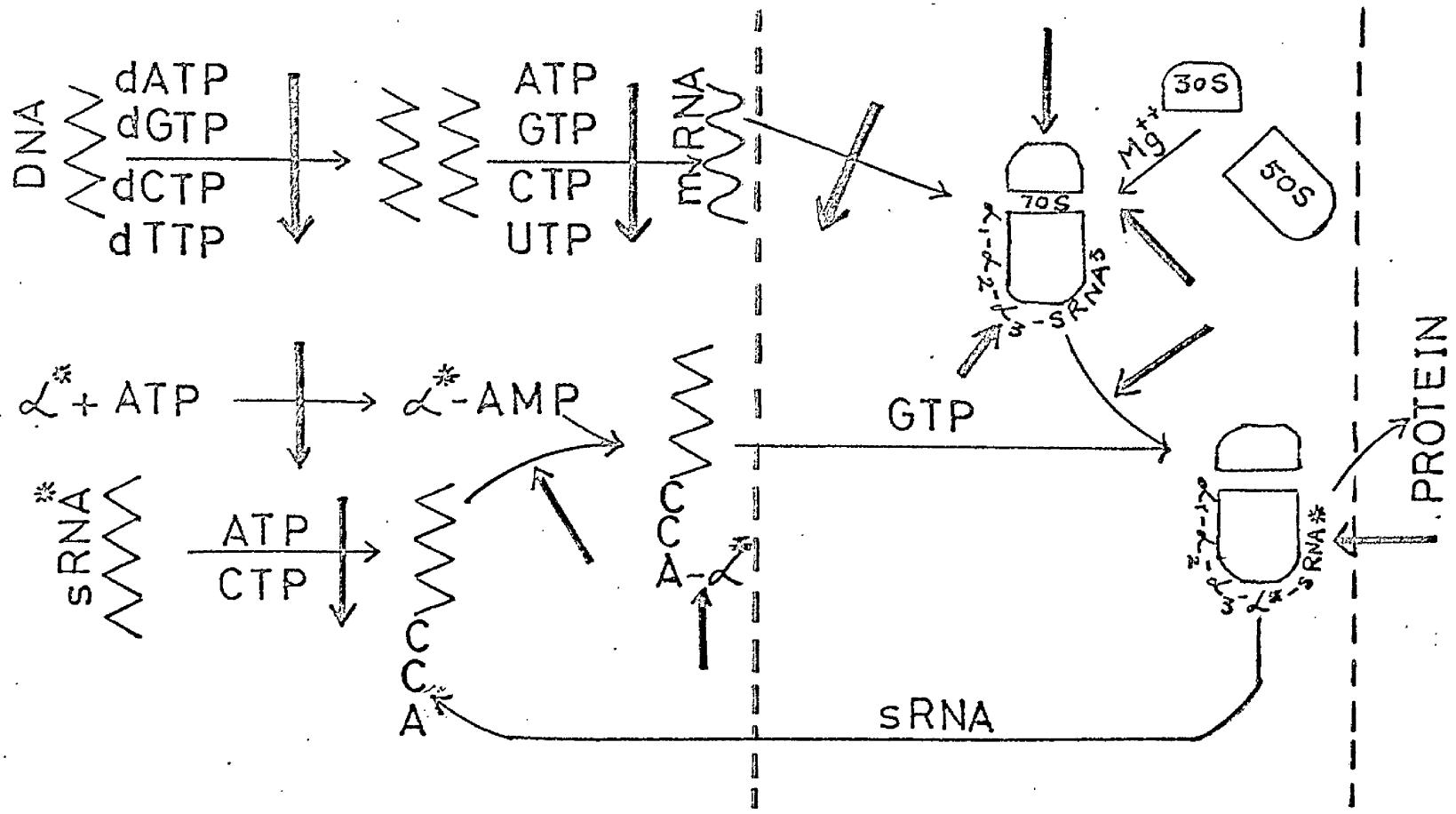

DNA
dATP
dGTP
dCTP
dTTP
ATP
GTP
CTP
UTP
mRNA
30S
50S
70S
Mg++
GTP
PROTEIN
sRNA
ATP
CTP
CCA
sRNA

Fig. 2

1. Chloramphenicol

Chloramphenicol inhibits the growth of blue-green algae and most gram positive and gram negative bacteria but has no activity against fungi, yeasts and some protozoa. Mammalian cells are affected only at high concentrations and after prolonged contact.

Chloramphenicol was found to inhibit protein synthesis (Gale and Folkes, 1953) while DNA and RNA synthesis continued. Chloramphenicol does not affect activation of amino acids (DeMoss and Novelli, 1956), the transfer of amino acids to sRNA (Lacks and Gros, 1959) or the binding of sRNA to ribosomes (Traut and Munro, 1964; Cannon, Krug and Gilbert, 1963) but it does block the transfer of amino acids from sRNA to ribosomes (Nathans and Lipman, 1961; Rendi and Ochoa, 1962). Since chloramphenicol is not active in mammalian protein synthesizing cell free systems this process must differ from that employed in bacteria. Gale and Folkes (1953) first showed that there is an increase in the rate of RNA accumulation due to chloramphenicol but that DNA synthesis is not significantly affected. Evidence has been presented in support of precursors of rRNA (Neidhard and Gros, 1957; Kurland and Maaløe, 1962) and of mRNA (Hahn and Wolfe, 1961) as the form of RNA that accumulates. The accumulation of RNA in presence of chloramphenicol is partly due to increased rate of synthesis of rRNA and partly due to the protection of mRNA from decay which follows normal mRNA detachment from ribosomes (Vazquez, 1966a). In cell free systems chloramphenicol binds to bacterial ribosomes or to the 50S subunit but not to the 30S subunit or other fraction or component of the cell (Vazquez, 1964). By binding to the bacterial ribosomes, chloramphenicol might inhibit

protein synthesis at a number of steps including: (1) formation of the m-RNA-ribosome-amino-acyl-sRNA complex, (2) growth of the peptide, (3) detachment of peptide (Vazquez, 1966). It is now believed that chloramphenicol blocks protein synthesis by preventing growth of the peptide on the ribosome (Julian, 1965).

A number of antibiotics of macrolide, lincomycin and streptogramin families are also inhibitors of protein synthesis and have a mode of action similar to chloramphenicol. Streptogramin B group, however, does not prevent binding of chloramphenicol to 70S ribosomes and it can be concluded that they have a different site of action (Vazquez, 1966b).

2. Tetracyclines

Tetracycline antibiotics are broad spectrum antimicrobial agents and occupy an important place among the chemotherapeutic antimicrobial drugs. They produce a variety of effects on susceptible bacteria and many of these result from their binding to metallic ions and thus attaching to their sensitive sites. The specific inhibition of protein biosynthesis by the tetracyclines seems to be responsible for their bacteriostatic properties (Franklin, 1966). Franklin also suggests that although the precise site of inhibition is not known the tetracyclines interfere with a reaction or reactions at the ribosomal level leading to the formation of peptide bond. Vazquez and Monro (1967) suggest that there are two binding sites of amino acyl tRNA to the ribosomes; one portion of tRNA molecule interacts with 30S subunit plus template and another portion reacts with 50S subunit. By study of tetracycline inhibition of various synthetic tRNAs such as polyphenyl-tRNA, these authors concluded that tetracyclines combine with ribosomes in such a way as to obstruct one of the two sites for the binding of amino acyl tRNA.

3. Puromycin

Puromycin is a broad spectrum antibiotic and is active against mammalian, protozoal and bacterial cells. Due to its toxicity to mammalian systems it is not important in chemotherapy but has been used in research as a specific and reversible inhibitor of protein synthesis. It was first shown by Creaser (1955) to inhibit induced enzyme synthesis. Later it was found that puromycin inhibits protein synthesis without affecting DNA or RNA synthesis. Yarmolinski and de la Haba (1959) first proposed that due to structural similarities, puromycin might act as analogue of the adenosine end of amino acyl tRNA. Nathans, Notani, Schwartz and Zinder (1962) suggested that puromycin substitutes for the incoming amino acyl tRNA at the ribosome and forms a peptide link between the terminal carboxyl group of the incomplete peptide chain and the amino group of the puromycin. Chloramphenicol inhibits puromycin action (Nathans, 1964) by blocking peptide bond formation.

4. Streptomycin

Streptomycin produces a wide variety of effects on sensitive cells and its real mode of action was not clear until recently. Streptomycin initiates a sequence of events which have been described by Brock (1966) as follows:

1) There is almost an instantaneous binding of streptomycin to the cells. The antibiotic is preferentially attached to the ribosomes both in vivo and in vitro in Bacillus megaterium (Hancock, 1962). Escherichia coli also binds streptomycin in vitro (Brock, 1962) but no in vivo studies have been done with this organism.

2) Another early effect is the induction of efflux of potassium ions in both Escherichia coli (Dubin and Davis, 1961; Dubin, Hancock and Davis, 1963) and in Bacillus megaterium (Hancock, 1964). This is attributed to a change in permeability of the membrane.

3) The next effect is inhibition of protein synthesis followed closely by loss of viability. RNA synthesis continues during this time (Dubin, Hancock and Davis, 1963).

4) All the other effects seen in streptomycin treated cells later than the inhibition of protein synthesis are cell death, metabolite leakage, increased permeability to β-galactosides, marked increase in ability to bind streptomycin, degradation of RNA and excretion of nucleotides, and the impairment of respiration (Dubin et al, 1963; Dubin and Davis, 1962; White and Flaks, 1962).

The mechanism of protein synthesis as known at present was summarised earlier. However, in view of the mechanism of action of streptomycin (misreading of the mRNA), further details are presented here (Maaløe and Kjeldgard, 1966). The specificity of the incorporation of an amino acid into a protein is determined through the recognition of a nucleotide sequence (anti-codon) of the sRNA by a specific nucleotide sequence (codon) of the mRNA or a polynucleotide. Under a given set of conditions, the precise amino acid incorporated is uniquely determined by the sequence of nucleotides in mRNA. The above hypothesis was given experimental proof through the brilliant work of Nirenberg and Matthaei in 1961, who devised a method for polypeptide synthesis in cell free system using synthetic polynucleotides. These workers also found that a high molecular weight polyribonucleotide containing only uracil (polyU) stimulated amino acid incorporation but only that of phenylalanine in presence of phenylalanine sRNA under appropriate conditions. From this result, assuming the validity of a triplet code, Nirenberg and Matthaei deduced that the code for phenylalanine at the mRNA level is UUU. Since their original discovery, elaborate experiments in the laboratories of Nirenberg and Ochoa have led to the determination of the genetic code for all the commonly occurring amino acids. It has been found that some amino acids have more than one group of code letters. This has been called the degeneracy in coding which means that there may be several codons each of which specifies a different sRNA which contains the same amino acid. A single nucleotide change can result in: 1) a new amino acid being inserted instead of the old; 2) no amino acid being inserted at all

(a nonsense mutation); or 3) the same amino acid being inserted, depending on the nature of the code, the nature of mutation, the extent of degeneracy, and the availability in the cell of a sRNA possessing the appropriate anti-codon for this new codon.

It is now generally believed that streptomycin causes misreading of mRNA and thus introduces ambiguities in the proteins synthesized. All the other effects produced by streptomycin are explicable on this basis (Brock, 1966). The exact mechanism of how the ambiguities are introduced is not clear as yet. It has been suggested (Davies, 1964) that attached mRNA blocks the attachment of streptomycin to the ribosomes. The studies of Flaks, Cox and White (1962) also suggest that one molecule of streptomycin binds to each ribosome. These and similar studies, together with the finding that it is the 30S subunit of ribosome which imparts resistance to streptomycin (Davies, 1964), have led to the hypothesis that streptomycin acts by altering the configuration of the 30S subunit, leading to errors in codon-anticodon recognition and thus introduces ambiguities in the protein synthesizing system. However, the binding of streptomycin to 30S ribosomes has not been shown directly. Streptomycin does not inhibit peptide bond formation but merely leads to the insertion of the wrong amino acid (Brock, 1966). Brock (1966) has also suggested that the effect of streptomycin on viability and growth of the cells may be due to induction of the synthesis of faulty proteins. When a sufficiently large amount of such proteins have accumulated, growth could no longer occur efficiently and cell division would slowly cease. The secondary effects of streptomycin (permeability alterations,

impairment of respiration, RNA degradation) might also arise from the miscoding induced by streptomycin, since it is conceivable that faulty proteins in various cellular structures such as membranes, ribosomes and electron-transport particles could lead to their malfunction. The killing induced by streptomycin may not be due to the production of faulty proteins as suggested by Gorini and Kataja (1964) who have shown that as much as 50-80% inactive protein can be formed without detectable killing. The killing induced by streptomycin might thus be an additional aspect of the antibiotic activity, perhaps resulting from the irreversible binding of streptomycin to the ribosomes (Brock, 1966).

Drugs affecting nucleic acid synthesis and function

Nucleic acids of a living cell perform two most important functions in its life processes. The first one is the transfer of genetic information from one generation to the other performed by the deoxyribonucleic acid (DNA). The second is the transfer of this information from DNA to the cell for controlled synthesis of specified proteins and other vital functions performed by ribonucleic acid (RNA). The subject of nucleic acid synthesis and function is being actively studied. The following brief summary of what is known about these processes at present has been derived from the monographs on "Control of Macromoleoular Synthesis" by O. Maaløe and N.O. Kjeldgard (1966), and on "The Biosynthesis of Macromolecules" by V.M. Ingram (1965).

The information necessary to specify the protein structure is thought to reside in the sequence of the four different bases in DNA (adenine, guanine, cytosine and thymine). The amino acid sequence of any protein is coded for in a sector of DNA, point for point, by its base sequence. The base sequence in the DNA molecule is replicated exactly every time a cell divides and this ensures genetic continuity. Any change in the base sequence spontaneously or due to an external influence may result in a mutation. In the transcription process the information contained in DNA is transferred to messenger RNA (mRNA). It is also an exact process in the sense that DNA nucleotide sequence is reflected in the base sequence of mRNA (the base uracil replaces thymine in RNA). The information coded in mRNA is then carried to the site of protein synthesis, the polyribosomes.

The DNA molecule is a double helical structure with two intertwined complementary and antiparallel deoxynucleotide chains. The complementary structure is maintained by complement base pairs adenine/thymine and guanine/cytosine which are held together by hydrogen bonds. In the replication process the two DNA strands separate and a complementary copy of new DNA is synthesized on each of the two separate parent strands so that the new DNA molecule consists of one old and one new strand. This mode of replication is called semiconservative and was first demonstrated by Meselson and Stahl (1958). Escherichia coli cells were grown for several generations in "heavy" medium containing $^{15}NH_4^+$ as the sole source of nitrogen. After transfer to "light" (^{14}N) medium, aliquots of cells were removed at intervals and the DNA was analyzed for specific density by centrifugation in a cesium chloride gradient. It was observed that, during the first generation of growth after transfer, heavy (^{15}N-^{15}N) DNA was gradually replaced by half-heavy (^{15}N-^{14}N) DNA. The quantity of half-heavy DNA present at the end of the first generation persisted in subsequent generations during which light (^{14}N-^{14}N) DNA accumulated. By density gradient centrifugation of heated, single-stranded DNA, it was shown that the half-heavy, or hybrid, DNA molecules always contained one heavy and one light strand. Kornberg and his associates have worked out the conditions for DNA synthesis in vitro. An enzyme has been isolated and purified from E. coli, the DNA-dependent DNA polymerase. This enzyme in presence of a DNA primer and the four deoxynucleotides dATP, dGTP, dCTP and dTTP and Mg^{++}, synthesizes new DNA with the simultaneous liberation of inorganic pyrophosphate. The base

sequence of newly formed DNA corresponds exactly to that of primer. However, the *in vivo* and *in vitro* DNA syntheses differ in processes initiating DNA synthesis. *In vitro* synthesis can probably begin wherever the proper free end group presents itself but *in vivo* initiation seems to be a very special process which probably can occur only at a single specific site on a DNA molecule. Auto-radiographs published by Cairns (1963) have shown continuous tracks of 1000 - 1200 μ long labelled DNA from *E.coli* cells. This enormously long DNA "molecule" is usually Y-shaped and the labelling indicates that the fork of the Y actually represents the "growing point" at which replication occurs. Meselson and Stahl (1958) in their studies of the semiconservative mechanism of DNA replication, showed that no light DNA appears until almost all the original heavy DNA had been transformed into hybrid DNA. These observations imply that successive rounds of replication are initiated at the same point on the bacterial genome to ensure replication of all parts of the genome before a new round of replication starts.

The RNA molecule, like DNA, is also composed of polynucleotide chains, but the base thymine is replaced by uracil. The secondary structure of RNA is not so well defined as DNA. RNA is found in the cell in three forms:

1) Ribosomal RNA (rRNA), the major RNA component of cell, which is found in ribosomes.
2) Messenger RNA (mRNA) which is the template for protein synthesis and presumably the synthetically specific part of the polyribosomes.
3) Soluble or transfer RNA (sRNA or tRNA) whose function is to act as amino acid adaptor molecules carrying specific amino acids into

their specific places on protein synthesizing template.

All RNA species seem to be copied from specific DNA regions (Maaløe and Kjeldgard, 1966). A polymerase that performs DNA dependent RNA synthesis in presence of DNA and four ribonucleotides (ATP, GTP, CTP and UTP) *in vitro* has been isolated from bacteria, plant and animal cells (Weiss and Gladstone, 1959; Steven, 1960; Hurwitz et al., 1961; Ochoa et al., 1961; Chamberlin and Berg, 1962).

Selective inhibition of DNA synthesis and not of RNA and protein synthesis results in "unbalanced growth". The phenomenon of unbalanced growth was first observed with a thymine auxotroph grown in absence of thymine (Cohen and Barner, 1954). It is believed that the cell by continued synthesis of RNA and protein in absence of DNA synthesis grows itself to death (thymine-less death). The phenomenon of thymine-less death has been studied in great detail to illuminate the synthetic interrelationships of the major macromolecules. The behaviour of *Escherichia coli* 15T$^-$, a thymine auxotroph of *E. coli* 15, when suspended in a thymine-less medium was described by Cohen (1957) in greater detail. This organism lost the ability to divide or "died" in absence of thymine in an otherwise complete medium, unlike other known auxotrophs (amino acid, purine or uracil requiring strains) which remain viable in the absence of their growth requirement. If other nutrients are omitted from the growth medium along with thymine, the killing was prevented. In other words, the metabolism of glucose, nitrogen and phosphate was essential to the thymine-less death. Omission of thymine alone produced a lag of 25-30 minutes during which the

viable count remained constant. This is followed by an exponential fall in viable count which continues for 4-5 decades. Protein and RNA of such cells at least doubled while DNA showed an increase of only a few per cent. These cells showed a great increase in their length and diameter. This increase in the cell size has been observed under other conditions of unbalanced growth.

Thymine-less death has been studied in greater detail recently in a multiple auxotroph of Escherichia coli 15TAU which requires thymine (T), arginine (A), and uracil (U) by Maaløe and coworkers. Maaløe has described the results in his monograph "Control of Macromolecular Synthesis" (1966). A medium which supports normal growth of this organism has been designated +TAU, medium lacking thymine only as -T +AU and a medium lacking arginine and uracil as +T -AU. By withdrawing thymine, arginine or uracil DNA, protein and RNA synthesis can be respectively controlled. A - T + AU medium shows the usual process of thymine-less death. However, if the cells are incubated in + T - AU medium RNA and protein synthesis stops but DNA synthesis continues until total DNA has increased by about 40%. This is attributed to the fact that cells which are already in the process of DNA replication, complete the replication but a new round of replication can not be started in absence of protein synthesis. Protein synthesis and not RNA synthesis requirement for initiation of DNA synthesis has been suggested by Frieson and Maaløe (1965) who showed that amino acid starvation in the presence of a high concentration of chloramphenicol inhibits initiation of DNA synthesis to the same extent as starvation alone, despite the fact that RNA synthesis is stimulated by the drug. If all the three factors are

withdrawn at once (-TAU medium), thymine-less death again takes place. However the cells have been found to be completely immune to thymine-less death if they have been incubated in a +T -AU medium for a sufficient time to allow completion of DNA synthesis already initiated. These observations have led to two suggestions that a) cells deprived of thymine during active replication suffer thymine-less death, whereas cells that have terminated one round of replication and not initiated a new one are immune, and b) that the act of initiation requires proteins synthesis.

A number of antibiotics are known at present to inhibit nucleic acid synthesis and function. Their mode of action is discussed under individual cases. The above description of thymine-less death was presented because their mode of action is often compared with thymine-less death.

1. Actinomycins

Actinomycins are a group of pigmented peptide containing antibiotics. Kirk (1960) first reported the preferential inhibition of RNA and protein synthesis while DNA synthesis was much less sensitive. She also found that actinomycin forms reversible complexes with DNA. Later work with actinomycin showed that it inhibits the synthesis of host cell RNA and the multiplication of a DNA containing virus but did not inhibit the replication of an RNA containing virus even at concentrations that gave complete inhibition of host cell RNA (Reich et al, 1961a; and Goldberg and Reich, 1964). This suggested that actinomycin inhibits DNA-dependent RNA synthesis. This hypothesis was further supported by work in cell free systems where actinomycin was found to be a potent inhibitor of RNA polymerase (Kirk, 1960; Hurwitz et al, 1962). The nature of the binding of actinomycin to DNA was suggested to be between peptide moiety of actinomycin and guanidine residue of DNA in the small groove of the DNA helix as shown by X-ray analyses (Hamilton, Fuller and Reich, 1963). The presence of guanine in a DNA template is necessary for the formation of complexes of drug with DNA has been shown by a number of workers (Goldberg, Rabinowitz and Reich, 1962; Reich, 1964a; Kahan, Kahan and Hurwitz, 1963). On the basis of the above and similar studies it is generally believed that actinomycin inhibits DNA-dependent RNA synthesis by combining in a very specific way with the DNA template. The binding site of the antibiotic lies in the small groove of the DNA-helix and that actinomycin competes with RNA-polymerase for these sites. The DNA-polymerase which is relatively

insensitive to antibiotic is thought to be bound to sites in the large groove of the DNA-helix (Cavalieri and Rosenberg, 1963). The inhibition of protein synthesis observed results from a lack of mRNA.

Due to the selective inhibition of DNA-dependent RNA synthesis, actinomycins have been widely used as a research tool to study the translation of genetic information from DNA template to mRNA.

2. Mitomycins

Mitomycins are a group of antibiotics which have both antibacterial and antitumour properties. Mitomycins cause selective inhibition of DNA synthesis without inhibition of RNA or protein synthesis in a variety of cell systems (Szybalski and Iyer, 1964). Treatment with mitomycin gives rise to filament formation in Escherichia coli (Reich et al, 1961a; Suzuki, Pangborn & Kilgore, 1967).

The bactericidal properties of mitomycins have been attributed to their irreversible covalent binding to DNA molecule resulting in the formation of cross links between complementary DNA strands (Iyer and Szybalski, 1963). The formation of cross links, presumably by changing the physical properties of DNA, interferes with replication of DNA molecule. Inhibition of DNA synthesis is accompanied by a breakdown of DNA and accumulation of precursors in the pool or in the medium in some, but not all, organisms (Kersten, 1962; Reich et al, 1961b). It was suggested that extensive breakdown of DNA in presence of mitomycins is caused by endonuclease activity associated with sRNA (Kersten et al, 1964) but Suzuki and Kilgore (1967) have proposed that degradation of nucleic acids is a result of lability of DNA due to alkylation by mitomycin rather than by endonucleases.

It is generally believed that mitomycin does not affect the synthesis of RNA and protein for a considerable period of time (Shiba et al, 1958). The lack of any direct effect of mitomycin on RNA synthesis is shown by studies on the formation of mRNA and inducible enzymes by inhibited cells. Since an early event in enzyme induction is believed to be synthesis of new mRNA any effect

on induced enzyme synthesis would indicate an effect on RNA-synthesizing capacity of the organism. mRNA produced by Escherichia coli treated with mitomycin appears to be normal when judged by its ability to form hybrids with DNA (Smith-Kielland, 1964). Cheer and Tchen (1962) have reported that addition of mitomycin to pre-induced E.coli leads to a temporary halt (2 - 3 minutes) followed by synthesis of β-galactosidase at a slightly reduced rate while the rate of enzyme synthesis by cells pretreated with mitomycin was inversely related to the duration of pretreatment. The rate of overall protein synthesis was unaffected. Suzuki and Kilgore (1967a) have recently reported that bactericidal concentrations of mitomycin C also cause a delayed inhibition of RNA and protein synthesis. They found that RNA was more conspicuously degraded than DNA. The capacity to synthesize β-galactosidase also diminished progressively. In view of the latest observation, it cannot be decided that the action of mitomycin is solely due to inhibition of DNA synthesis.

3. Proflavins and Ethidium

Some drugs have been found to be inserted between adjacent base-pairs of double helix of DNA (Lerman, 1964; Warings, 1964; Ward, Reich and Goldberg, 1965). This phenomenon has been termed "intercalation" and has been studied in great detail with acridines (particularly proflavin) and a phenanthridine, ethidium. Intercalation requires local unwinding of DNA helix. The space occupied by an intercalated molecule is the same as a normal base pair so that the adjacent base pairs are separated by twice their normal distance (Lerman, 1961). These drugs are usually bacteriostatic (Albert, 1960), and DNA synthesis is the process most profoundly affected leading to the formation of filamentous cells. Significant inhibition of RNA synthesis is also observed (Tomchick and Mendel, 1964).

Proflavin has been suggested to cause inhibition of DNA-directed RNA synthesis, as it inhibits inducible β-galactosidase synthesis by Escherichia coli (Kepes, 1963).

4. Phenethyl Alcohol:

Phenethylalcohol (PEA) was first reported by Berrah and Konetzka (1962) to be a selective and reversible inhibitor of DNA synthesis. Due to its bacteriostatic properties, it was used by many workers as a selective and reversible inhibitor of DNA synthesis (Jacob, Brennen and Cuzin, 1963; Konetzka and Berrah, 1962). However, some workers found it difficult to selectively inhibit DNA synthesis while protein and RNA synthesis continue (Lester, 1965; Prevost and Moses, 1966; Rosenkranz, Carr and Ross, 1965). More observations have appeared which are inexplicable if PEA primarily affects DNA synthesis. These include reports of inhibition of an RNA phage (Nonoyama and Ikeda, 1964); inhibition of germination of spores (Lester, 1965; Slepecky, 1963) under conditions where DNA synthesis does not occur even in absence of PEA and inhibition of mRNA synthesis and enzyme induction (Rosenkranz, Carr and Rose, 1965; Prevost and Moses, 1966). Treick and Konetzka (1964) suggested that PEA may inhibit DNA synthesis in Escherichia coli by altering the membrane site of the initiation of DNA synthesis. Silver and Wendt (1967) examined the effects of PEA on cell permeability of E.coli and concluded that the primary action of PEA is at the level of the cell membrane with resultant breakdown of the cellular permeability barriers. The effect on DNA synthesis could be due to a coupling of the initiation of DNA replication to the membrane as suggested by Treick and Konetzka (1964) or it could be a secondary consequence of the initial change in cell permeability (Silver and Wendt, 1967). As a result of these

studies the exact mode of action of PEA is still not clearly understood and further work is required to finally establish the site sensitive to PEA.

5. Hydroxyurea:

Hydroxyurea is known to be an inhibitor of DNA synthesis in mammalian and bacterial cells. In mammalian cells it has been shown to interfere with reduction of ribonucleotides to deoxyribonucleotides by the enzyme ribonucleotide redutase (Adams, Abrams and Lieberman, 1966; Young and Hodas, 1964). Partial successes have been claimed for reversal of drug action in mammalian systems by addition of deoxyribonucleotides (Adams and Lindsay, 1967; Young et al, 1967). Rosenkranz and Levy (1965) first reported inhibition of synthesis of bacterial DNA by hydroxyurea. Further detailed studies in the same laboratory (Rosenkranz, Garro, Levy and Carr, 1966) showed hydroxyurea to be a bacteriostatic agent which reversibly inhibited DNA synthesis with no effect on protein and RNA synthesis. The ribosomes, rRNA and mRNA from treated cell appeared to be normal and functional. DNA from treated cells is neither degraded nor cross-linked. The inhibition of DNA synthesis was accompanied by appearance of filamentous cells. However, the various effects were studied only up to 100 minutes of hydroxyurea treatment. Prolonged exposure of E. coli to hydroxyurea (Rosenkranz and Carr, 1966) resulted in a rapid loss of viability which was accompanied by depolymerization of DNA. By the use of various inhibitors and mutants these workers were also able to show that protein synthesis but not RNA synthesis was required for lethal action of hydroxyurea. They have suggested that either some lethal proteins are being made on the normal control of protein synthesis has been abolished. It is thus possible that loss of viability is caused by both continued unbalanced growth and degradation of genetic material.

6. Nalidixic acid

Nalidixic acid, a substituted naphthyridine, is a broad spectrum antibacterial agent especially active against gram negative bacteria (Deitz, Bailey and Froelich, 1964). A series of papers (Goss, Deitz and Cook, 1964, 1965; Deitz, Cook and Goss, 1966; Cook, Deitz and Goss, 1966; Cook, Goss and Deitz, 1966) have been published from the same laboratory on the mechanism of action of nalidixic acid on Escherichia coli. These workers have shown that nalidixic acid is lethal for growing cultures of E.coli. Treatment with nalidixic acid results in the appearance of long filamentous cells. There is no detectable effect on integerity and permeability of cell membrane. No leakage of intracellular material could be demonstrated at least in the beginning. The respiration of the cells is not affected. Active growth is a pre-requisite for lethal action. Macromolecular synthesis studies show that DNA synthesis is immediately inhibited while there is no effect on RNA and protein synthesis up to at least 60 minutes of treatment. Nucleic acids are degraded, DNA degradation is more extensive than RNA. DNA degradation is evident before cells become nonviable. Degradation of DNA continues during the period of loss in viability. Moreover, DNA degradation was observed only under conditions which permitted bactericidal action of nalidixic acid. The drug is not firmly bound in the cell as its action can be reversed by washing and resuspending in drug free medium.

Thymine-less death is observed to the same extent in presence or absence of nalidixic acid in Escherichia coli TAU. In absence

of arginine and uracil, no effect of nalidixic acid on the viability is observed. Nalidixic acid exerts its lethal effect only in a full medium. The lethal effect of nalidixic acid can also be controlled by addition of dinitrophenol, an uncoupler of oxidative phosphorylation and chloramphenicol which inhibits protein synthesis. Thus the action of nalidixic acid differs from thymine-less death in one respect that it does not have a lethal effect in absence of protein and RNA synthesis while thymine-less death continues at a reduced rate under similar conditions.

On the basis of above results, Cook, Deitz and Goss (1966) have concluded that nalidixic acid rapidly inhibits DNA synthesis in growing cells of Escherichia coli. This inhibition renders the DNA of such cells vulnerable to attack by endogenous nucleases and the genetic material of the cell is ultimately destroyed with a resulting loss of viability.

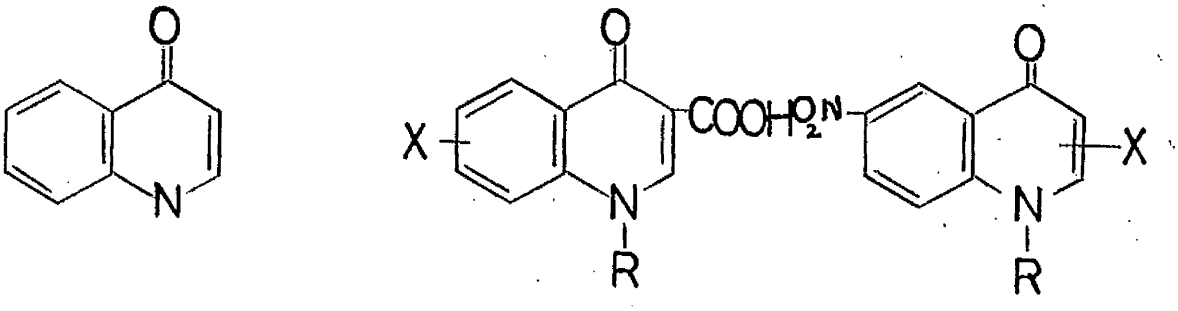
The brief discussion above of some of the well known antibiotics and antimicrobials shows that they inhibit the growth and multiplication of the sensitive organism in a number of ways. The primary target of these antimicrobials is known only in a few cases and those too are still subject to criticism and controversies. This is not surprising since the growth and multiplication of a living cell is such a complex phenomenon that knowledge about it is still fragmentary and far from complete. Brock (1966) has pointed out, " . . . growth is not a single process, but an extremely complex series of integrated and highly interdependent processes, none of which can be completely defined in the absence of others." If any of these processes is disturbed by a drug, its effects may not be observed at the primary site but at a distant site, connected in some manner to the primary site. Thus it is very difficult to relate the observed effects of a drug to its primary site of action unless all the inter-relationships in the cell are known. This is well exemplified in the case of streptomycin, where all the secondary effects were observed before the primary effect of misreading the mRNA at the ribosomal level was discovered. Brock (1966) has suggested that we must not study the mode of action of a drug by finding the first detectable effect but rather the sequence of events which occurs after the drug is added.

In spite of these difficulties in understanding the exact mode of action of antimicrobial drugs, it is certain that they all require growing cells as a pre-requisite for their lethal action (with the possible exception of surface active antimicrobials). There is

overwhelming evidence in support of the statement that only growing cells are sensitive to antibiotics. In most of the cases it has been experimentally shown that nongrowing cells are insensitive to the drug, but become sensitive as soon as the conditions permitting growth are provided. This has been found not only *in vitro* but also *in vivo*. For example, the bactericidal action of penicillin in pneumococcal pneumonia (Smith and Woods, 1956a) and on subcutaneous abscesses (1956b) has been shown by histological examination to be greatest when the rate of bacterial cell division is greatest. Hobby and Lenert (1958) obviously impressed by these observations remarked, ". . . studies with streptomycin, polymyxin, neomycin, oxytetracyclin, isoniazid and other agents have indicated that these antimicrobials . . . are most effective during active multiplication. This gradually increasing body of evidence has suggested that cell multiplication may be requisite to the activity of all antimicrobial drugs".

Quinolones:

Quinolones form a large group of substituted heterocyclic antibacterials synthesized at the ICI laboratories. Routine screening of the quinolone series showed both *in vivo* and *in vitro* activities (Holms, unpublished results). It was found that benz-halogen-3-carboxyl-4-quinolones and the 6-nitro-4-quinolones were most active but 6-nitroquinolones were toxic *in vivo*. The basic ring structure and the two derivatives are shown below:



Quinolone Ring Structure | 3-carboxyl derivatives (carboxy Q) | 6-nitro derivatives (Nitro Q)

Both these derivatives have an alkyl substituent at the ring nitrogen (position R). The 3-carboxyl derivatives have halogen or alkyl substitution in the benz ring at 6 or 7 carbon (position X). The 6-nitro derivatives can have a variety of substitutions at carbon 2 or 3 of the quinolone ring e.g. alkyl, amino, hydroxymethyl and aldehyde. It was also shown with 3-carboxyl series that (a) N-ethyl substitution shows the highest activity, (b) Fluoro- is more active than chloro- which is superior to bromo-and iodo- compound

which have very low activity and (c) substitution of position 7 gives higher activity than that obtained at position 6.

The 6-nitro derivatives were very active but unlike the 3-carboxyl derivatives did not give sharp and reproducible end-points when tested against Escherichia coli in vitro using turbidity measurements as an index of growth. Further examination showed this to be due to continuation of growth after the control had reached stationary phase.

On the basis of the above results, two compounds N-ethyl-3-carboxyl-7-chloro-4-quinolone (carboxy Q) and 1, 2 dimethyl-6-nitro-4-quinolone (nitro Q) were tested for their activity against Escherichia coli in vitrokinetically. (These drugs are referred to by the abbreviations i.e., carboxy Q and nitro Q hereafter). It was found that carboxy Q is bactericidal only towards dividing cells and after a period of induction and the response of the cells changes rapidly with concentration reflecting a narrow distribution of cells resistant to a given concentration of drug. Nitro Q, on the other hand, kills nondividing cells very rapidly and dividing cells more slowly without a period of induction. Moreover there is a strong tendency for overgrowth of cells resistant to the concentration of nitro Q being used.

The most unique action of nitro Q on nondividing cells aroused interest in the comparative study of these two broad groups of quinolones viz. carboxylic and noncarboxylic quinolones. Carboxylic quinolones like all the other known antimicrobial compounds are active only against growing cells while the noncarboxylic quinolones are active against both growing and nongrowing cells.

If both the groups have the same mode of action but differ in their activities according to the physiological state of the sensitive cells, this would provide an ideal system for comparison since in the same test system altered responses are obtained with essentially the same compounds differing only in the substituted groups. The present study of antibacterial action of quinolones was taken up to elucidate the differences in the antibacterial action of carboxylic and noncarboxylic quinolones and if possible to find the mode of action of quinolones as a whole. Carboxy Q was selected as representative of carboxylic quinolones and nitro Q as the non-carboxylic quinolone in the present study.

MATERIALS AND METHODS

ORGANISMS

Three strains of *Escherichia coli* were used:

1. *E. coli* ATCC 11229 strain 198

This is a strain used for testing germicide activity in The Walter Reed Army Medical Centre, U.S.A. Most of the work was done with this strain.

2. *E. coli* NCIB 8114 strain 15 (ATCC 9723)

Parent wild strain of the thymineless mutant used.

3. *E. coli* NCIB 8583 strain 70-462 (ATCC 9723h)

Thymineless mutant isolated from *E. coli* 8114.

E. coli 11229/198 was obtained from American Type Culture Collection (ATCC) and *E. coli* 8114 and 8583 from National Collection of Industrial Bacteria (NCIB, Torry Research Station, Aberdeen, Scotland).

Maintenance of Cultures

Ampoules of freeze dried cultures were broken aseptically and a few drops of nutrient broth added. This suspension was then inoculated into 6 x 8 ml. nutrient broth in tablet bottles and incubated overnight at 37°.

These nutrient broth cultures were streaked on nutrient agar plates. The plates were also incubated overnight at 37°. Next day single isolated colonies were picked up and subcultured into nutrient broth. These nutrient broth cultures were tested for homogeneity by microscopic examination. One of these was then used to inoculate:

1. Cooked meat medium (20 ml. in Universal containers)

2. Nutrient agar slopes.

3. Nutrient broth (8 ml. in tablet bottles).

The above three media were incubated overnight at 37° and stored as stock cultures at 4°.

Media

Composition

The following media were used:

1. Cooked meat medium (oxoid)
2. Nutrient broth (oxoid)
3. Nutrient agar (oxoid)
4. Buffered complex agar medium for viable count.
5. Glucose ammonium salts (GA) medium.

1. Cooked meat medium

This is an Oxoid preparation and has the following composition (g/l. distilled water).

Peptone (Oxoid L.37)	10 g.
"Lab Lemco" Beef Extract	10 g.
Neutral Heart Tissue	30 g.
Sodium Chloride	5 g.

pH approximately 7.6

One tablet was soaked in 10 ml. distilled water for 15 minutes in a universal container.

Autoclaved at 15 lb. per square inch (p.s.i.)

2. Nutrient broth

This was also obtained from Oxoid and had the following composition (g/l. distilled water).

"Lab Lemco" Beef Extract	1 g.
Yeast Extract (Oxoid L.20)	2 g.
Peptone (Oxoid L.37)	5 g.
Sodium Chloride	5 g.

pH approximately 7.4

25 g. were dissolved in 1 l. of distilled water, mixed well and dispensed 8 ml. each into tablet bottles of 16 ml. capacity.

Autoclaved at 15 lb. p.s.i.

3. Nutrient agar

It had the same composition as nutrient broth but in addition had 15 g. of agar/l. 28 g. were soaked in 1 l. of distilled water for 15 minutes. The solution was steamed for an hour in a boiler (Burco Ltd.). It was then dispensed 200 ml. each into screwcapped bottles for pouring plates or 8 ml. each into tablet bottles (16 ml. capacity) for making slopes.

Autoclaved at 15 lb. p.s.i.

4. Buffered complex medium for viable count

Composition (g./l. distilled water)

Trishydroxymethylaminomethane (Reagent grade, Sigma)	2.017 g.
Maleic acid	1.93 g.
Difco Bacto Peptone	30 g.
Difco Yeast Extract	50 g.
Ion-agar No. 2 (Oxoid)	6 g.

5N NaOH ⟶ pH 7.2

All the components except agar were dissolved first in distilled water. The agar was added and the medium steamed for 30 minutes or more until agar dissolved. It was dispensed 400 ml. each into screwcapped bottles.

Autoclaved at 5 lb. p.s.i.

5. Glucose ammonium salts (GA) medium

This medium was used for all growth experiments and for preparation of inocula. It was made up in two parts:

Part A: g./l. distilled water

Potassium dihydrogen orthophosphate	9 g.
Ammonium sulphate	2 g.
5N NaOH ⟶ pH 7.2	approximately 10 ml.

Autoclaved at 5 or 15 lb. p.s.i.

Part B: g./l. distilled water

Glucose	30 g.
Magnesium sulphate	1 g.

Autoclaved at 5 lb. p.s.i.

Part A and Part B were autoclaved separately. After cooling, 3 parts of A were aseptically mixed with 2 parts of B.

Additions to GA medium

(1) Inhibitors:

0.5 or 1 mM solutions of quinolones were made up in "A" part of the GA medium. The solutions were sterilised by autoclaving at 5 lb. p.s.i. and added to growth flasks in appropriate volumes (replacing the same volume of A part) to give required final concentrations.

Chloramphenicol stock solution (2 mmg./ml.) was made up in medium "A" and sterilised by autoclaving at 5 lb. p.s.i.

(2) Thymine, thymidine and other deoxyribonucleosides.

1 mM. solutions were prepared in "A" part of GA medium and added in appropriate amounts to growth flasks to get the required final concentration. They were sterilized at 5 lb. p.s.i. Paper chromatography by the method of Buchanan (1951) of autoclaved solutions showed no difference between sterilised and unsterile solutions.

Sterilisation:

Sterilisation was carried out in Manlove-Alliot autoclave (Manlove Alliot and Co. Ltd., Engineers, Nottingham, England) to which steam was supplied from a Speedylec electrode boiler (Bastian & Allen Ltd., Harrow, England). Sterilization was done at 5 or 15 lb. p.s.i. for a length of time depending on the volume of medium. The efficiency of sterilization was also checked by Browne's tubes.

Microscopy:

Smears prepared from young cultures of E. coli 11229 grown in presence of quinolones were stained by Crystal Violet and photographed with Watson's eyepiece camera on Ilford FP3 film. The eyepiece camera was attached to a Watson microscope model phase 60.

Bacterial size measurements were done by Watson Image Shearing Eyepiece (W.I.S.E.). The optics of this eyepiece allow the image of any object to be sheared into a red and a green image. The two images are sheared until they just touch one another. The distance

moved by the images is equal to their length or diameter or any other dimensions as the case may be. The two images are sheared by rotating a micrometer drum. The divisions on this micrometer are calibrated in terms of microns (μ) by a stage micrometer for each objective used. I used a 3.6 mm. fluorite oil immersion objective lens. With this lens each division on the micrometer was found to be equal to 1.13 μ. Both the length and the diameter of the bacterial cells were measured and the volume (v) calculated by the formula:

$$v = \frac{\pi d^2 (3l-d)}{12}$$

where d = diameter, l = length assuming the cell to be a circular cylinder with hemispherical ends (Powell and Errington, 1963).

Size distribution

Coulter Counter model F(Coulter Electronics) was used to study the size distribution of E. coli 11229 under the influence of quinolones.

Coulter Counter model F is so designed that by means of a mercury siphon (manometer), a specific volume of a suspension of particles in an electrolyte is forced to flow through an aperature (orifice) of specific dimensions. An aperture current exists between an electrode inside the aperture tube and another electrode outside the aperture tube. As a particle passes through the aperture it displaces electrolyte, thus changing the resistance between the two electrodes, producing a voltage drop whose magnitude is proportional to the volume of the particle. The voltage pulses are fed into a threshold circuit which discriminates between them by generating count pulses for only those particles that exceed the threshold level, thus counting the number of particles in passage.

The magnitude of electrical pulses (representing the particles traversing through the aperture) are directly proportional to the amount of aperture current and system amplification. The sensitivity of the entire system may be controlled by adjusting the following controls:

1) Attenuation control. The attenuation controls the overall sensitivity of the instrument with regard to electronic amplification.

2) Aperture current control. The aperture current changes the current passing between the two electrodes. On increasing the aperture current, a particle will produce a larger pulse height for a given size. Decreasing the aperture current will decrease the

pulse height.

3) Threshold dial. The threshold dial controls the level above which particle pulses will be counted. It is possible to adjust the threshold control so that only the desired range of particles will be counted. The particle volume is proportional to threshold setting.

The aperture current and attenuation switch settings are selected to bring a desired particle size within the threshold range. The proper ratio of gain to aperture current has to be found for each particular system by experimenting with different dial settings.

The electrolyte is also selected for a user's particular need and should be completely free from any dust particles or debris as they clog the aperture tube. Debris in electrolyte must also be avoided to get as low a background count as possible.

I have used a 30 μ aperture tube and a setting of 8 for attenuation and 0.25 for aperture current. Buffered saline of the following composition was used as an electrolyte:

Buffer:	
di-sodium hydrogen orthophosphate (anhydrous)	42.6 g.
sodium dihydrogen orthophosphate	9.35 g.
distilled water	1 litre.
Saline:	
sodium chloride	9 g.
distilled water	1 litre.

To each litre of saline 5 ml. of the above buffer and 1 ml. of formalin (40% formaldehyde W/V) were added. Formalin was added to prevent any bacterial growth. The whole solution was mixed and filtered through millipore filters of 0.22 μ porosity. This was necessary to remove any particles in the range of bacterial size and thus keep the background counts to a minimum. The lowest threshold level used was 4 because below this level the electrical noise generated within the machine interferes with the actual particle count.

Calibration of Threshold Units

The threshold control is divided into arbitrary units of 0-105. These units can be calibrated in terms of volume by reference to standard mono size particles. Many types of these standard particles are available. I have used polystyrene or polytoluene latex spheres (Bioproducts Department, The Dow Chemical Company, Midland, Michigan, U.S.A.). The three sizes used had an average diameter of 0.796, 1.305 and 2.68 μ.

The size of any particle can be determined by taking a series of counts with increasing threshold units. If these counts are plotted against threshold units, there is usually a plateau at low threshold units. As the threshold level is increased, some of the smaller particles are not counted and so there is a fall in the number of particles counted. This fall in count continues until the largest particle is counted and then the count again levels off. The slope of this line depends on the distribution of sizes in a particular suspension. If the suspension is composed of monosized particles this slope is very sharp. With a wider particle size range this slope is not very sharp. The midpoint of this slope represents the average size of all the particles in the suspension.

The corresponding threshold unit at the midpoint of the slope is equal to the average volume of the particles. With standard particles of known size the value of one threshold unit can thus be calculated. Table 1 shows the calibration with the various sizes of the standard particles. Since the two sensitivity controls, attenuation and aperture current, have to be adjusted to get a proper pulse picture with different sized particles, the results

TABLE 1

CALIBRATION OF COULTERCOUNTER FOR VOLUME MEASUREMENTS

Particle Diameter μ	Particle Volume μ^3	Settings Used Att. x Ap.Cur. = P	Value of 1t μ^3	Value of 1t at P=2 μ^3	Average Value of 1t at P= 2 μ^3
0.796 ± .0083	0.265	.25 x 4 = 1	.0165	.033	
1.305 ± .0158	1.162	.25 x 8 = 2	.033	.033	
		0.354 x 8 = 2.83	.0465	.0326	.0312
2.68 ± .0149	10.18	1 x 16 = 16	0.23	.0288	
		1 x 32 = 32	0.465	.0288	

Abbreviations: Att. = Attenuation Switch Setting

Ap. Cur. = Aperture Current Setting

P = Product of above two Settings

t = Threshold Unit

can only be compared if the product of the two controls is the same in all cases. Therefore, although the actual measurements were made at products ranging from 1 to 16, they were converted to their equivalent values at product 2. Product 2 was selected because the bacterial size measurements were made at this product. The results show that 1 threshold unit at product 2 is equal to .0312 μ^3. Multiplication of this value by the given threshold unit gives the volume in terms of cubic microns (μ^3). In all cases corrections were made for background count.

The size distribution of Escherichia coli 11229 obtained by Coulter Counter was also compared with direct microscopic measurements by image shearing eyepiece in order to ensure that correct settings were being used with the Coulter Counter. Figure 3 shows a plot of size against number of E. coli 11229.

FIG. 3 : Size distribution of stationary phase Escherichia coli 11229.

●——● By Coulter Counter

○——○ By Watson's Image Shearing Eyepiece.

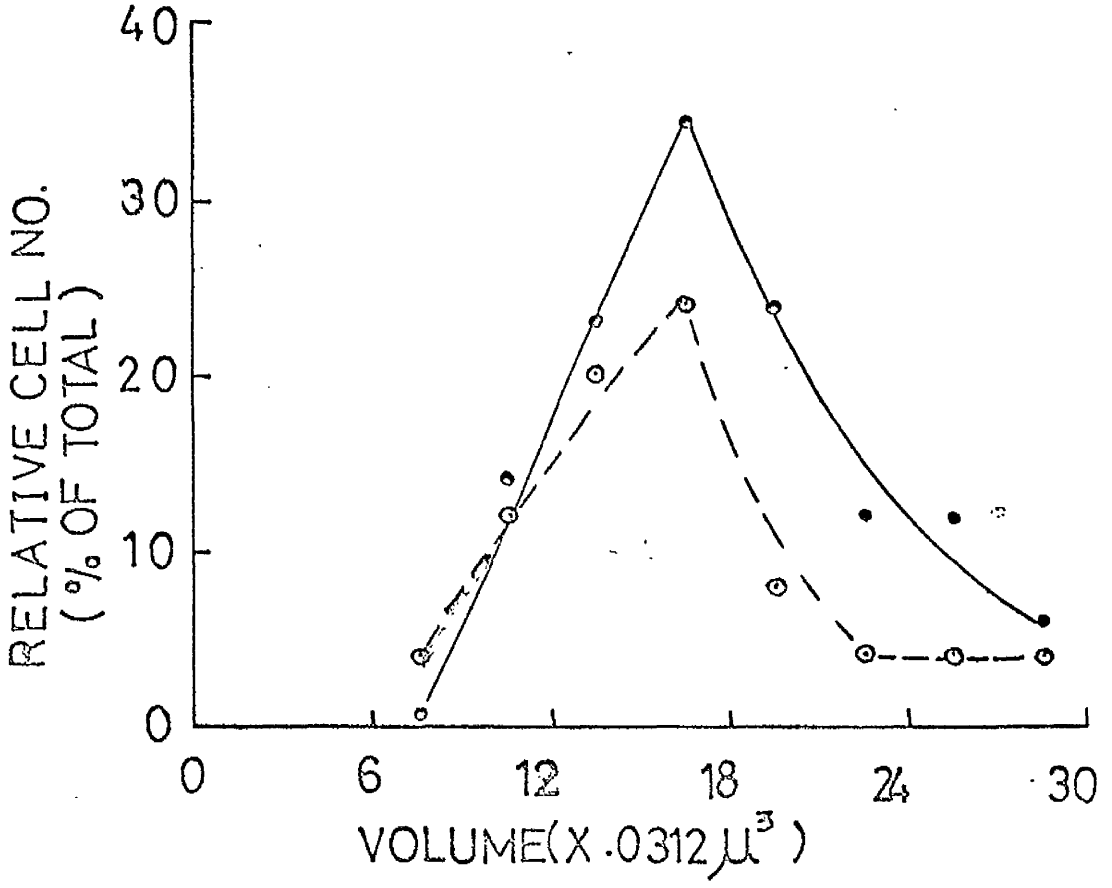

RELATIVE CELL NO.
(% OF TOTAL)
40
30
20
10
0
0
6
12
18
24
30
VOLUME(X .0312 μ³)

Fig. 3.

Growth Studies

Preparation of inoculum:

Starter cultures in nutrient broth were periodically obtained by subculturing from stock culture in cooked meat medium. This was done to minimise any physiological change due to increased number of transfers from stock culture.

The inoculum for any growth experiment was prepared as follows:

(1) Starter culture in nutrient broth from stock culture in cooked meat medium. Incubated overnight at 37°.

(2) From nutrient broth 0.1 ml. was inoculated into 100 ml. GA medium in 500 ml. conical flask and incubated overnight at 37° on a rotary shaker (L.H. Engineering Co., Bells Hill, Stoke Poges, Bucks., MK 1). This is the 1st passage.

(3) From 1st passage, 1 ml. was inoculated into another 100 ml. GA medium, and incubated at 37° for 8 hours on the rotary shaker (2nd passage). This culture was stored overnight at 4°.

Further passages were grown in the same way as 2nd passage. For an actual growth experiment 4th to 6th passage was used as 1% inoculum. This procedure allowed maintenance of reproducibility from one experiment to another.

In the case of *E. coli* 8583, the thymine auxotroph, the GA medium was supplemented with 5 μM (0.63 μg/ml) thymine which can support full growth (Table 2). The inoculum was prepared in the same way as above except that 1st passage was grown without shaking. The reason for this change was that if 1st passage was grown with

TABLE 2

THYMINE AND THYMIDINE REQUIREMENT OF ESCHERICHIA COLI 8583

Thymine Concn. µg/ml	ε350	Thymidine Concn. µg/ml	ε350
0	.045	0	.03
0.2	.65	0.2	.24
0.4	.67	0.4	.52
0.6	.74	0.6	.70
0.8	.80	0.8	.70
1.0	.84	1.0	.70
2.0	.78	2.0	.75
4.0	.90	4.0	.66
6.0	.80	6.0	.825
8.0	.83	8.0	.75
10.0	.80	10.0	.80

A series of test tubes with various concentrations of thymine and thymidine were set up. Tubes were incubated overnight at 37° and the ε350 recorded.

shaking, the 2nd passage showed a very long lag or no growth at all.

A fully grown culture of E. coli 11229 in GA medium has an extinction of about 1.2 - 1.6 at 500 mμ and contains approximately 2-4 x 10^9 viable cells per ml. The mean generation time (M.g.t.) is about 45 minutes.

A fully grown culture of E. coli 8114 in GA medium has an extinction of about 1.0 - 1.6 at 500 mμ and has about 2 x 10^9 viable cells/ml. The M.g.t. is about 48 minutes.

A fully grown culture of E. coli 8583 in GA medium has an extinction of 0.8 - 1.2 at 500 mμ and a viable count of 0.5 - 1.0 x 10^9 viable cells/ml. The M.g.t. is about 51 minutes.

When washed cell suspensions were used as inoculum, the cultures were spun down, washed twice in phosphate buffer pH 7.2 (same concentration as in Medium GA) and resuspended in the same phosphate buffer.

The cells of E. coli 8583 were pretreated with EDTA in some experiments. The pretreatment with EDTA was essentially the same as described by Lieve (1965).

In one experiment where anaerobic conditions were used, pure nitrogen at a rate of 300 - 400 ml./min. was passed into the growth flask to replace all the oxygen. Amount of dissolved oxygen in water under similar conditions as measured by an oxygen electrode attached to a Beckmen Oxygen Analyser Model 777 was found to be nearly zero.

Growth Experiments

Experimental cultures were grown in 800 ml. of GA medium in 1 l. flasks provided with side arms to facilitate sampling. The flasks were vigorously agitated by a magnetic stirring device which has been described (Fewson, 1967)(Figure 4). The breaking of vortex at the bottom produced tiny air bubbles spreading throughout the medium. This method has been shown to be an efficient aeration system (Leggate, 1967). In some experiments where a larger number of conditions were used, the cells were grown in 250 ml. conical flasks with 100 ml. medium in a shaking water bath. All growth experiments were performed at 37°.

Growth was followed by withdrawing 4 ml. samples. Viable count was performed by a special method as described below. The cells were then killed by adding a drop of formalin and the extinction of the sample measured at 350 or 500 mμ wavelength in a spectrophotometer (Spectronic 20 Bausch & Lomb). The samples were read in optically matched tubes (105 x 13 m.m.) with distilled water blank. When extinction was recorded at 350 mμ, corrections were made for absorption due to quinolones at this wavelength.

FIG. 4 : Magnetically stirred growth apparatus used for growth studies.

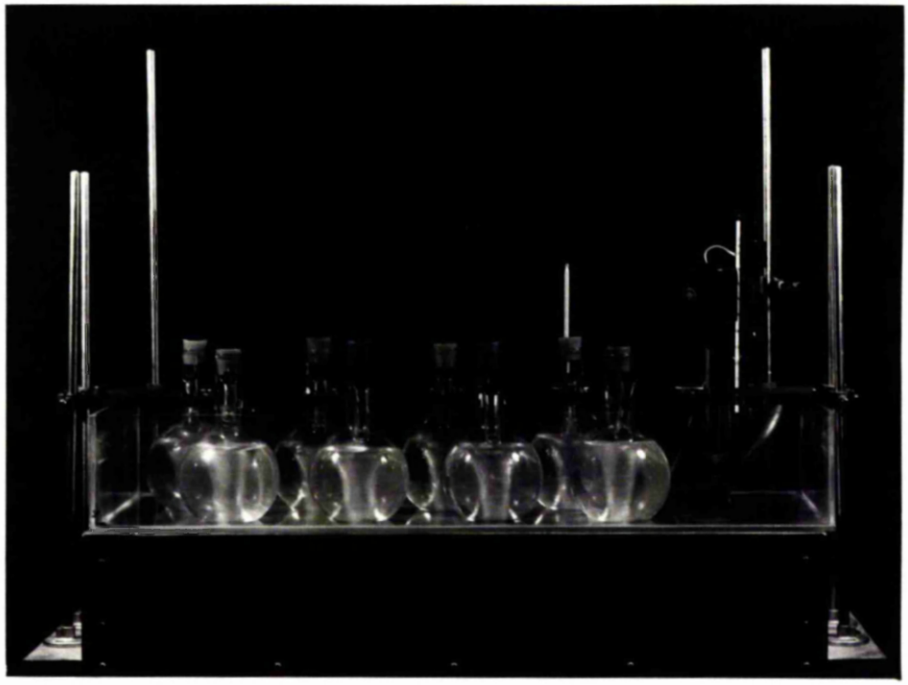


Fig. 4

Viable count method

Viability was measured by a special counting method (Holms, unpublished work). This method consists of making serial dilutions directly in tubes containing a buffered complex medium at 48°. The tubes are immediately solidified by cooling. After incubation at 37°, discrete colonies appeared in the tubes. These were enumerated with a set of prepared standards calibrated by pour plate technique.

The buffered complex medium was stored at 4° and melted before use by boiling. The melted medium was transferred in 6 ml. amounts to sterile 150 x 16 mm. rimless tubes by an automatic syringe pipette (Froud & Sons). This pipette itself was sterilised by boiling in a small instrument steriliser (Macarthy's Hospital Equipments). The tubes of agar were stored overnight in water baths at 48° before being used the next day.

The dilutions of the sample were done by special 0.1 ml. pipette assemblies (Figure 5) which had been made water repellent by silicone treatment. Four such pipettes were used at a time. They were sterilised by immersing the glass part in a boiling water bath. They were used in rotation so that while one was being used two were being sterilised and the fourth was being cooled. 0.1 ml. sample was withdrawn, mixed with 6 ml. of buffered complex medium thoroughly by a vortex mixer (Scientific Industries International Incorporated U.K. Ltd.). Successive dilutions were made in the same manner. The pipette was rinsed with boiling water between transfers. A maximum of five tubes were used for a fully grown culture of E. coli. When dilutions were complete the pipette was again suspended in

FIG. 5 : Special pipette assembly used for making dilutions of cell suspensions for viable count by tube method.

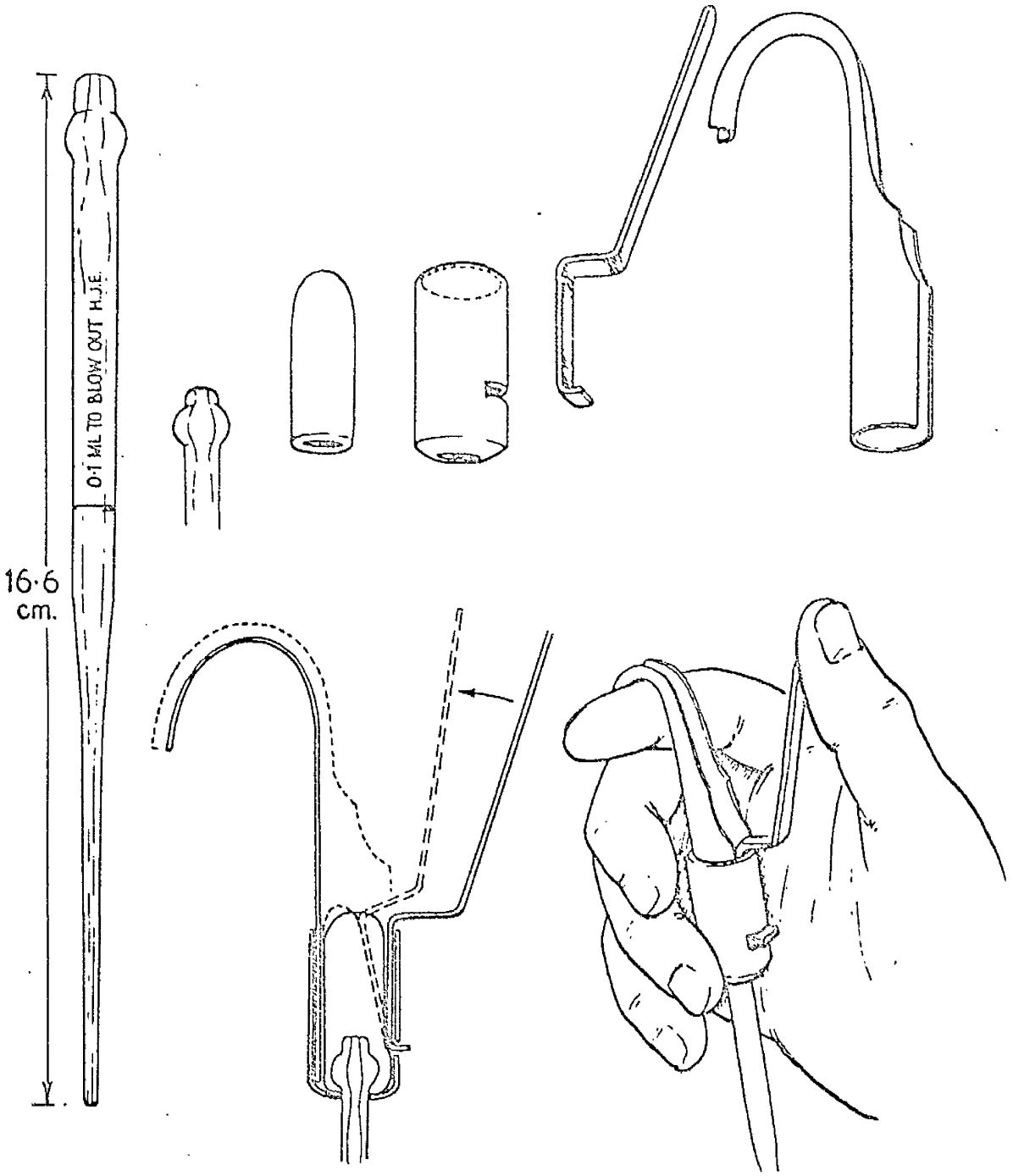


Fig. 5

boiling water. The agar tubes were immediately solidified by placing in an iced water bath. The tubes were incubated at 37° overnight when discrete colonies developed. Each set of tubes was then compared with the standards and $\log_{10}$ read off from a table on the basis of the dilution ($1/60$, $1/60^2$ etc.) which matched the individual tube in the set of standard tubes.

Preparation and Calibration of Standard Tubes

A fully grown culture of E. coli 11229 in GA medium was diluted by a series of 1/10 dilution five times. This served as the master suspension which was further diluted by a series of logarithmic dilutions so as to have a difference of 0.1 of a $\log_{10}$ unit between successive dilutions. These dilutions were made very carefully and as accurately as possible. Each of these bacterial suspensions was counted by adding one ml. to 5 ml. of buffered complex medium in replicates of ten. Viable count was also taken by standard pour plate method in triplicate on nutrient agar. After incubation at 37° the plates were counted and the average number of viable cells in each suspension was calculated. At the lower end of scale, the colonies developing in the tubes were also counted which agreed fairly well with the plate counts of the corresponding bacterial suspensions (Table 3). The tubes with higher number of cells were assigned the same number as that obtained by plate counts. Three best tubes out of the ten replicates for each dilution were selected. These three sets were preserved with formalin and sealed. Two sets were stored at 4° as spares and one was used for taking counts. The standards were kept in a special viewing box which was illuminated from the bottom with a fluoroscent light (Figure 6).

The unknown sample is counted by matching the tube with appropriate number of colonies to one of the standard tubes. Once an unknown tube has been matched to a standard, the viable count of the culture from which the unknown has been derived is easily calculated from a knowledge of the number of 1/60 dilutions performed and the known number of colonies in the standard. A table was constructed

TABLE 3 :

A fully grown culture of Escherichia coli 11229 in GA medium was suitably diluted by a series of logarithmic dilutions so as to have a difference of 0.1 of a $\log_{10}$ unit between successive dilutions. Each of these dilutions was then counted by both pour plate and tube dilution techniques as described in the text. Plate count is an average of three plates and tube count is an average of ten tubes.

T A B L E 3

CALIBRATION OF TUBE COUNT BY PLATE COUNT

DILUTION NUMBER	PLATE COUNT		TUBE COUNT Average No. per ml.
	Average No. per ml.	Log. No. per ml.	
1	27	1.43	26
2	32	1.50	32
3	38	1.58	37
4	43	1.63	42
5	65	1.81	50
6	77	1.88	-
7	113	2.05	-
8	133	2.12	-
9	168	2.25	-
10	211	2.32	-
11	263	2.42	-
12	388	2.58	-
13	491	2.69	-
14	635	2.78	-
15	800	2.90	-
16	1015	3.01	-
17	1206	3.08	-
18	1633	3.21	-
19	1993	3.30	-
20	2426	3.38	-
21	3013	3.48	-
22	3722	3.57	-

FIG. 6 : Viewing box for standard counting tubes.

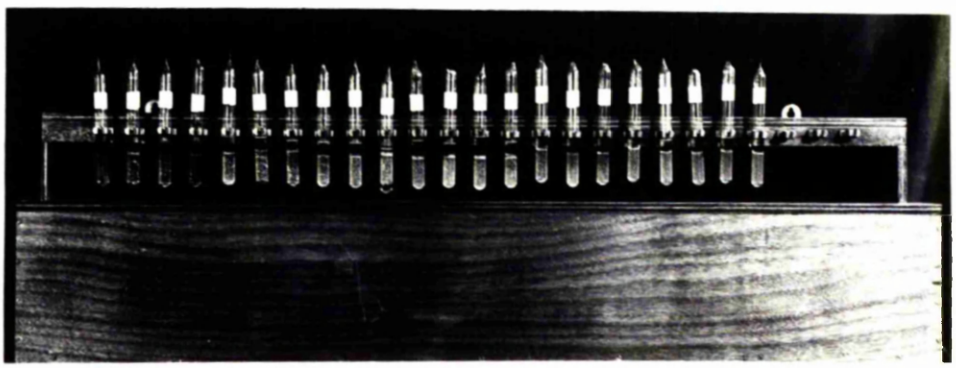


Fig. 6

TABLE 4 : Table for direct reading of viable count by tube dilution method.

Column 2 indicates the number of colonies in each standard tube and the corresponding log values are recorded in column 3. Dilution numbers refer to each successive $^{1}/_{60}$ dilution.

TABLE 4

No. of Tube	No. of Cells	log N	← DILUTION NO. →				
			1	2	3	4	5
1	27	1.43	2.43	4.21	5.99	7.77	9.55
2	32	1.50	2.50	4.28	6.06	7.84	9.62
3	38	1.58	2.58	4.36	6.14	7.92	9.70
4	43	1.63	2.63	4.41	6.19	7.97	9.75
5	65	1.81	2.81	4.59	6.37	8.15	9.93
6	77	1.88	2.88	4.66	6.44	8.22	10.00
7	113	2.05	3.05	4.83	6.61	8.39	10.17
8	133	2.12	3.12	4.90	6.68	8.46	10.24
9	168	2.22	3.22	5.00	6.78	8.56	10.34
10	211	2.32	3.32	5.10	6.88	8.66	10.44
11	263	2.42	3.42	5.20	6.98	8.76	10.54
12	390	2.59	3.59	5.37	7.15	8.93	10.71
13	490	2.69	3.69	5.47	7.25	9.03	10.81
14	614	2.79	3.79	5.57	7.35	9.13	10.91
15	800	2.90	3.90	5.68	7.46	9.24	11.02
16	1015	3.01	4.01	5.79	7.57	9.35	11.13
17	1206	3.08	4.08	5.86	7.64	9.42	11.20
18	1630	3.21	4.21	5.99	7.77	9.55	11.33
19	1993	3.30	4.30	6.08	7.86	9.64	11.42
20	2420	3.38	4.38	6.16	7.94	9.72	11.50
21	3013	3.48	4.48	6.26	8.04	9.82	11.60
22	3720	3.57	4.57	6.35	8.13	9.91	11.69

(Table 4) from which the viable count can be directly read off.

Evaluation of the tube dilution method

The validity of the calibration was tested by determination of viable counts with both the pour plate and tube dilution method. A fully grown culture of E.coli 11229 was suitably diluted to give a wide range of density. Both methods were employed to perform 10 replicate counts on each of several suspensions. Table 5 gives the results by both methods. There is an excellent correlation between the two methods.

All viable counts methods devised so far are not absolutely reliable. Their precision is affected by many factors such as dilution, sampling and technical errors. In the tube count used here, it is clear that dilution errors are considerably reduced because of the low number of dilutions carried out. Five dilution steps at the most are needed as compared to nine or ten with plate count for a fully grown culture. The lowest count that is assessed by tube count is $1/60$ of the maximum density in the calibration series and thus reduces the sampling error. The technical error is also reduced due to the low number of dilutions necessary and the use of automatic pipettes for dispensing the agar and sample.

The other advantage of this method is that it does not require a prediction of the number of cells in the sample. In a study of antibacterials a very wide range of number of cells in the sample is encountered which cannot be predicted beforehand. Therefore this method is very suitable for the enumeration of a culture which

TABLE 5

VIABLE COUNTS OF ESCHERICHIA COLI 11229

BY POUR PLATE AND TUBE METHODS

PLATE COUNT		TUBE COUNT	
Mean ($\log_{10}$)	Standard Deviation	Mean ($\log_{10}$)	Standard Deviation
8.480	± 0.157	8.402	± 0.059
8.371	± 0.056	8.392	± 0.053
8.023	± 0.107	8.035	± 0.154
7.845	± 0.077	7.817	± 0.022
7.747	± 0.098	7.755	± 0.041
7.715	± 0.162	7.662	± 0.056
4.487	± 0.071	4.455	± 0.069
3.685	± 0.034	3.632	± 0.089
3.444	± 0.045	3.510	± 0.032
2.926	± 0.087	2.983	± 0.022

is undergoing rapid changes as found in the quinolone treated cultures.

The increased accuracy and reproducibility of the tube counting method due to larger samples and fewer dilutions together with ease and convenience both in actual operation and counting make this method preferable to all the other standard techniques for measuring viability.

Respiration studies

Standard manometric techniques (Umbreit, Burris & Staufer, 1957) were employed to study rate of oxygen uptake/mgm. protein/hour (Qo_2). Each Warburg vessel received 5 mgm. of bacterial cells suspended in 100 m moles of phosphate buffer (pH 7.0), 25 m moles of glucose in side arm and 0.2 ml. of 20% KOH in the centre well in a total volume of 2 ml. The oxygen uptake was studied at 37°. The Warburg apparatus was supplied by B. Braun Apparatebau, Melsungen, Western Germany, Models V85 and VL85.

In one experiment Warburg flasks contained 150 m moles ammonium sulphate, 15 m moles magnesium sulphate, 25 m moles glucose, 10 m moles phosphate buffer, 0.2 ml. 20% KOH in centre well and 1%, 5% and 10% inoculum cells in the side arm. Rate of oxygen uptake was followed during growth of the cells in the Warburg vessels.

Proteins were measured by the method of Lowry et al (1951).

Biochemical Analysis

The analysis of the cells was carried out by Schneider (1957) fractionation method. The cultures were treated with 5% trichloroacetic acid overnight at 4°. Next day the samples were centrifuged, washed once with water and resuspended at known wet wt./ml. The suspensions were digested with 0.66N NaOH overnight at 30° and proteins estimated in the alkaline digest by Folin Ciocalteu reagent method described by Lowry et al (1951).

Nucleic acids were extracted from the cell suspensions by twice hot 0.5N perchloric acid treatment (70° for 20 minutes). The supernates were combined and DNA was estimated by modified Burton diphenylamine method (Burton, 1956). RNA was estimated by orcinol reaction (Ashwell, 1957).

Materials

All chemicals used were BDH Chemicals of analar grade except trishydroxymethylaminomethane (reagent grade), thymine, thymidine, deoxyadenosine, deoxycytosine and deoxyguanosine which were obtained from Sigma Chemical Company.

The quinolones* were synthesized at the research laboratories of Imperial Chemical Industries (I.C.I.) in Cheshire, England. They were kindly supplied as a gift.

Chloramphenicol was obtained from Parke Davis & Co. Ltd., Hounslow, Nr. London.

Oxygen free nitrogen was obtained from British Oxygen Co., Glasgow.

Footnote:

* Abbreviations used for quinolones:

1. CarboxyQ: N-ethyl-3-carboxy-7-chloro-4-quinolone.
2. NitroQ: 1, 2 dimethyl-6-nitro-4-quinolone.

R E S U L T S

GROWTH INHIBITION BY QUINOLONES

(a) Effect of CarboxyQ on Escherichia coli 11229

Growth of E. coli 11229 in presence of various concentrations of carboxyQ is shown in Figures 7a and 7b. Very low concentrations of carboxyQ (1.25 and 2.5 μM) do not have an appreciable effect on the growth. There is slight inhibition of the rate of multiplication (Figure 7a) but the rate of increase in turbidity is not reduced and in fact it is slightly enhanced (Figure 7b). With higher concentrations (5 and 10 μM) there is no appreciable effect on either cell number or mass up to two hours. After this time with 5 μM the viable count remains more or less constant for another hour. Thereafter there is some fall in viable count and further slowing down of the rate of increase in turbidity. Doubling the concentration to 10 μM results in a rapid decrease in viable count after two hours of incubation. The turbidity is maintained at a constant level after an initial increase up to three hours. Increasing the drug concentration to 20, 40, 80 and 160 μM does not have an additional effect on the rate of killing but they do inhibit the rate of increase in turbidity proportionately (Figures 8a, 8b). It should be noted that a period of growth elapses before even high concentrations of drug exert any effect.

FIG. 7 : Effect of carboxyQ on the growth of *Escherichia coli* 11229.

(a) Viability

(b) Turbidity

o—o Control in GA medium

△—△ GA + 1.25 µM carboxyQ

□—□ GA + 2.5 µM carboxyQ

●—● GA + 5 µM carboxyQ

▲—▲ GA + 10 µM carboxyQ

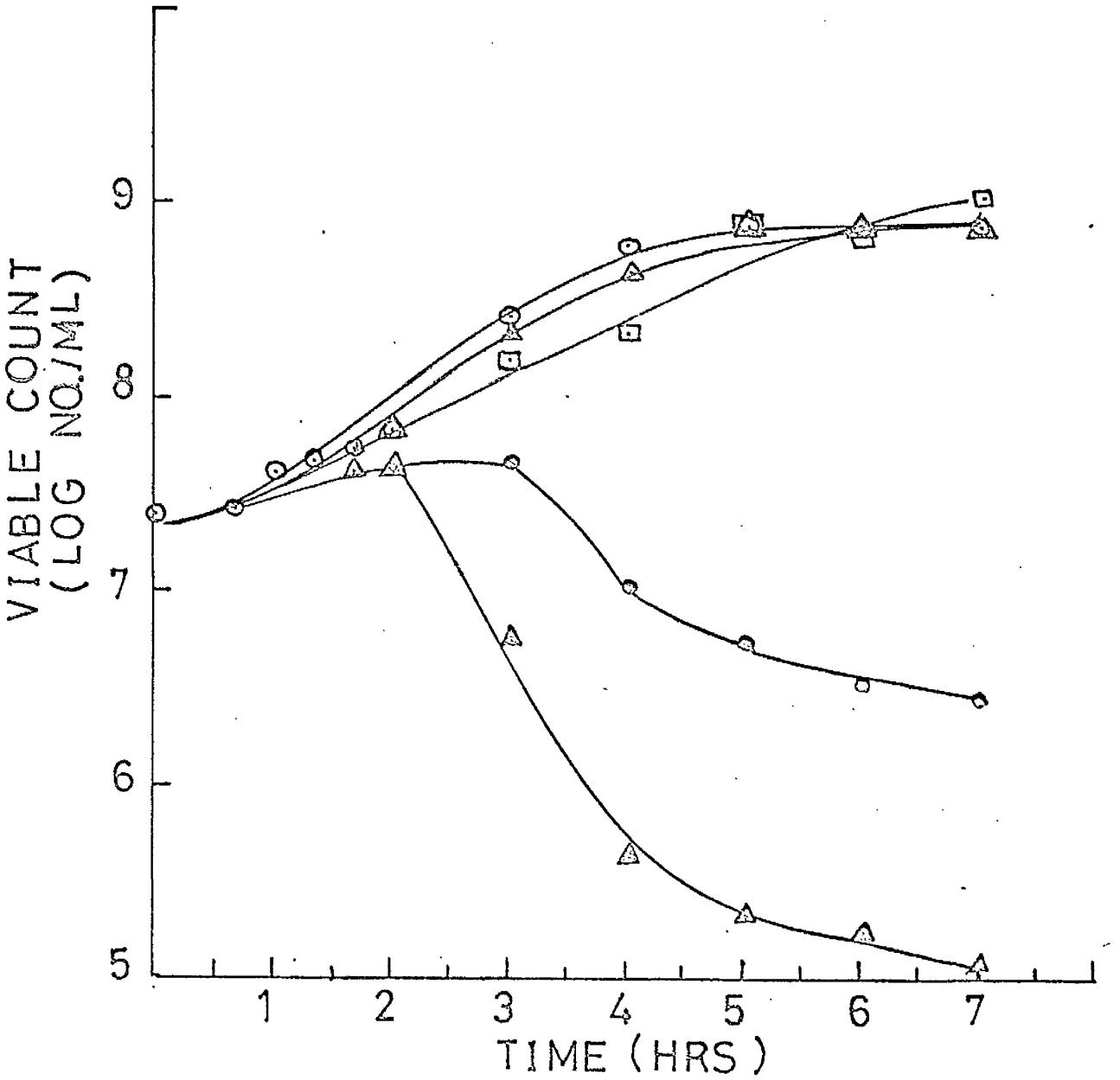


Fig. 7a

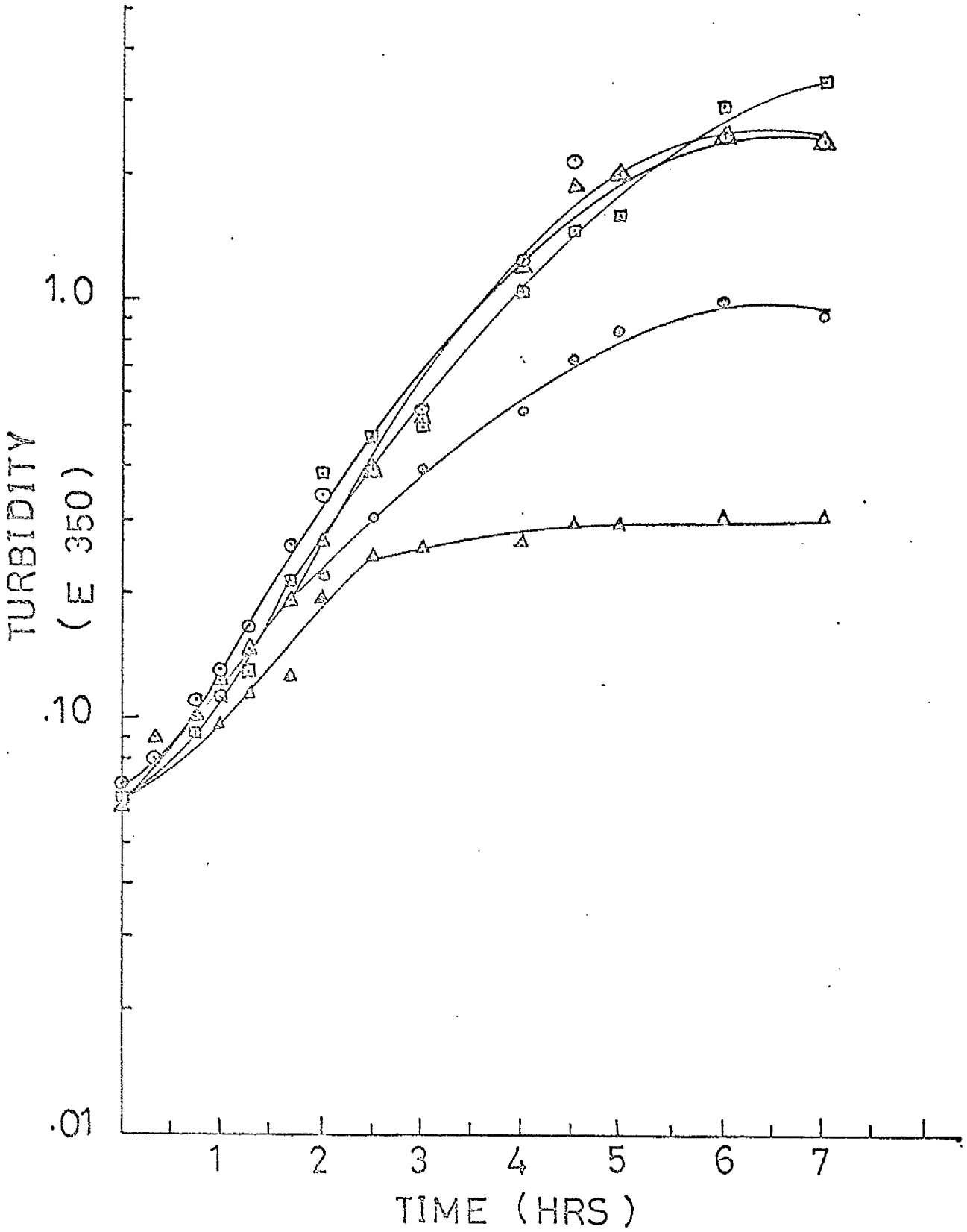


Fig. 7b

FIG. 8 : Effect of high concentrations of carboxyQ on the growth of Escherichia coli 11229.

(a) Viability

(b) Turbidity

o — o Control in GA medium.

Δ — Δ GA + 10 μM carboxyQ

□ — □ GA + 20 μM carboxyQ

● — ● GA + 40 μM carboxyQ

▲ — ▲ GA + 80 μM carboxyQ

■ — ■ GA + 160 μM carboxyQ

NOTE: Double pointed arrows in the viable count plot indicate the range with various concentrations of drug. The dots (●) represent the mean of all the points.

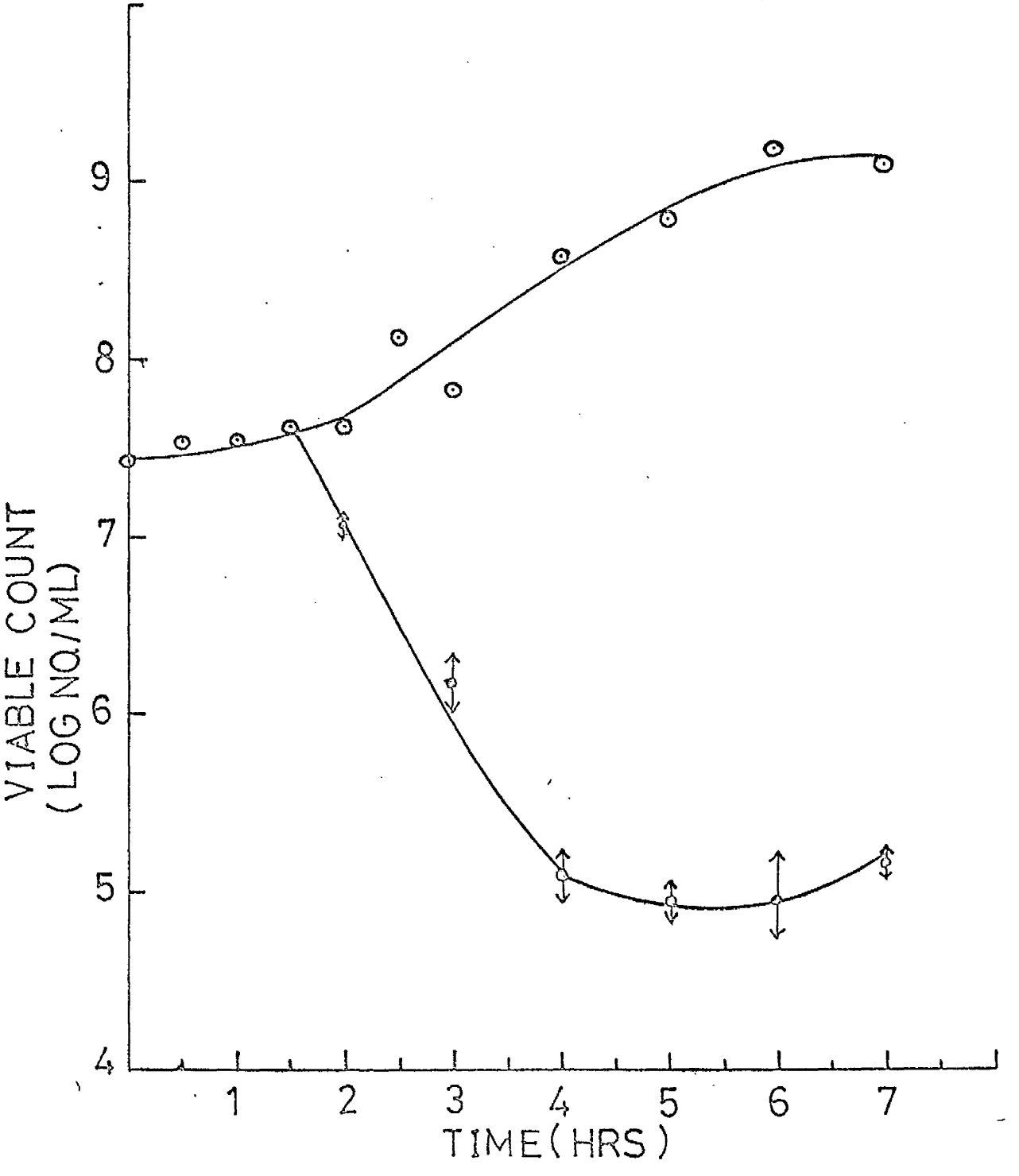


Fig. 8a

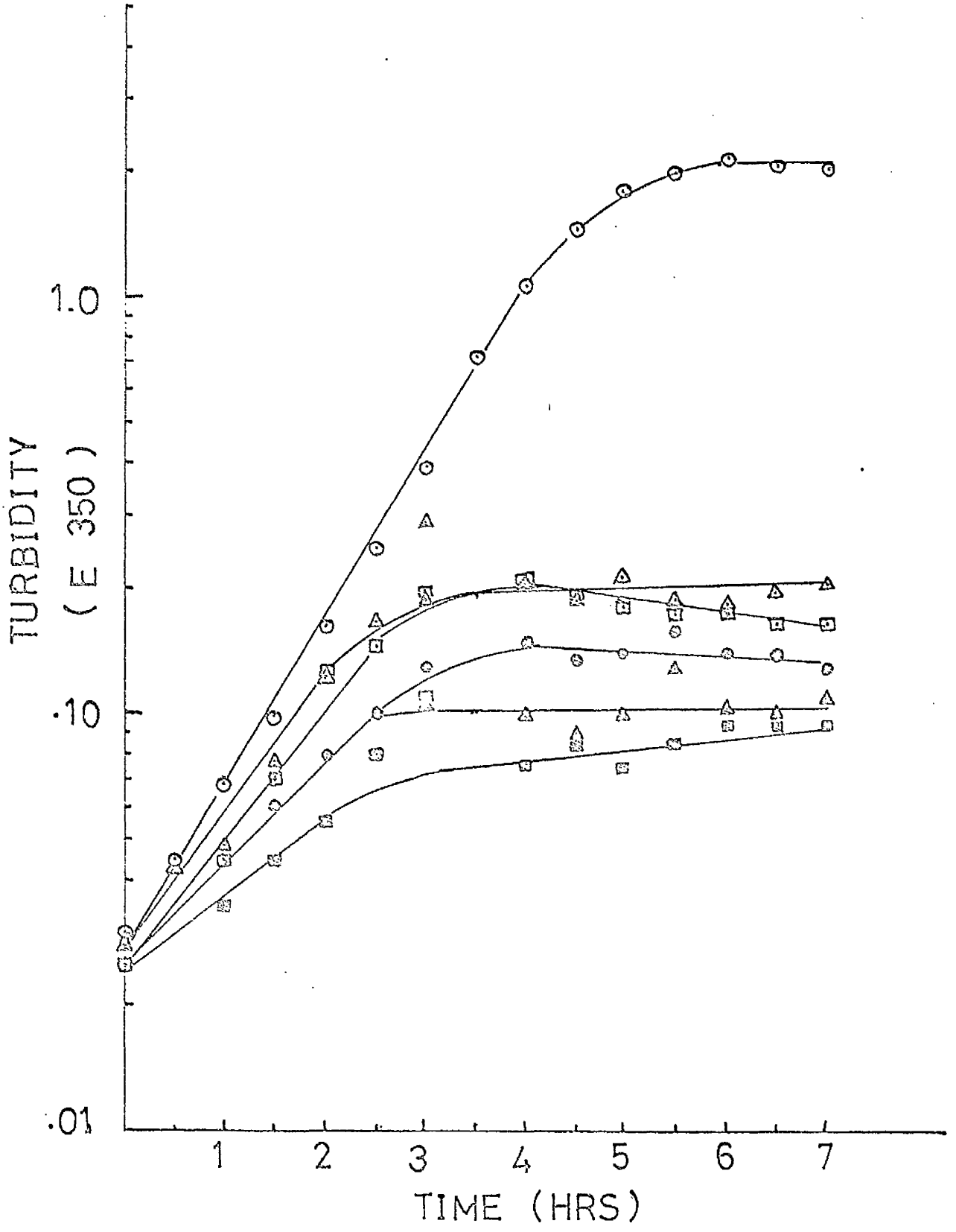


Fig. 8b

(b) Effect of nitroQ on Escherichia coli 11229

Figures 9a and 9b show the effect of 5, 10, 20 and 40 μM concentrations of nitroQ. There is a slight loss in viability (Figure 9a) with 5 μM up to one hour after which the culture shows a steady increase in viable count though at a slower rate than the no drug control. The turbidity curve of 5 μM (Figure 9b) also shows a slower rate of increase up to three hours after which it increases at a higher rate but is lower than the control at the end of experimental period. Doubling the concentration to 10 μM shows an immediate lethal effect up to 40 minutes after which the viable count remains constant up to three hours. At this time the number of viable cells begins to increase. Turbidity also shows a slow increase followed by more rapid increase. Further increase in drug concentration to 20 and 40 μM is rapidly bactericidal for the first hour after which the killing continues at a slower rate up to four or five hours. At about six hours an increase in viable count is observed. The turbidity remains constant with 20 and 40 μM after a slight increase in the beginning. The population that appears at about six hours represents cells that are drug resistant and not merely slow growing cells (refer to section on drug resistance on page 109). The proportion of resistant population in the inoculum varies from experiment to experiment and thus the response to a particular concentration of drug is slightly variable in different experiments. However the rapid loss in viability during the first hour of incubation is always observed and it depends on the length of the lag phase of the control culture. The longer the lag phase, the more pronounced is the initial rapid lethal effect of nitroQ.

FIG. 9 : Effect of nitroQ on the growth of *Escherichia coli* 11229.

(a) Viability

(b) Turbidity

o — o Control in GA

△ — △ GA + 5 µM nitroQ

□ — □ GA + 10 µM nitroQ

● — ● GA + 20 µM nitroQ

▲ — ▲ GA + 40 µM nitroQ

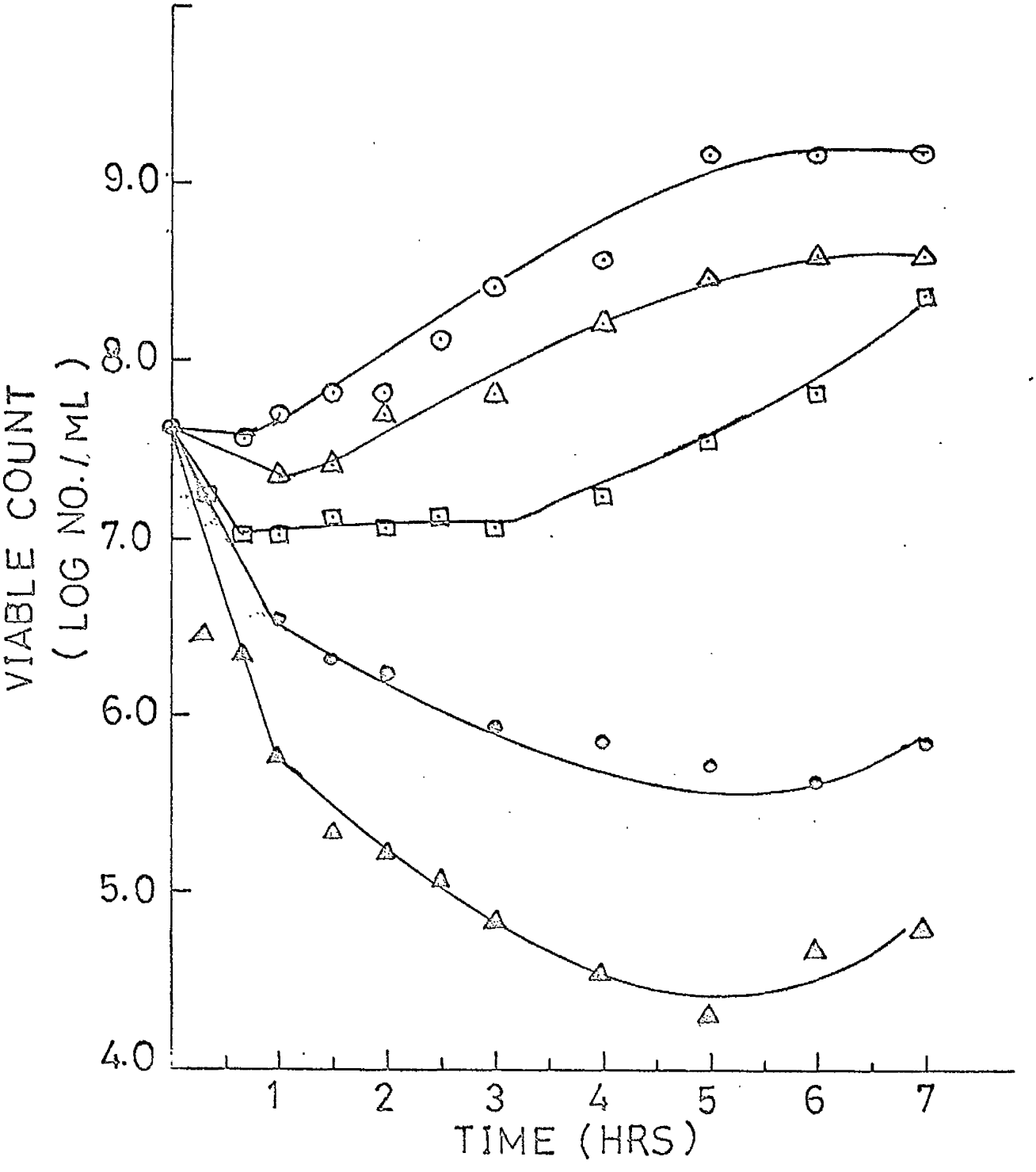


Fig. 9a

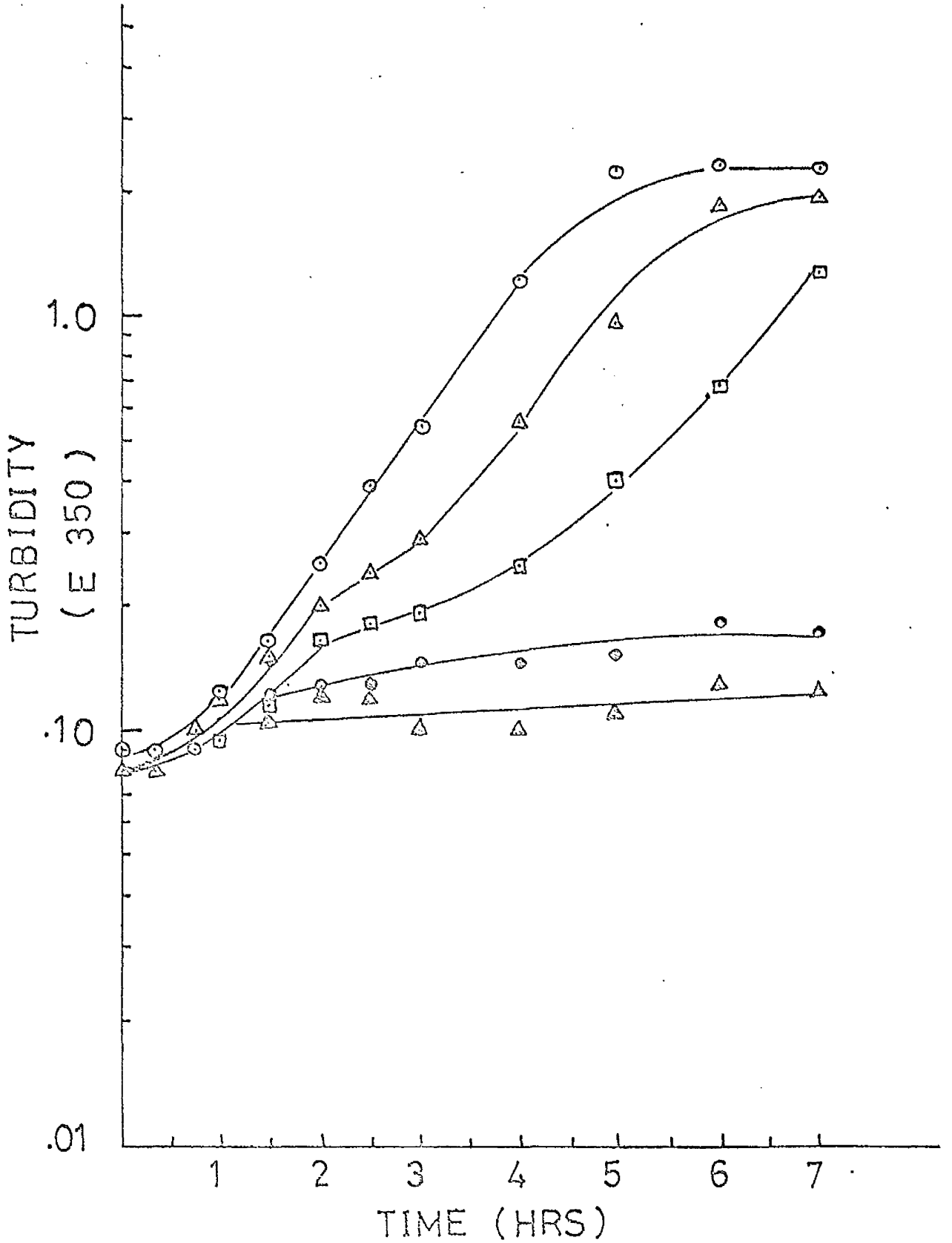


Fig. 9b

With 20 um drug concentration, about 90% of the population is usually nonviable within the first hour of incubation. Higher drug concentrations show a relative increase in the sensitivity of the cultures.

(c) Effect of carboxyQ on Escherichia coli 8114

A range of drug concentrations (2.5, 5, 10, 20 and 40 μM) were employed to study the sensitivity of this strain (Figures 10a, 10b). As with E. coli 11229 2.5 μM is slightly bacteriostatic since it shows an increase in both cell number and turbidity at a slower rate than the control culture. Response to higher concentrations of the drug differs from the response of E. coli 11229. After a period of slight increase in number, there is some loss of viability followed by a period of two hours during which the count remains constant. Then again a rapid loss of viability is observed. This response of E. coli 8114 to higher concentrations of carboxyQ was found to be reproducible. The effect on turbidity, however, is very similar to that observed with E. coli 11229.

FIG. 10 : Effect of carboxyQ on the growth of *Escherichia coli* 8114.

(a) Viability

(b) Turbidity

o — o Control in GA

△ — △ GA + 2.5 μM carboxyQ

□ — □ GA + 5 μM carboxyQ

● — ● GA + 10 μM carboxyQ

▲ — ▲ GA + 20 μM carboxyQ

■ — ■ GA + 40 μM carboxyQ

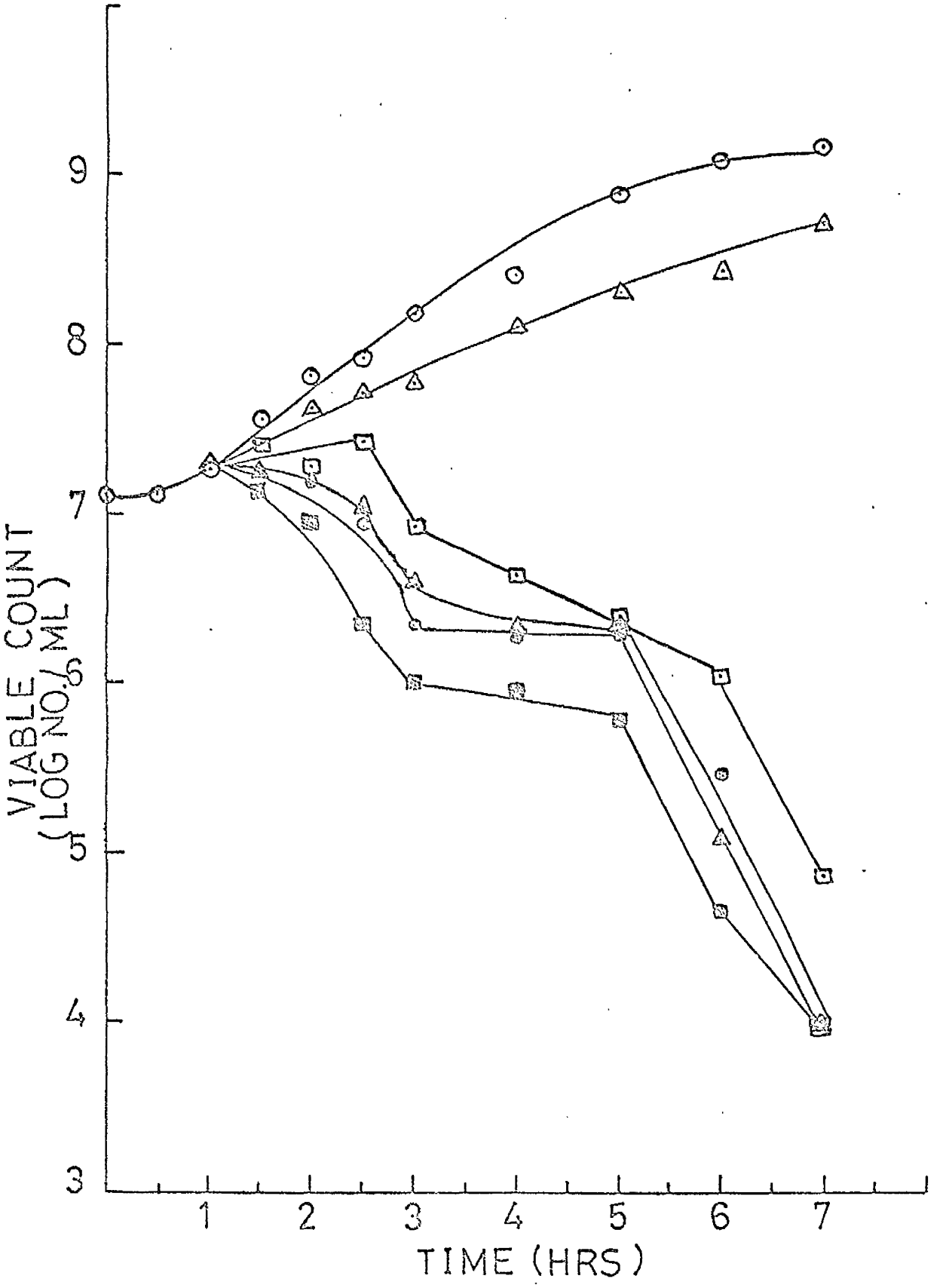


Fig. 10a

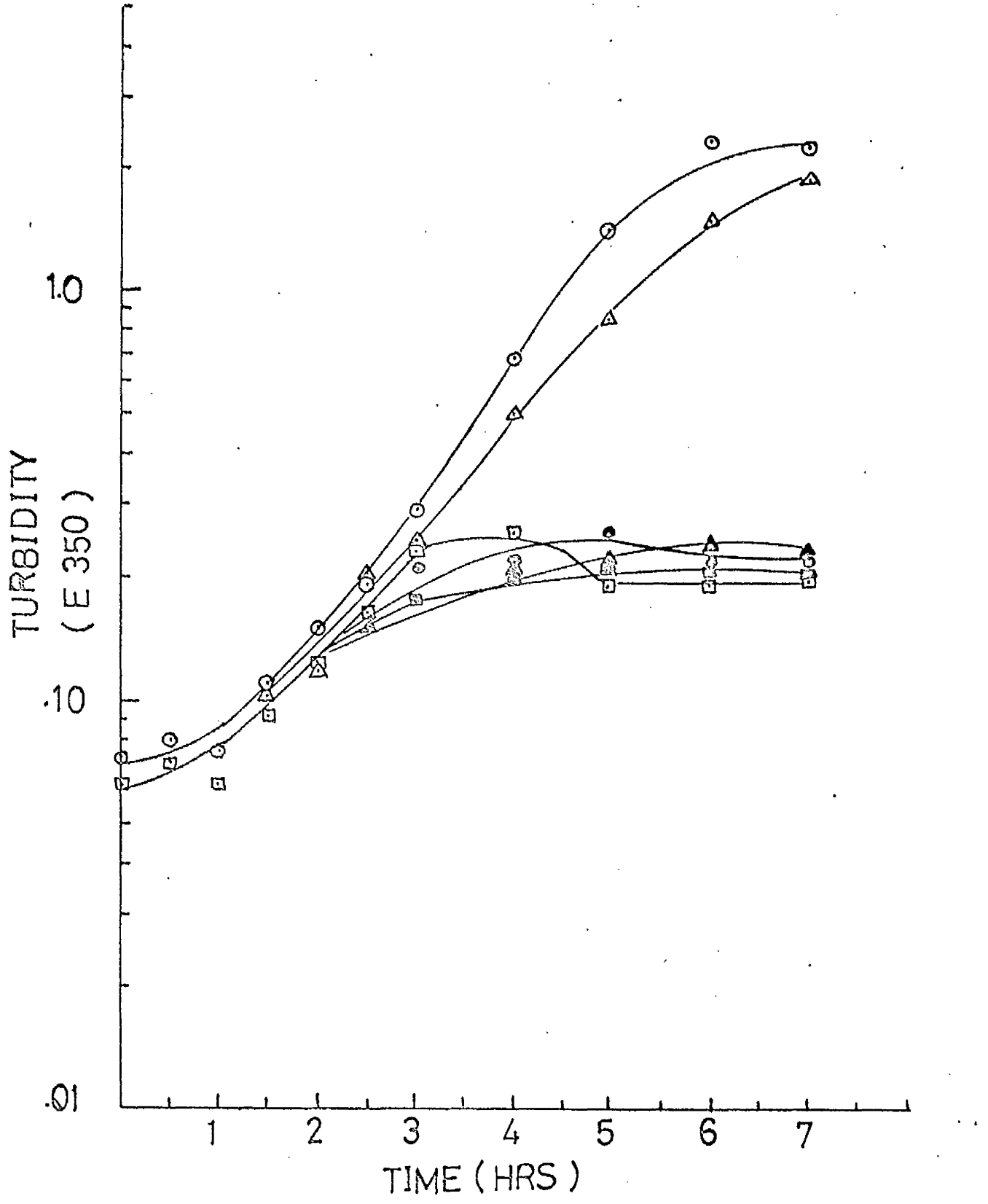


Fig. 10b

(d) Effect of nitroQ on Escherichia coli 8114

This strain is slightly less sensitive to nitroQ as compared to E. coli 11229 (Figures 11a,11b). Lower concentrations (2.5 and 5 μM) show a slower rate of increase in both cell number and turbidity as compared to the control. After seven hours the cell number is almost the same as control but turbidity does not reach the same level. With 10 μM drug concentration the viable count remains more or less constant up to four hours after which it begins to rise. On the other hand the turbidity shows an increase throughout the experimental period though at a slow rate. With 20 μM drug concentration, after a rapid loss in viability up to an hour the viable count shows a slow but steady decrease up to seven hours. Turbidity remains at a constant level from four hours onwards after an initial increase. Higher concentration (40 and 80 μM) show an immediate rapid loss in viability followed by a slower fall up to about five hours when a rise in viable count is observed similar to that observed with E. coli 11229. The turbidity in these cases shows little or no increase throughout the experimental period.

FIG. 11 : Effect of nitroQ on the growth of *Escherichia coli* 8114.

(a) Viability

(b) Turbidity

o — o	Control in GA
△ — △	GA + 2.5 μM nitroQ
□ — □	GA + 5 μM nitroQ
● — ●	GA + 10 μM nitroQ
▲ — ▲	GA + 20 μM nitroQ
■ — ■	GA + 40 μM nitroQ
◑ — ◑	GA + 80 μM nitroQ

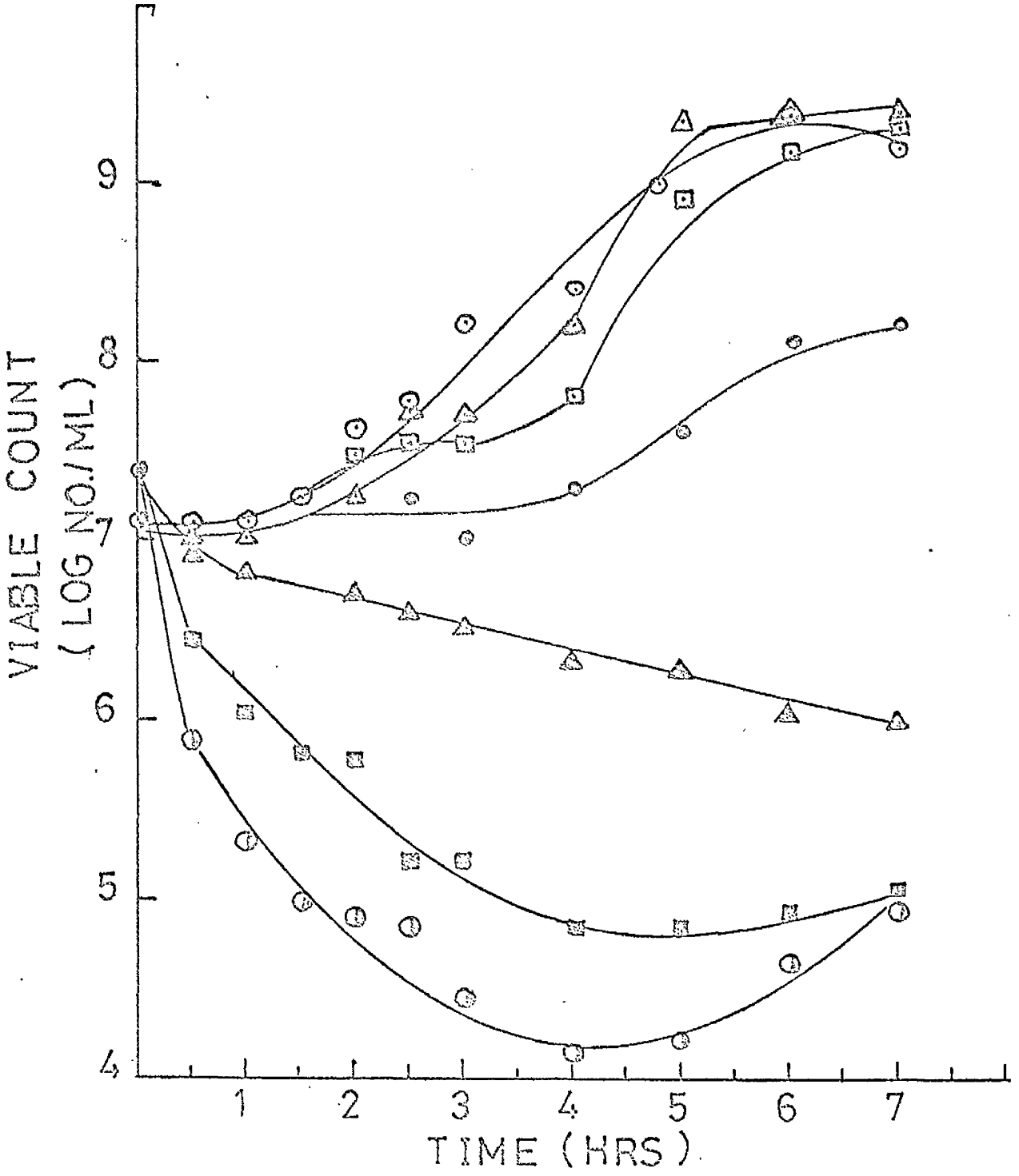


Fig. 11a

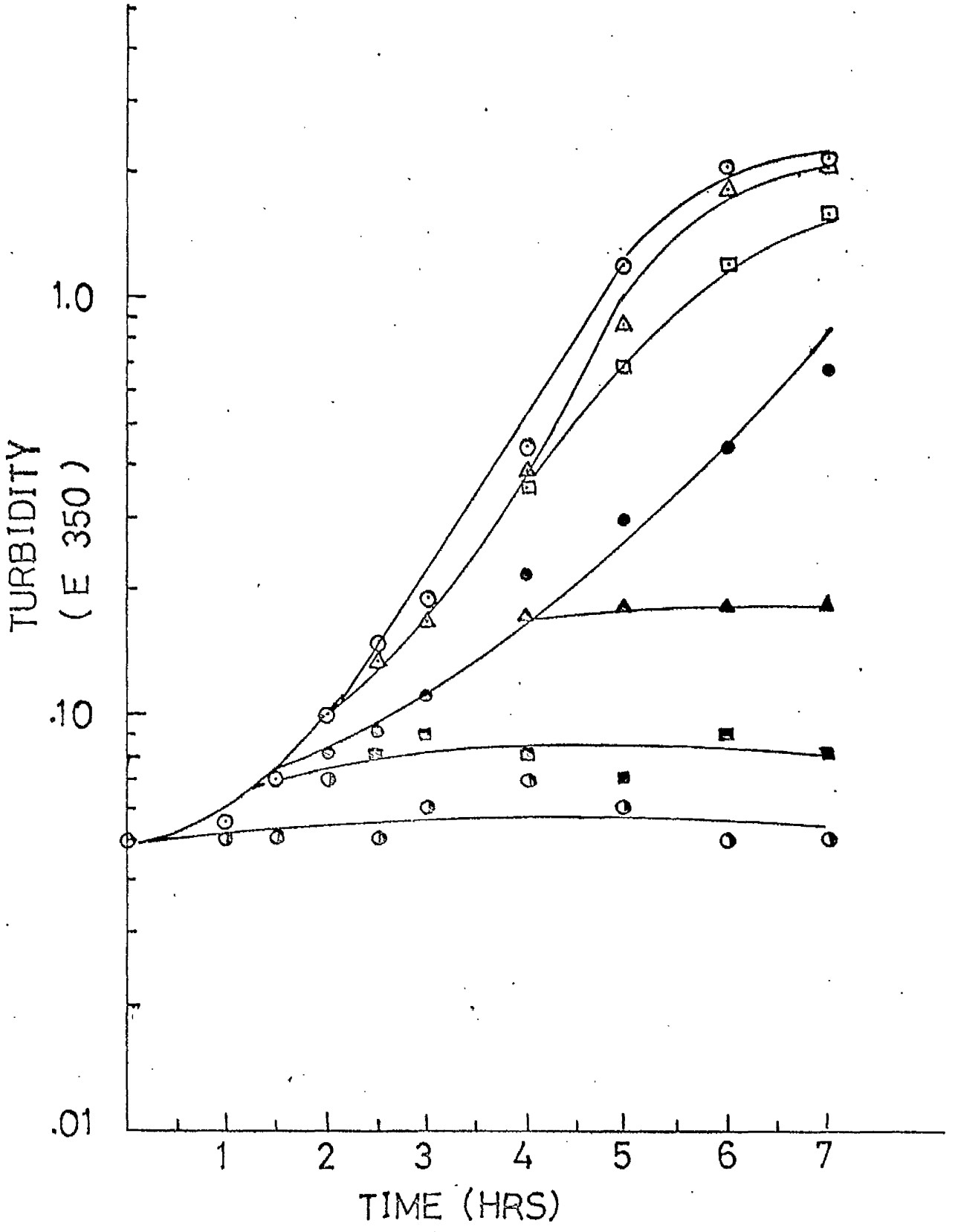


Fig. 11b

(e) Effect of CarboxyQ on Escherichia coli 8583

E. coli 8583 is the thymine auxotroph derived from E. coli 8114. This strain is much more sensitive to carboxyQ than the other two strains (Figures 12a, 12b). At 2.5 μM after a slight loss in viability a slow increase in cell number is observed. Turbidity also increases slowly and after seven hours of incubation is about tenfold less than the control. With 5 and 10 μM the viable count remains constant up to 4 and 2 hours respectively, after which a rapid loss in viability is observed. At still higher concentrations of 20, 40 and 80 μM after a lag of 30 mins., there is a fall in viable count at a slower rate up to 3 hours followed by a higher rate of killing. The turbidity with 5, 10, 20, 40 and 80 μM concentration shows little or no increase during the whole experimental period.

FIG. 12 : Effect of carboxyQ on the growth of *Escherichia coli* 8583.

(a) Viability

(b) Turbidity

o — o Control in GA

△ — △ GA + 2.5 μM carboxyQ

□ — □ GA + 5 μM carboxyQ

● — ● GA + 10 μM carboxyQ

▲ — ▲ GA + 20 μM carboxyQ

■ — ■ GA + 40 μM carboxyQ

◑ — ◑ GA + 80 μM carboxyQ

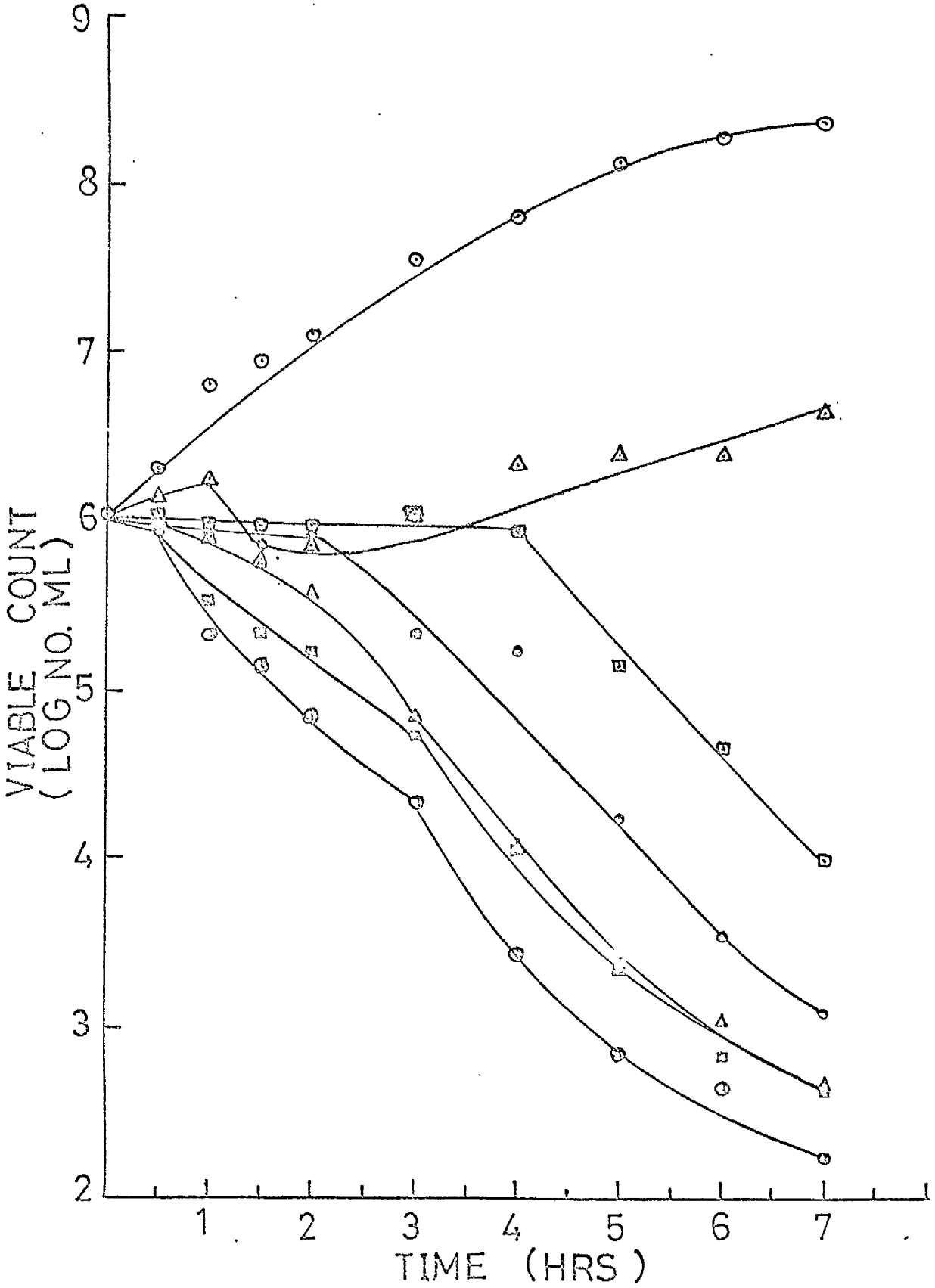


Fig. 12a

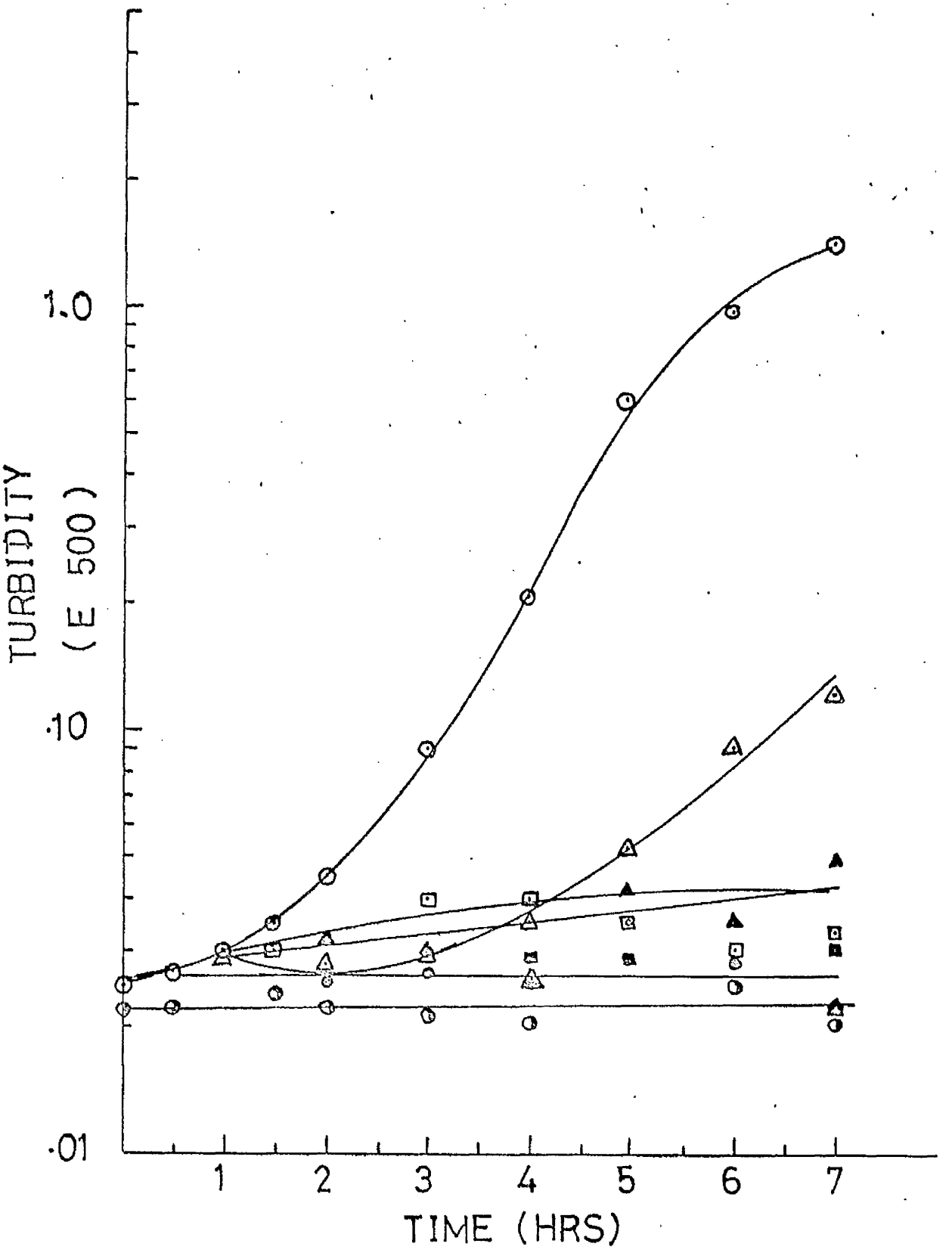


Fig. 12b

(f) Effect of nitroQ on Escherichia coli 8583

Figures 13a and 13b show the effect of 2.5, 5, 10, 20, 40 and 80 μM concentrations of nitroQ on E. coli 8583. At 2.5 μM the viable count remains constant up to five hours after a slight initial increase. After five hours the cell number rises rapidly. During this time turbidity shows a continuous rise though at a slower rate than the control. With 5 and 10 μM, after an initial loss of viability, the viable count increases slowly up to six hours when the cell number suddenly goes up presumably due to the growth of a resistant population. The turbidity also shows a steady increase throughout the experimental period which is inversely proportional to drug concentration. The cultures with 20, 40 and 80 μM show a typical bactericidal curve as observed with E. coli 11229. Here also resistant cells begin to appear at about five or six hours. There is very slight or no increase in turbidity observed with 20, 40 and 80 μM drug concentrations

FIG. 13 : Effect of nitroQ on the growth of *Escherichia coli* 8583.

(a) Viability

(b) Turbidity

o—o	Control in GA
△—△	GA + 2.5 µM nitroQ
□—□	GA + 5 µM nitroQ
●—●	GA + 10 µM nitroQ
▲—▲	GA + 20 µM nitroQ
■—■	GA + 40 µM nitroQ
◑—◑	GA + 80 µM nitroQ

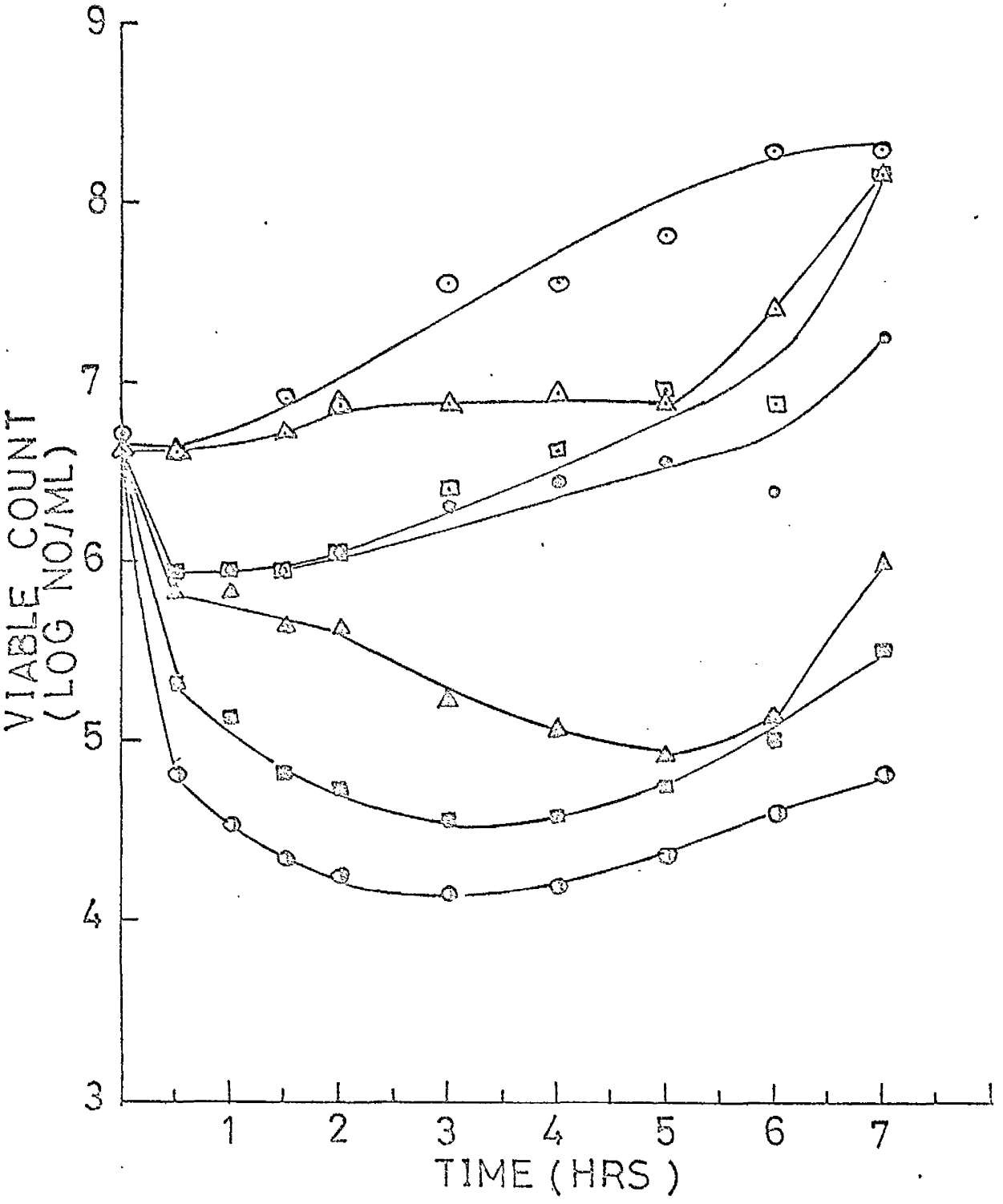


Fig. 13a

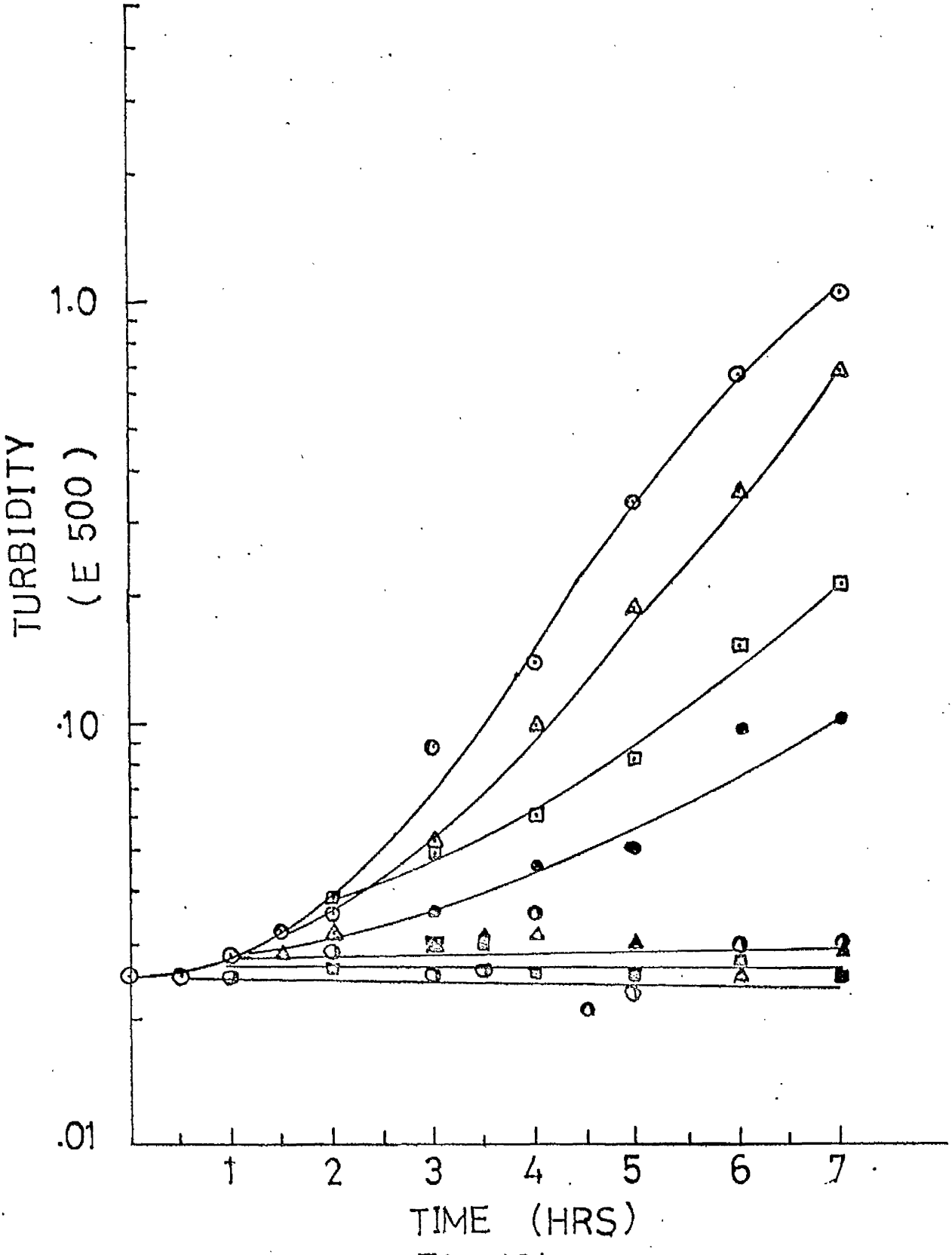


Fig. 13b

Effect of quinolones on the average cell size of Escherichia coli 11229

Both carboxyQ and nitroQ induce the formation of filaments from normal short rodshaped cells of E. coli 11229. Figure 14 shows photomicrographs of young cultures (3 - 4 hours old) grown without drug and in presence of 2.5, 5, 10 and 20 μM carboxyQ and nitroQ. The bacteriostatic concentrations (2.5 and 5 μM) give rise to longer cells as compared to 10 and 20 μM which are bactericidal. The appearance of the filamentous cells is associated with "unbalanced" growth"due to a number of physical and chemical agents. Most of these agents such as UV and X-ray irradiations, mitomycins, hydroxyurea, nalidixic acid, phenethyl alcohol and thymine deficiency give rise to such long forms due to selective inhibition of DNA synthesis while allowing RNA and protein synthesis. At bactericidal concentrations of quinolones the cells do not experience unbalanced growth to the same extent as at lower concentration since most of the population is killed within a short time. With bacteriostatic concentrations the cells are not killed but allowed to grow without any controlled macromolecular synthesis resulting in the formation of long filamentous cells.

FIG. 14 : Morphological changes induced in cells of *Escherichia coli* 11229 by quinolones. Smears were prepared from 4 hr. old cultures, stained with crystal violet and photographed with a Watson phase 60 microscope. Magnification is the same in every case.

(a) CarboxyQ treated cultures.

(b) NitroQ treated cultures.

(A) Control

(B) 2.5 µM drug

(C) 5 µM drug

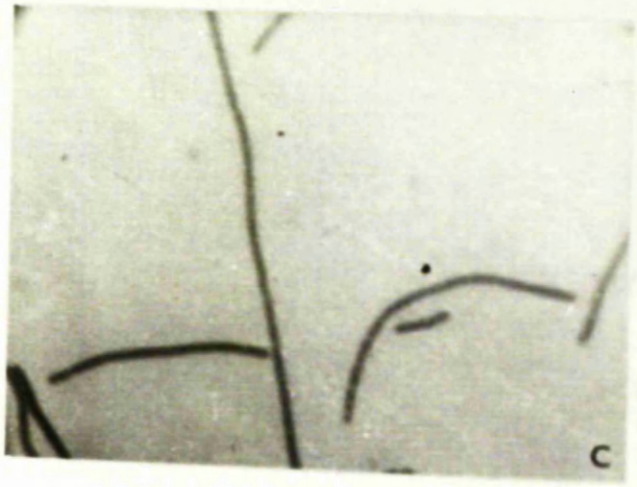
(D) 10 µM drug

(E) 20 µM drug

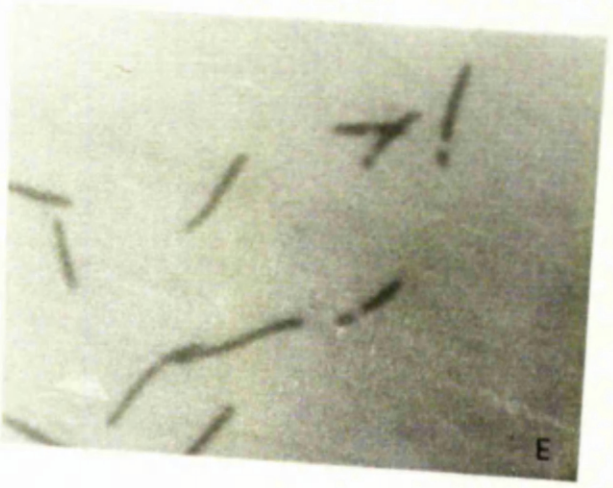
(F) 40 µM drug

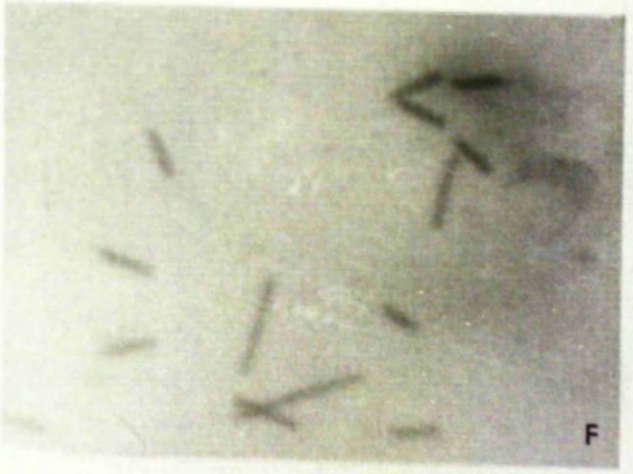

A

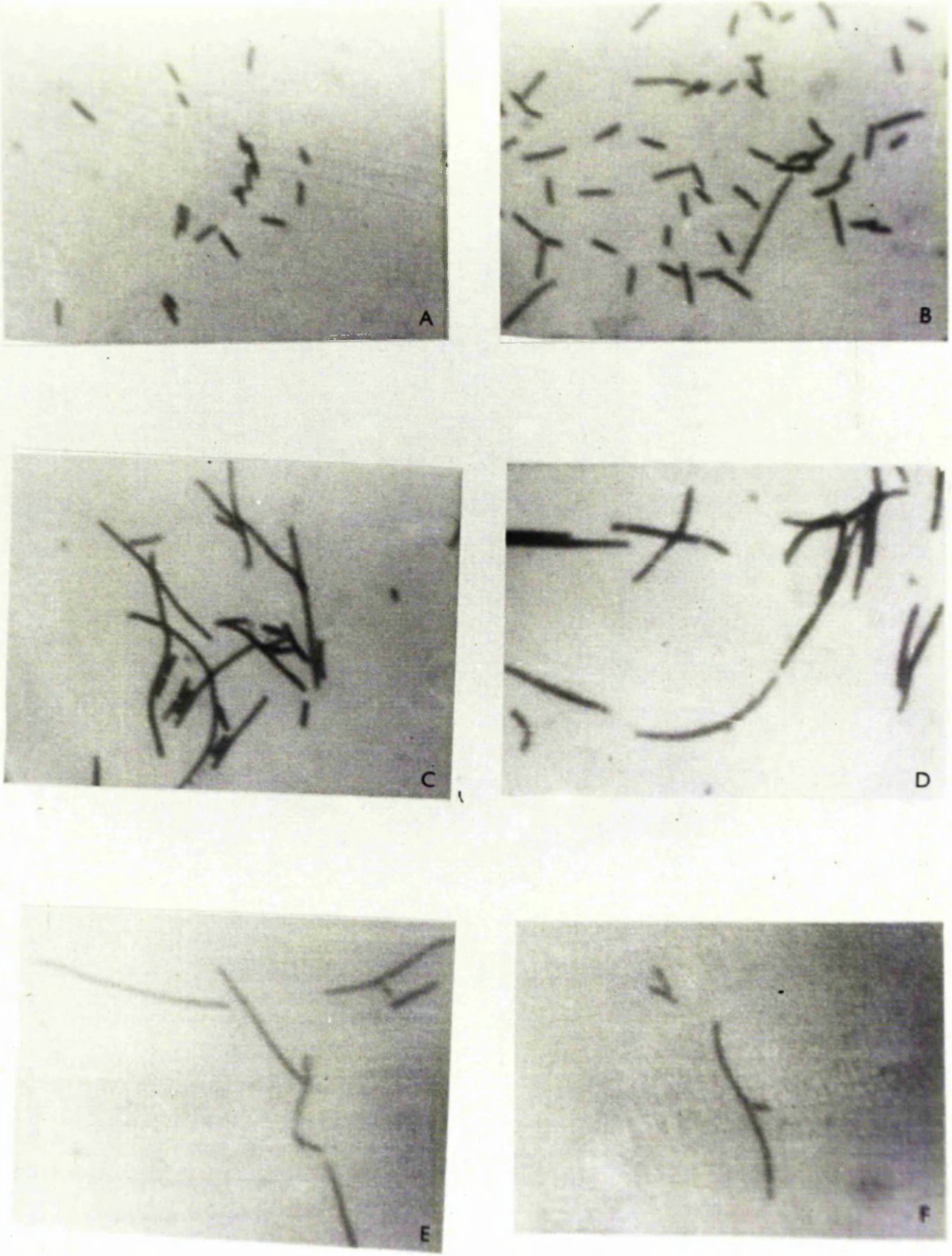

B


C


D


E


F


A
B
C
D
E
F

Size variation during growth of Escherichia coli 11229 with and without quinolones

The average cell size changes during the growth cycle of a bacterial culture. During the lag phase and early log phase the onset of growth is accompanied by a gradual increase in size. The increase in size continues until the cell divides into two. The daughter cells also increase in size and so the number of cells increases with time during log phase. In this phase of active multiplication the average cell size is appreciably increased. When the culture approaches stationary phase, the cells stop to increase in size but divide so that the average cell size is again reduced to the original value.

Quinolones were found to give rise to enormously long filamentous cells. It was interesting to determine the change in average cell size when grown in presence of quinolones. Coulter counter, which measures the cell volume and thus cell size electronically, was used for size distribution studies when E. coli 11229 is grown with and without quinolones. Table 6 and Figure 15 shows the average volume per cell during growth cycle of E. coli 11229 with 0, 2.5, 5, 10 and 20 μM quinolones. The control culture shows a gradual increase in average size until it reaches a maximum at three or four hours which is the middle of log phase. As the culture approaches stationary phase, the size is again reduced gradually to nearly the initial size. The same pattern is observed with 2.5 μM carboxyQ but the average cell size remains higher than the control during later stages of growth. At 5 μM

TABLE 6 :

Escherichia coli 11229 was grown with 0, 2.5, 5, 10 and 20 μM carboxyQ and nitroQ. Samples were taken every hour and total numbers were determined by Coulter Counter, Model F. Average volume per cell was determined by calculating total volume and dividing it by total number in each case.

TABLE 6

Effect of quinolones on average volume/cell of Escherichia coli 11229 during a growth cycle.

TIME (HOUR)	← CONCENTRATION OF CARBOXYQ →									
	0 μM		2.5 μM		5 μM		10 μM		20 μM	
	Total No. $x10^{-7}$	Average Vol/Cell (μ^3)	no. $x10^{-7}$	Average Vol/Cell (μ^3)	Total No. $x10^{-7}$	Average Vol/Cell (μ^3)	Total No. $x10^{-7}$	Average Vol/Cell (μ^3)	Total No. $x10^{-7}$	Average Vol/Cell (μ^3)
Zero	2.27	.679	-	-	-	-	-	-	-	-
1	3.60	.727	3.67	.696	3.24	.709	3.35	.702	3.36	.690
2	6.90	.827	5.87	.722	3.84	1.240	3.53	1.285	3.91	1.180
3	16.80	.976	12.9	2.097	5.96	2.145	4.08	2.227	3.97	1.791
4	46.10	.913	31.9	1.263	7.30	3.284	4.02	2.617	3.93	2.066
5	76.34	.851	56.21	1.151	10.48	5.138	4.09	2.693	3.69	2.007
6	102.14	.752	80.97	.798	9.99	4.606	4.00	2.404	4.11	1.920
7	109.25	.712	94.56	.998	11.09	5.131	4.39	2.619	3.88	1.902

TIME (HOUR)	← CONCENTRATION OF NITROQ →									
	0 μM		2.5 μM		5 μM		10 μM		20 μM	
	Total No. x10^{-7}	Average Vol/Cell (μ^3)	Total No. x10^{-7}	Average Vol/Cell (μ^3)	Total No. x10^{-7}	Average Vol/Cell (μ^3)	Total No. x10^{-7}	Average Vol/Cell (μ^3)	Total No. x10^{-7}	Average Vol/Cell (μ^3)
Zero	2.75	.659	-	-	-	-	-	-	-	-
1	3.20	.735	3.00	.776	-	-	2.68	.825	2.46	.909
2	5.66	.808	4.43	1.102	3.55	1.175	3.03	1.205	2.60	1.032
3	14.70	.924	13.50	.822	7.20	1.142	4.00	1.467	2.22	1.523
4	44.00	.924	32.00	1.020	16.90	1.280	7.50	1.835	3.50	1.590
5	84.80	.837	72.00	.974	46.80	1.053	15.20	1.904	3.45	2.076
6	121.00	.712	75.00	.867	68.00	.829	25.00	1.054	4.77	1.847
7	114.00	.729	77.40	.833	73.00	.822	52.20	.996	4.30	1.137

FIG. 15 : Effect of quinolones on average volume per cell of Escherichia coli 11229 during a growth cycle.

(a) CarboxyQ

(b) NitroQ

o—o	Control in GA
Δ—Δ	GA + 2.5 μM drug
□—□	GA + 5 μM drug
●—●	GA + 10 μM drug
▲—▲	GA + 20 μM drug

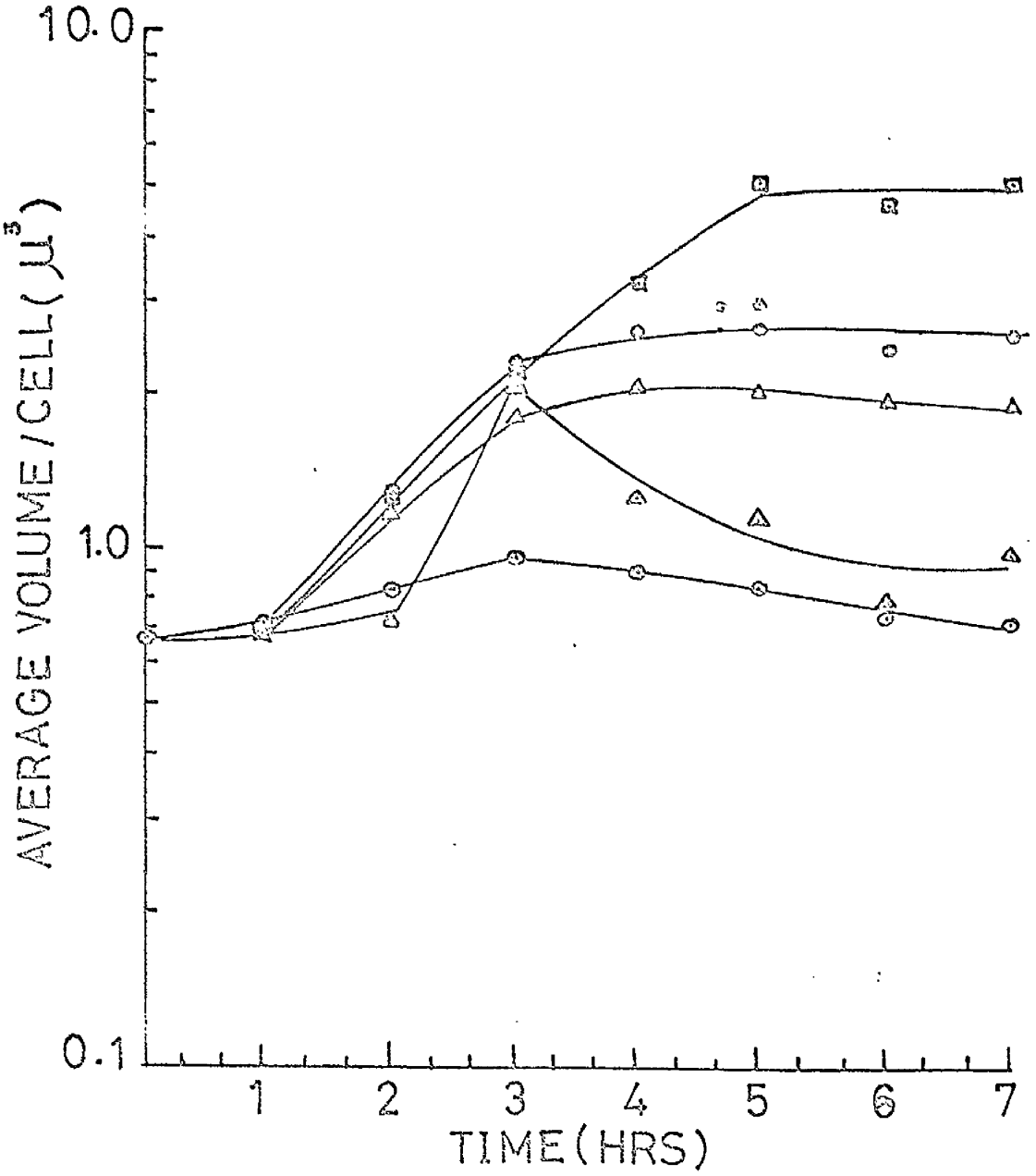


Fig. 15a

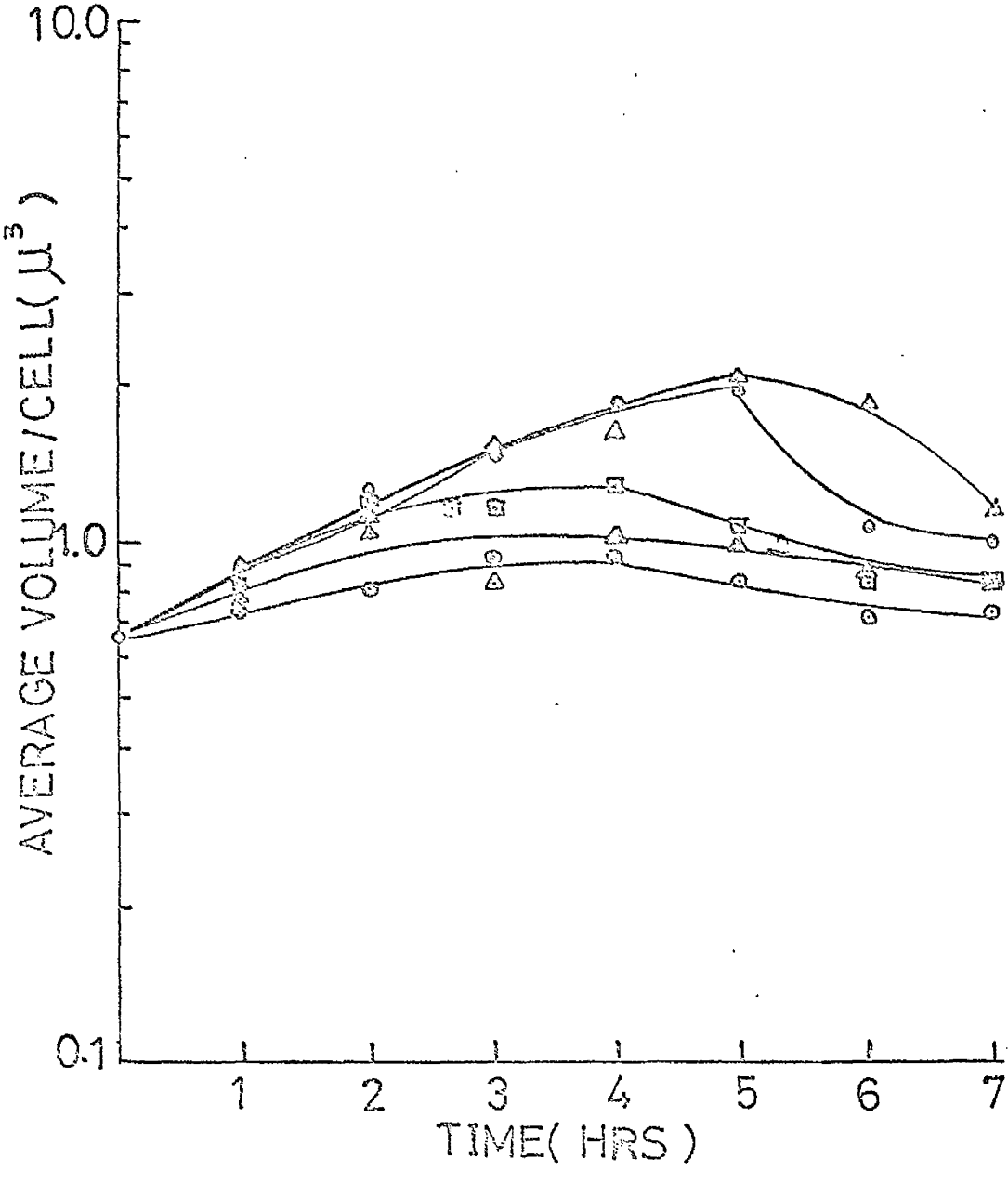


Fig. 15b

concentration of carboxyQ the average cell size increases enormously and does not fall in the stationary phase. The same pattern is observed with 10 and 20 μM but the maximum size achieved is inversely proportional to the drug concentration. At the bactericidal concentrations, the killed population which remains at normal size contributes greatly to the average cell size and thus the overall size does not increase appreciably.

NitroQ also causes an increase in the average cell size but not to the same extent as carboxyQ. Here the maximum size observed is only twice that of control and is proportional to drug concentration (Table 6). There is a sudden decrease in average cell size at about five hours when the viable count generally shows an increase presumably due to growth of resistant cells of normal size.

Influence of cell density on the action of quinolones on Escherichia coli 11229

Figures 16a and 16b show the effect of inoculum size on the action of carboxyQ. A 1% inoculum was used to inoculate flasks with no drug and with 40 μM carboxyQ. Duplicate sets of flasks with 0, 40 and 160 μM drug concentrations were inoculated with 5% and 10% inocula respectively. Flasks with 1% inocula showed a typical response both in viability and turbidity.
The flask with 5% inoculum and no drug showed a slower increase in viable count and turbidity and reached stationary phase earlier than the 1% control. The flasks with 40 and 160 μM and 5% inocula showed no significant difference in the rate of killing but there was a difference in the rate of increase in the turbidity and the final levels. The 10% inoculum control showed slowest rate of increase in cell number and turbidity and also reached stationary phase earliest. In the flasks with 10% inocula and 40 and 160 μM drug fall in viability was proportional to drug concentration. The turbidity remained constant after an initial increase. The loss of viability was observed only where the control culture was growing and there was no further loss of viability after the control culture had reached stationary phase.

The effect of cell density on the action of nitroQ was studied in the following experiment. A fully grown culture of E. coli 11229 was spun down and resuspended at 1/10th of its original volume in fresh CA medium. This thick suspension was then

FIG. 16 : Effect of high cell density on the action of carboxyQ on Escherichia coli 11229

(a) Viability

(b) Turbidity

o—o	Control in GA	1% inocula
△—△	GA + 40 µM carboxyQ	
●—●	Control in GA	5% inocula
▲—▲	GA + 40 µM carboxyQ	
■—■	GA + 160 µM carboxyQ	
◑—◑	Control in GA	10% inocula
◭—◭	GA + 40 µM carboxyQ	
◧—◧	GA + 160 µM carboxyQ	

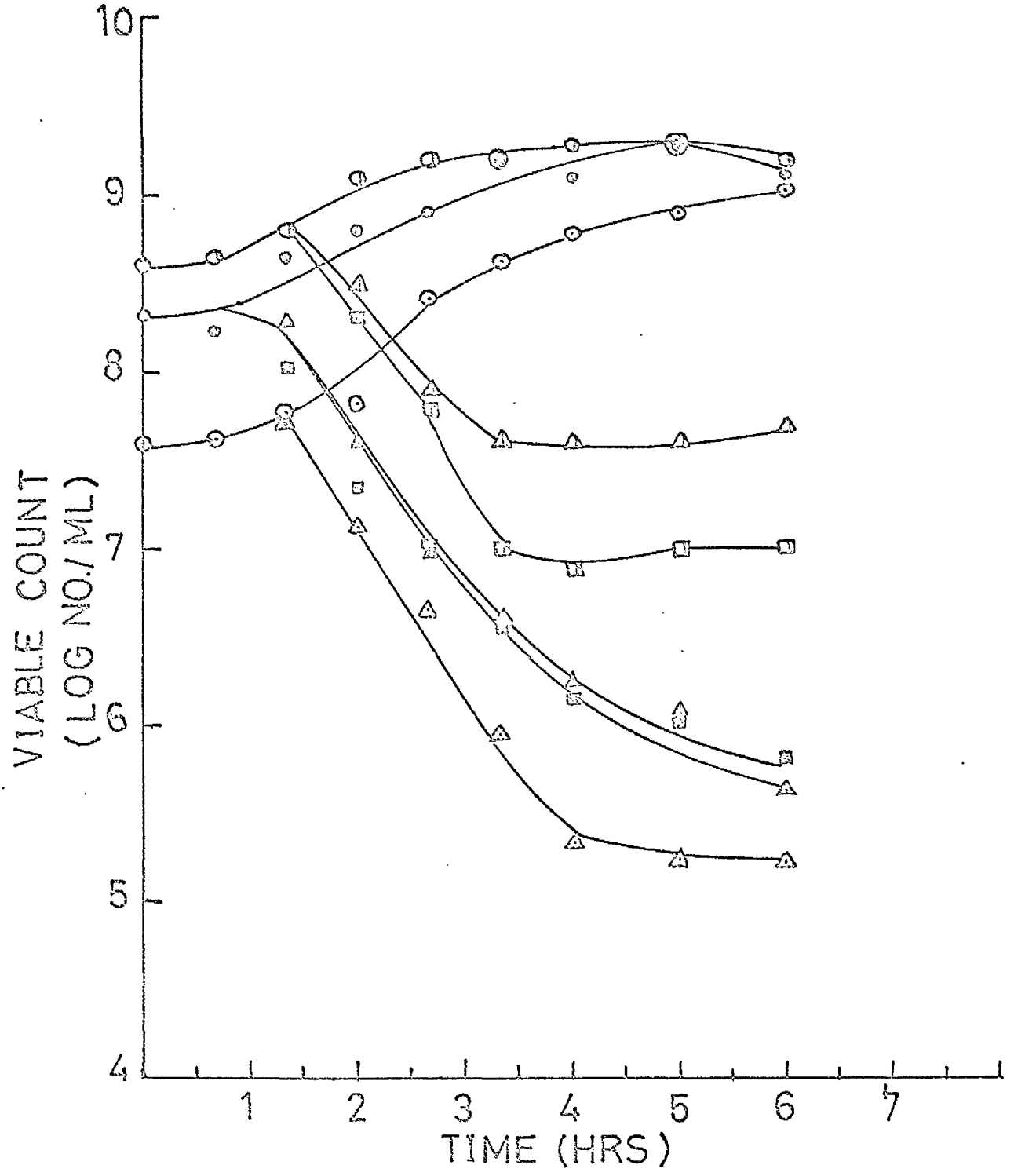


Fig. 16a

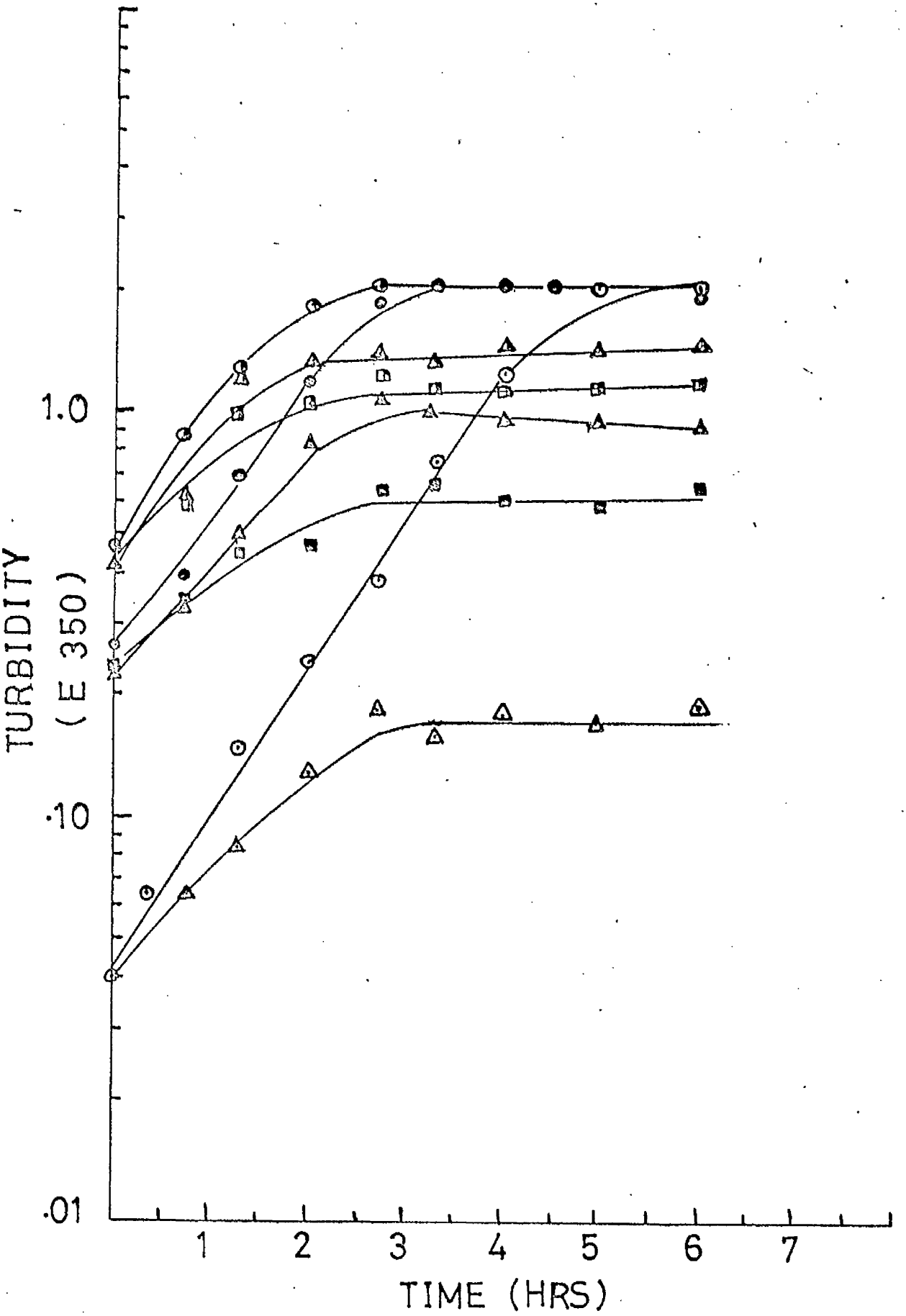


Fig. 16b

inoculated into sets of flasks with no drug and 40 μM nitroQ to give final cell densities, approximately 1%, 10% and 100% of the original culture. Figures 17a and 17b show the growth with and without 40 μM nitroQ at these cell densities. The 1% inoculum showed a typical growth curve in flask without drug. The flask with 40 μM drug showed killing at a uniform rate from zero minutes up to five hours instead of the biphasic response observed when unwashed cells are used as inoculum. The 10% inoculum grows and reaches stationary phase at three hours in the control. In the flask with drug viability followed a more or less typical pattern while the turbidity remained constant after a slight increase. The culture with the same density as the inoculum (100% cell density) and no drug showed a slight increase in the viable count and turbidity up to about two hours. No change was observed during the rest of the experimental period. The culture with 40 μM nitroQ after a slight initial fall in viable count showed some increase in cell number before being maintained at a constant level. No effect on turbidity was observed.

FIG. 17 : Effect of high cell density on the action of nitroQ on Escherichia coli 11229.

(a) Viability

(b) Turbidity

□—□ Control in GA

■—■ GA + 40 µM nitroQ

1 % inocula

△—△ Control in GA

▲—▲ GA + 40 µM nitroQ

10% inocula

○—○ Control in GA

●—● GA + 40 µM nitroQ

100% inocula

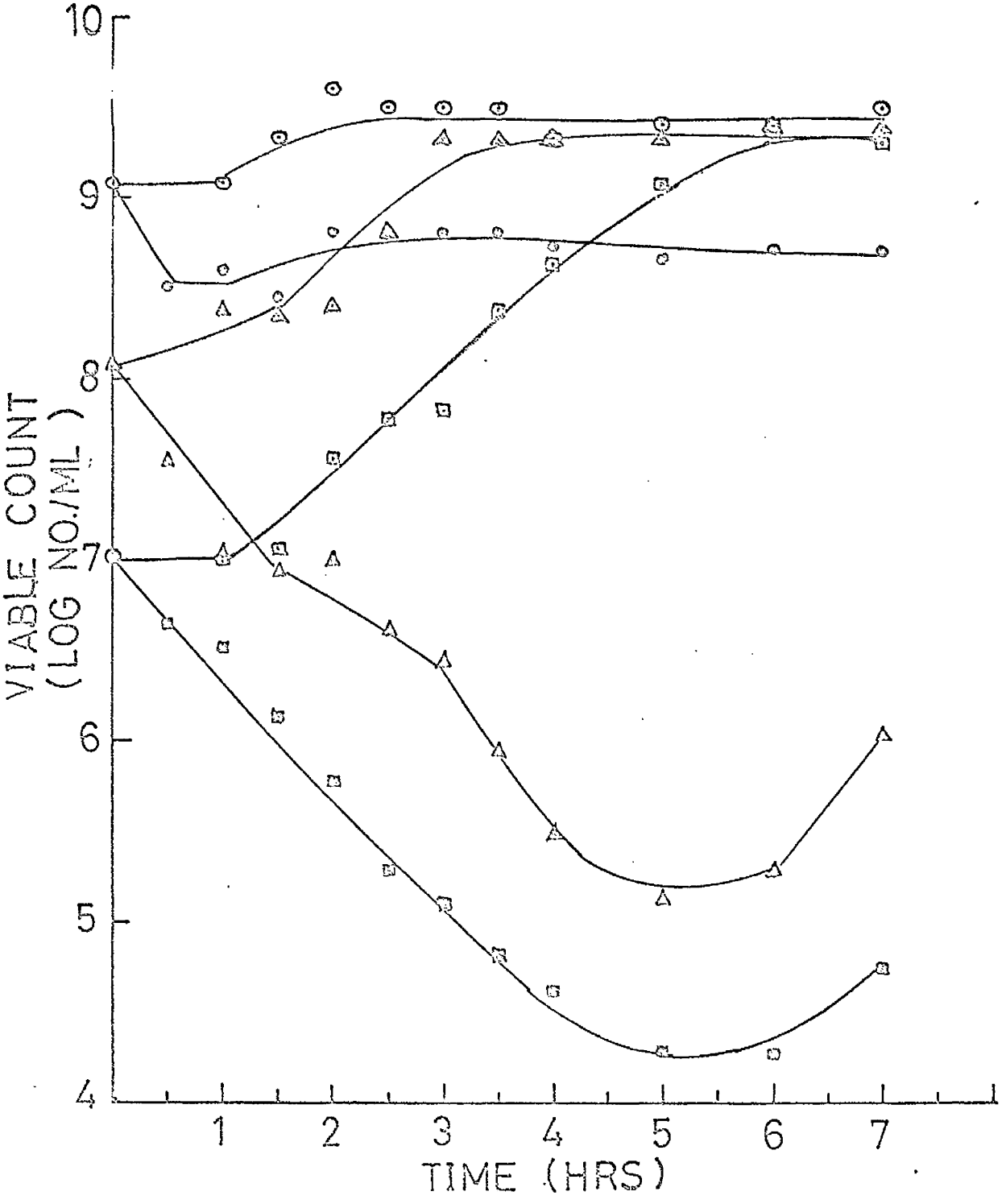


Fig. 17a

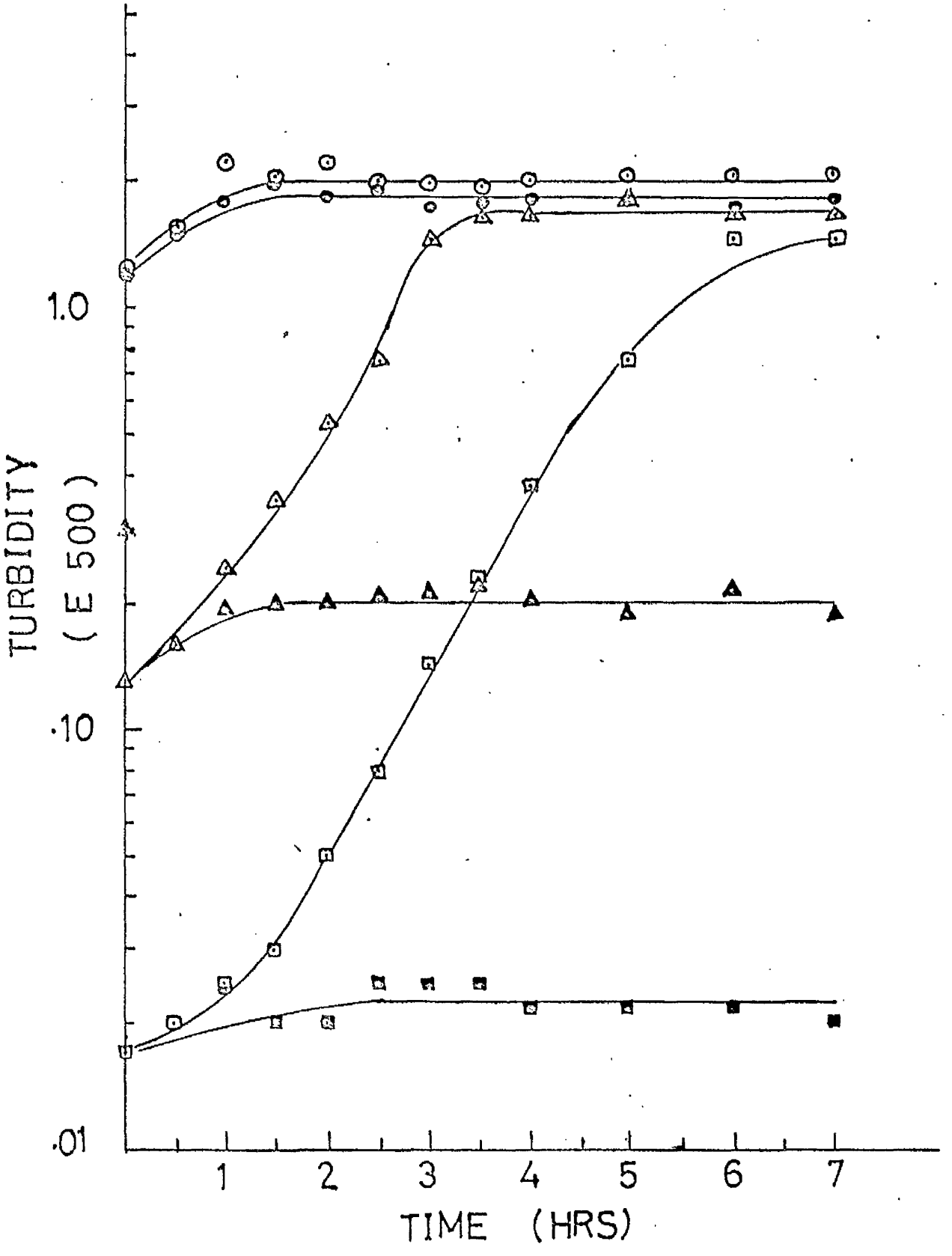


Fig. 17b

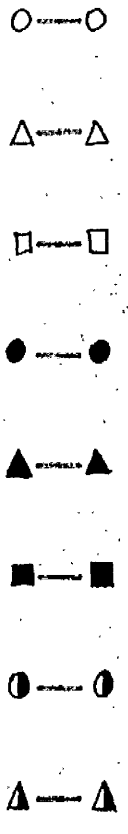
Effect of nitroQ at different times during the initial stages of growth of Escherichia coli 11229

To test the possibility that sensitivity to nitroQ varies with different stages of growth and not the density of the culture, 20 μM nitroQ (final concentration) was added to replicate flasks of cultures growing without drug (Figures 18a and 18b). The no drug control after a lag phase of 30 minutes to an hour entered the logarithmic phase and stationary phase was reached at about six hours. The drug was added at 30 minutes intervals from zero to three hours. There was very little difference in the response of the cultures when drug was added at zero or 30 minutes. Both viability and turbidity followed more or less the same pattern. When the drug was added later than 30 minutes up to three hours, there was a progressive loss of sensitivity. All the cultures to which drug was added later than 30 minutes started to proliferate after a time lapse which was inversely proportional to the time of addition of drug. This suggests that the cells are most sensitive to nitroQ during the lag phase. When the cells enter the logarithmic phase the sensitivity to quinolones is progressively reduced according to the age of cells.

FIG. 18 : Effect of nitroQ during the initial stages of growth of Escherichia coli 11229.

(a) Viability

(b) Turbidity



Control in GA

GA + 20 µM nitroQ at 0′

GA + 20 µM nitroQ at 30′

GA + 20 µM nitroQ at 1.00′

GA + 20 µM nitroQ at 1.30′

GA + 20 µM nitroQ at 2.00′

GA + 20 µM nitroQ at 2.30′

GA + 20 µM nitroQ at 3.00′

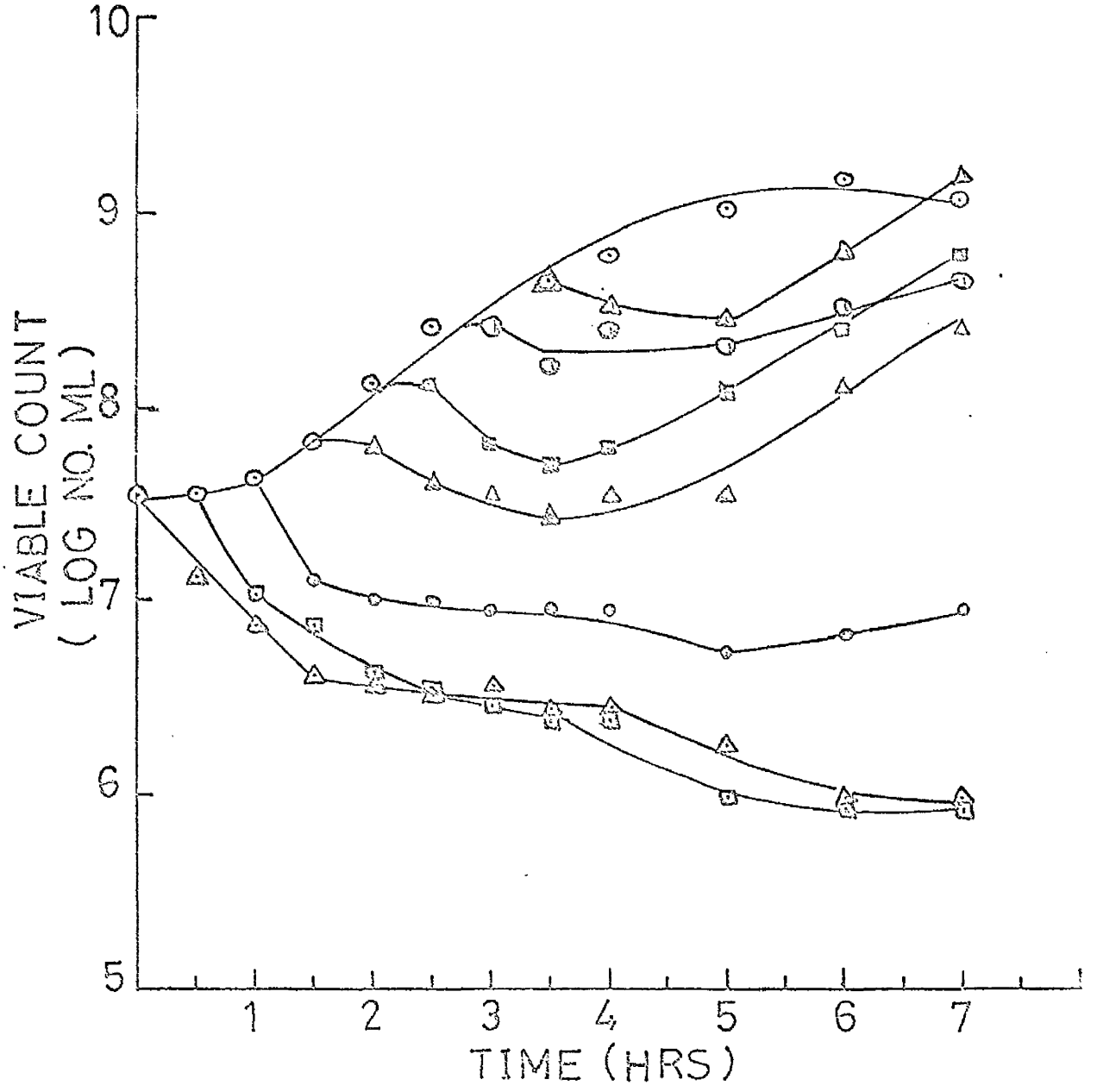


Fig. 18a

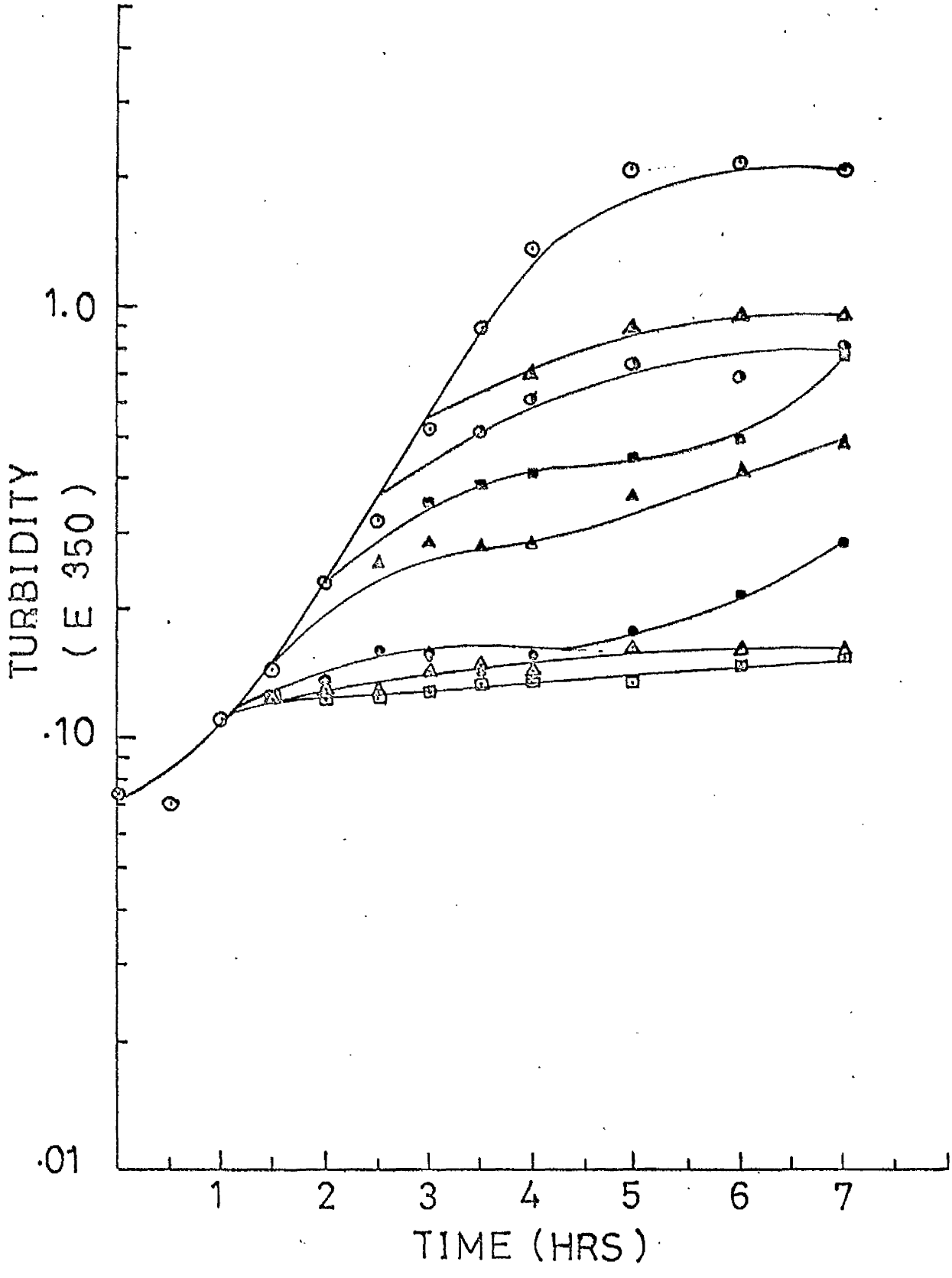


Fig. 18b

Effect of inhibition of protein synthesis on the action of quinolones on Escherichia coli 11229

The carboxyQ is not active during the lag phase and fall in viable count is observed only after a period of growth. On the other hand, nitroQ is more active in the initial stages of growth cycle when the cells are not dividing. Chloramphenicol inhibits protein synthesis and thus growth of sensitive cells. In order to see the effect of inhibition of protein synthesis, 20 µg/ml chloramphenicol was added to one portion of cultures which had been exposed to either carboxyQ or nitroQ for two hours. Figures 19a and 19b show the result of such an experiment. This concentration of chloramphenicol is only bacteriostatic. Addition of chloramphenicol reduced the activity of carboxyQ appreciably. This suggests that carboxyQ requires protein synthesis for full activity. Addition of chloramphenicol to nitroQ treated culture did not reduce the activity but in fact slightly enhanced it suggesting that protein synthesis is not indispensible for the activity of nitroquinolones.

FIG. 19 : Effect of chloramphenicol on the action of quinolones on Escherichia coli 11229.

(a) Viability

(b) Turbidity

o —o	Control in GA
●—●	GA + 20 µg/ml chloramphenicol (CAP)
△—△	GA + 20 µM carboxyQ
▲—▲	20 µg/ml chloramphenicol added at 2.00′ to the above
□—□	GA + 20 µM nitroQ
■—■	20 µg/ml chloramphenicol added at 2.00′ to the above

Arrow indicates the time of addition of chloramphenicol.

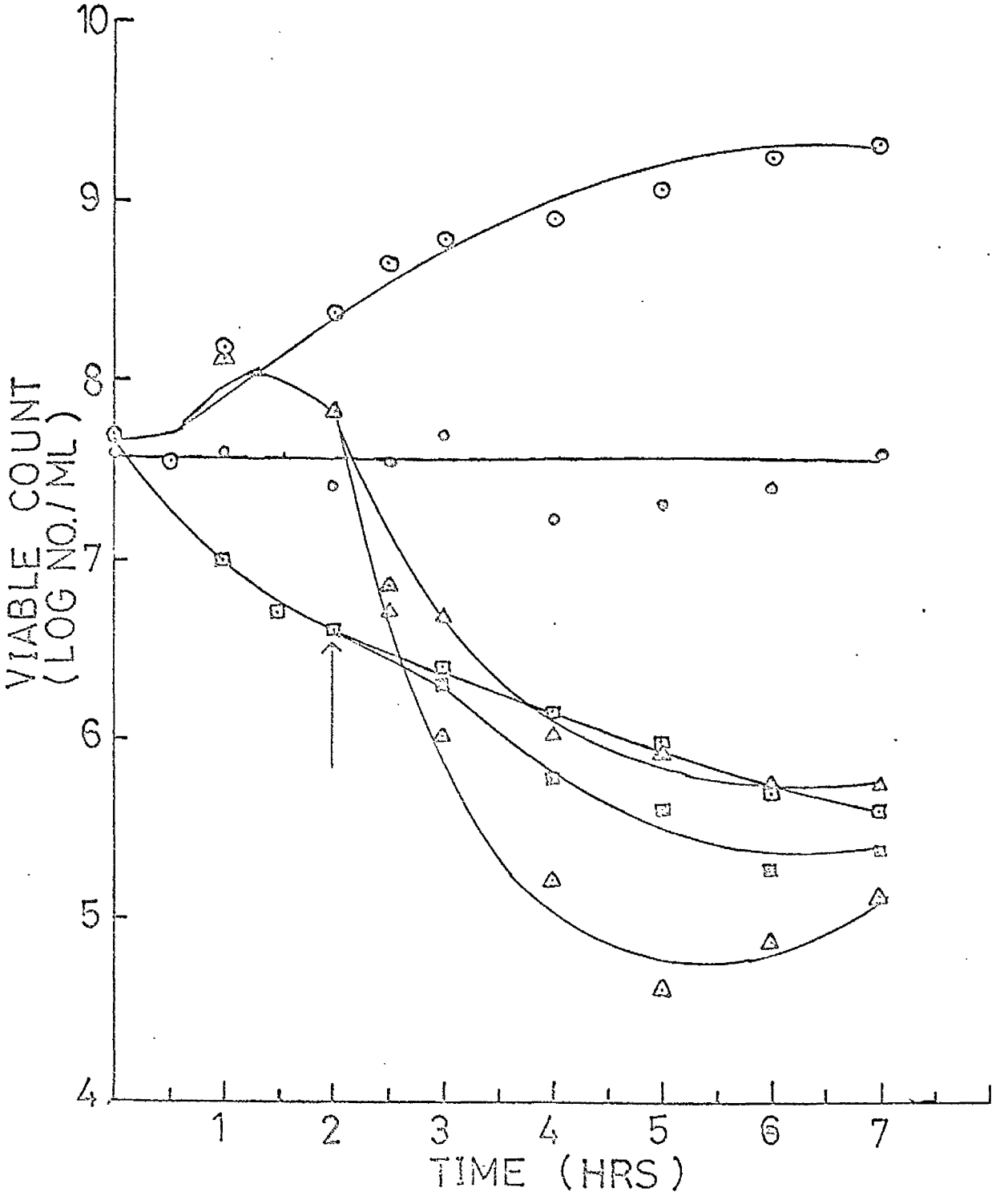


Fig. 19a

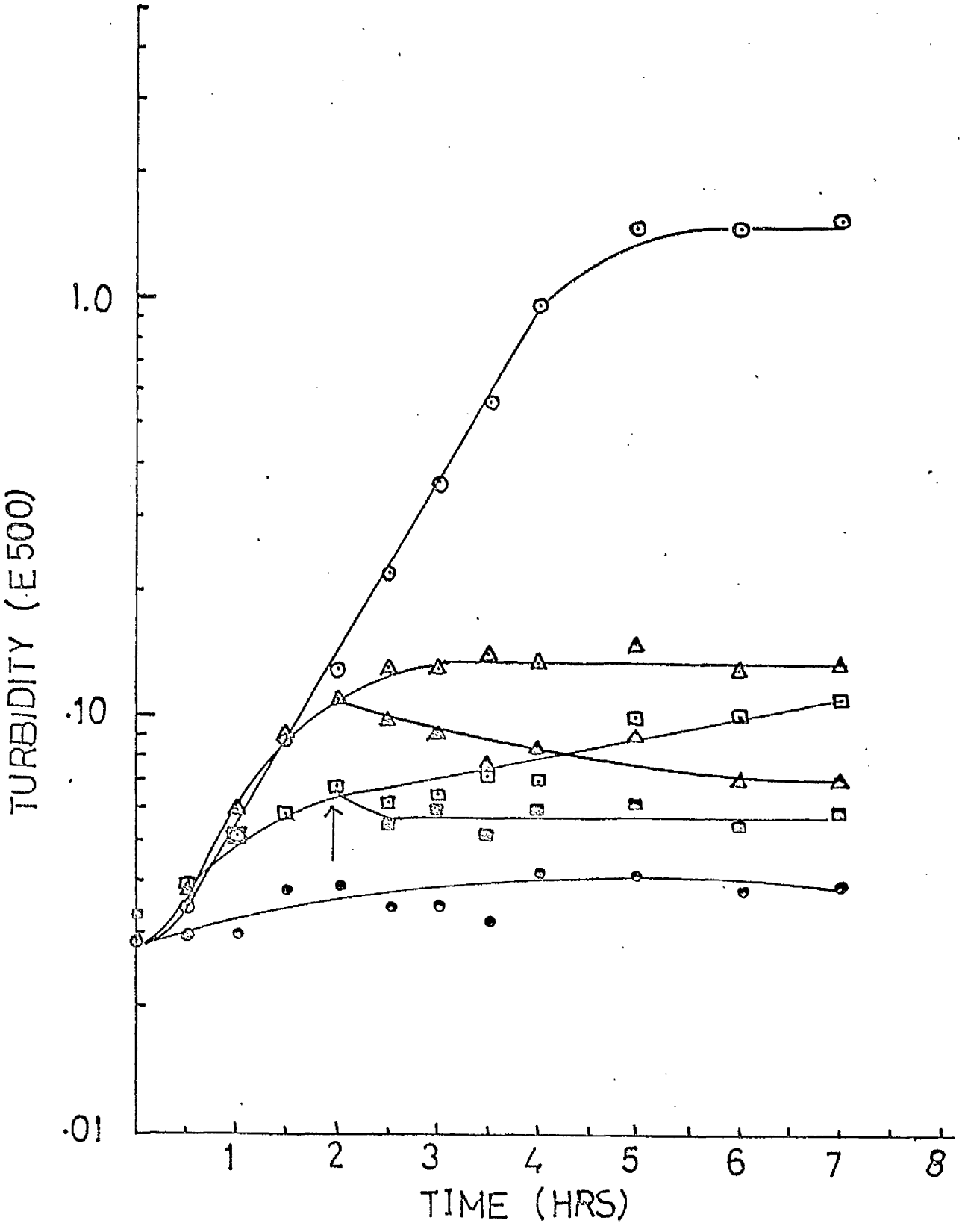


Fig. 19b

Effect of aeration conditions on the action of quinolones on Escherichia coli 11229

Growth of E. coli under anaerobic conditions occurs after a long lag and with a reduced rate of growth. The activity of carboxyQ is manifested only when the cells start to grow. On the other hand nitroQ is more active during lag phase. It was interesting to see whether the long lag and reduced rate of growth under anaerobic conditions affect the activity of quinolones or not. Anaerobic conditions were maintained by passing nitrogen at the rate of 300 - 400 cc. per minute. Figures 20a and 20b show the effect of 20 μM quinolones under both aerobic and anaerobic conditions. The aerobic cultures showed more or less a typical response both in respect of viability and turbidity. The control under anaerobic conditions showed a lag of about three hours as compared to 30 minutes or an hour lag under aerobic conditions. The mean generation time was also increased from 45 minutes aerobically to about 100 minutes anaerobically. No appreciable difference was noted in the sensitivity to nitroQ under aerobic and anaerobic conditions. However, carboxyQ was completely without effect during the three hour lag phase anaerobically and the loss of viability observed after the control had started to grow was proportional to the rate of growth of the control.

FIG. 20 : Effect of aeration conditions on the action of quinolones on Escherichia coli 11229.

(a) Viability

(b) Turbidity

○—○	Control in GA	Aerobic
△—△	GA + 20 µM carboxyQ	
□—□	GA + 20 µM nitroQ	
●—●	Control in GA	Anaerobic
▲—▲	GA + 20 µM carboxyQ	
■—■	GA + 20 µM nitroQ	

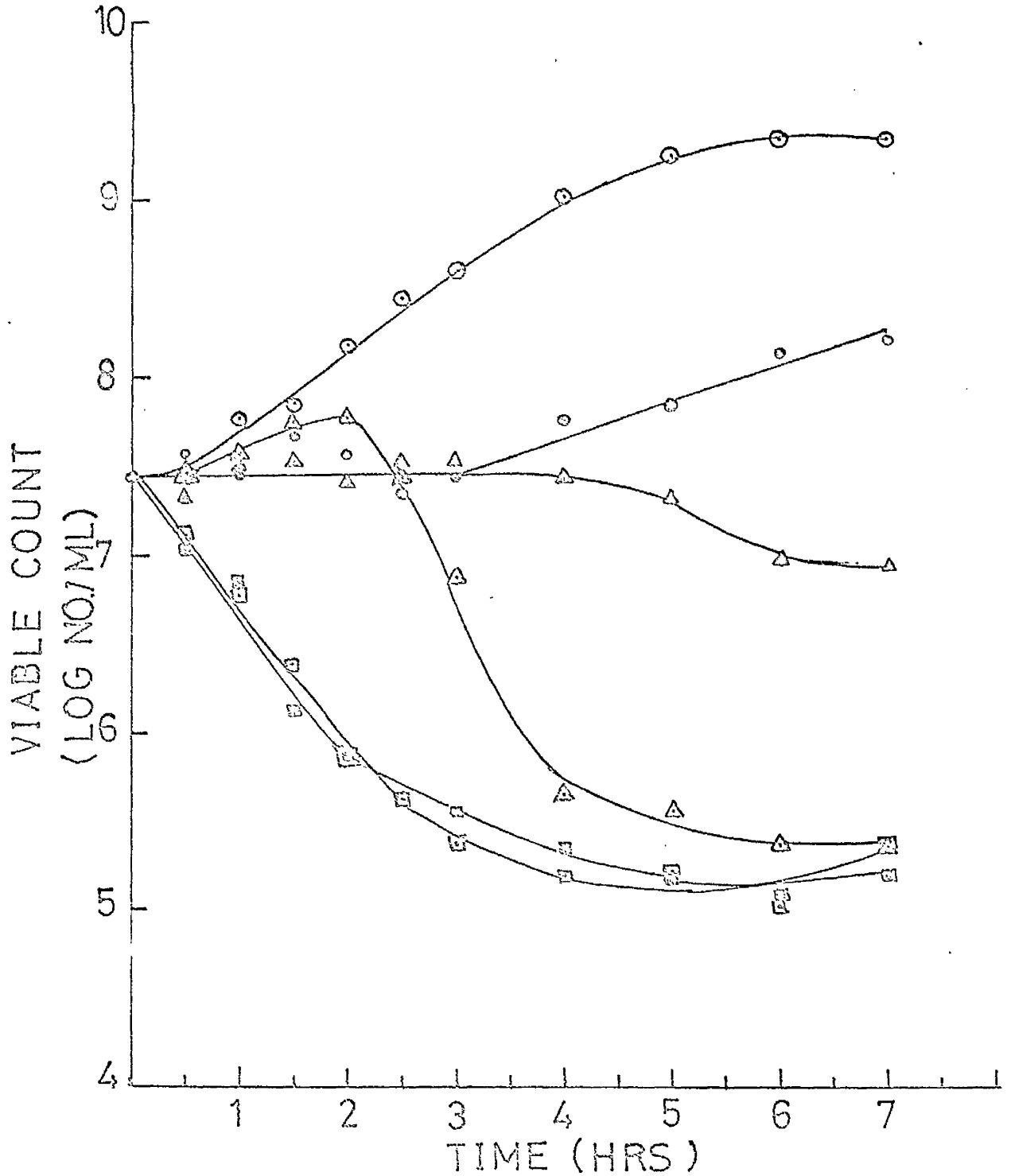


Fig. 20a

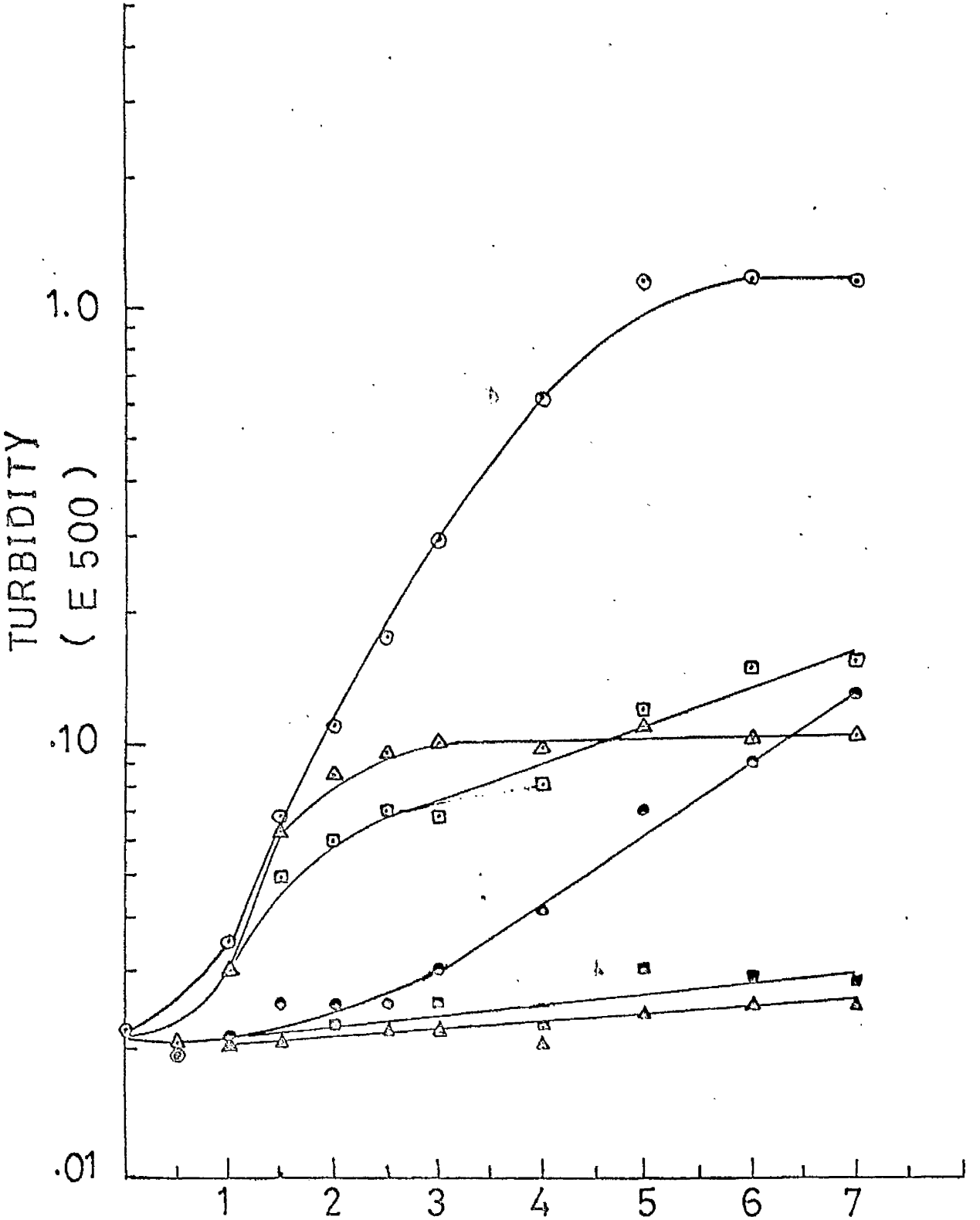


Fig. 20b

EFFECT OF CHANGES IN GROWTH MEDIUM ON THE ACTION OF QUINOLONES ON ESCHERICHIA COLI 11229

(a) Effect of quinolones in media deficient in glucose or ammonium (NH_4^+)

The carboxylic quinolone (carboxyQ) is lethal only when the culture has started to grow while the noncarboxylic quinolone, nitroQ is more active during lag phase when the cells are not actively dividing. Experiments were carried out when growth was inhibited by lack of either nitrogen or carbon/energy source or both in the GA medium. In all the experiments with deficient media, the inoculum was washed twice in phosphate buffer as described in Methods. Figures 21a and 21b show the effect of both quinolones (20 μM) in GA medium less glucose. The turbidity remained constant throughout the experimental period in the three cultures with no drug and with 20 μM each of both the quinolones. The viable count of both no drug control and carboxyQ culture remained constant. The culture with nitroQ, however, showed some fall in viability up to two hours after which the count remained constant. This loss of viability was later shown to be probably due to endogenous energy sources since when nitroQ is added after two hours of incubation in medium deficient in energy source, no fall in viability could be observed.

FIG. 21 : Effect of quinolones in GA medium deficient in glucose on Escherichia coli 11229.

(a) Viability

(b) Turbidity

○—○ Control in GA

●—● Control in GA less glucose

△—△ GA less glucose + 20 μM carboxyQ

□—□ GA less glucose + 20 μM nitroQ

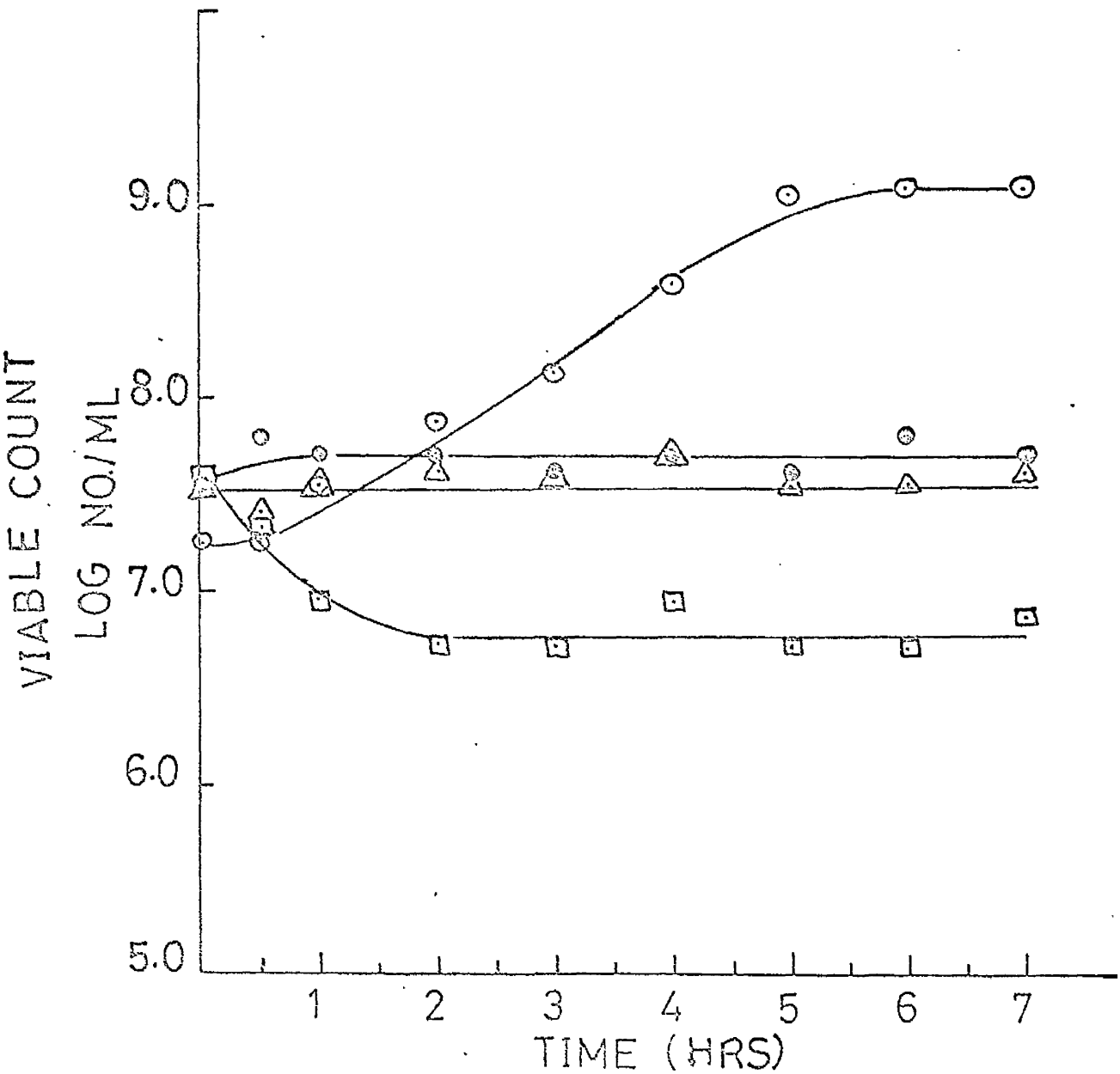


Fig. 21a

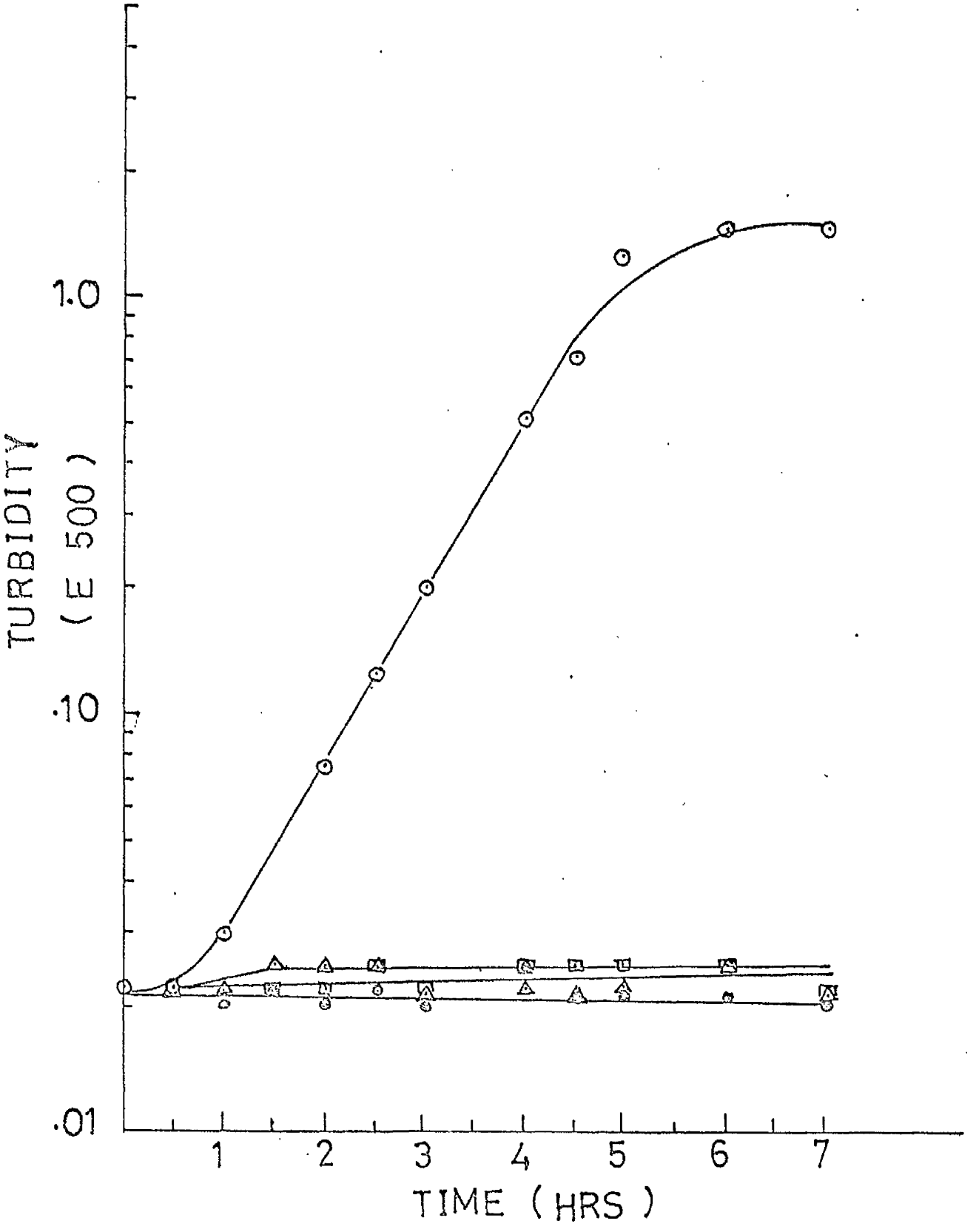


Fig.21b

Figures 22a and 22b show the action of quinolones in whole GA medium and GA medium less NH_4^+. Both the quinolones were 20 μM final concentrations. In the NH_4^+ deficient media with no drug and with carboxyQ after about a doubling, the viable count remained constant for the rest of the experimental period. However there was a rapid loss of viability in presence of nitroQ in this deficient medium. The rate and the total loss of viability was much more than in the whole medium. The final count was almost hundredfold less in deficient medium than in whole medium. This high sensitivity of the cells in absence of nitrogen source is reproducible.

FIG. 22 : Effect of quinolones in whole GA medium and GA medium less NH_4^+.

(a) Viability

(b) Turbidity

○—○ Control in GA

△—△ GA + 20 µM carboxyQ

□—□ GA + 20 µM nitroQ

●—● Control in GA less NH_4^+

▲—▲ GA less NH_4^+ + 20 µM carboxyQ

■—■ GA less NH_4^+ + 20 µM nitroQ

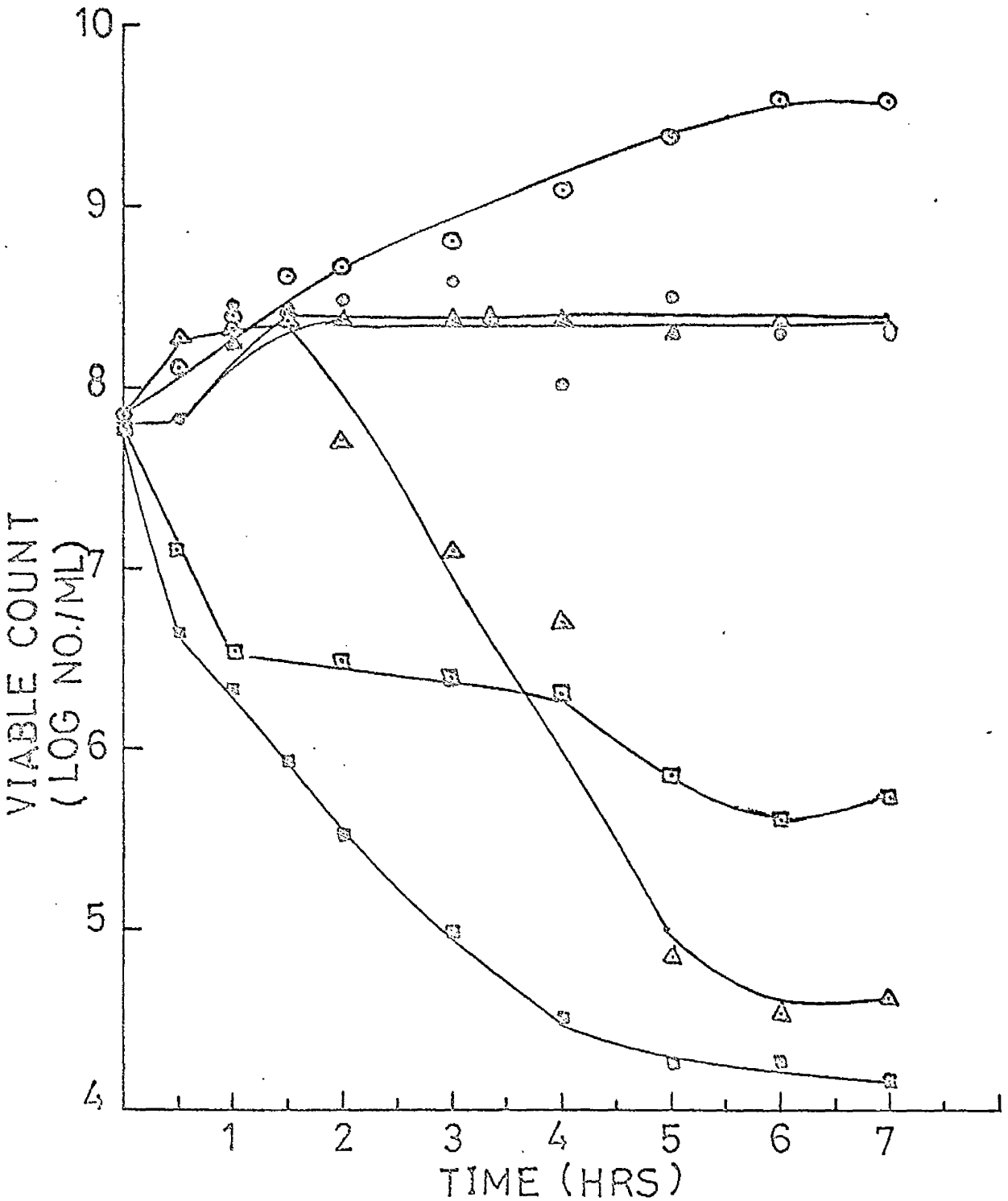


Fig. 22a

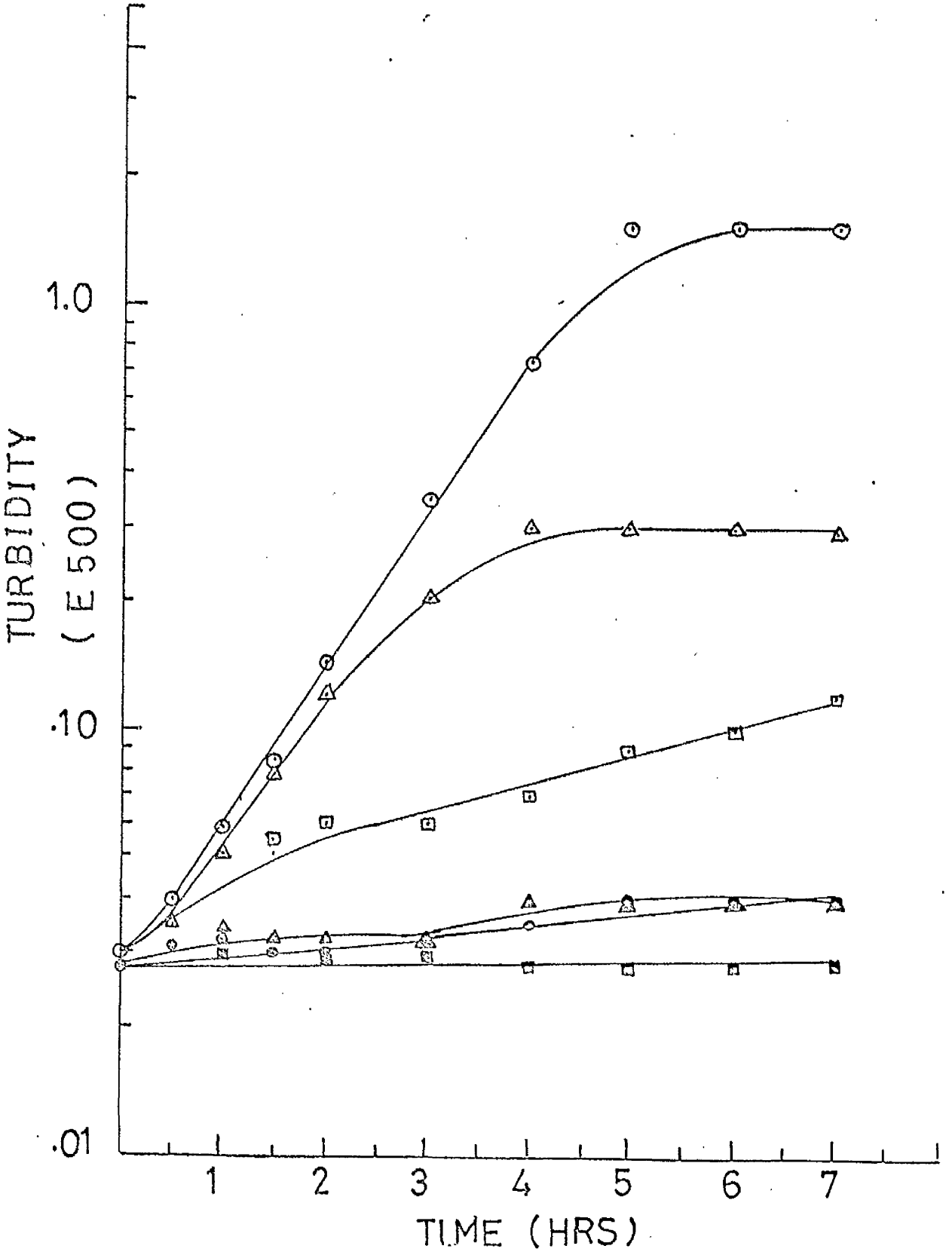


Fig. 22b

(b) Effect of adding glucose to cells in deficient media on the action of quinolones

Since nitroQ is active only in presence of glucose (energy source), glucose was added to cells exposed to 20 μM nitroQ in GA medium deficient in glucose (Figures 23a and 23b). If the drug is added at zero minutes to deficient medium there is some loss of viability presumably due to endogenous energy source. However, if the cells are incubated for about two hours in absence of drug the small source of energy is used up and there is no effect of drug added at this time in absence of glucose. Therefore, drug was added to three such cultures at two hours and glucose was added at two, three and four hours to each of them. There is an immediate and rapid loss of viability as soon as glucose is added and almost 99.9 per cent population is killed in about two hours after the addition of glucose in each case.

The same experiment was done with 20 μM carboxyQ (Figures 24a and 24b). In this case, however, there was not an immediate effect of glucose as loss in viability was noticed after about an hour. This suggests that some time must elapse in full medium before any lethal effect can be exerted by carboxyQ.

FIG. 23 : Effect of adding glucose to GA medium deficient in glucose on the action of nitroQ on Escherichia coli 11229.

(a) Viability

(b) Turbidity

○—○ Control in GA

△—△ GA + 20 μM nitroQ

●—● Control in GA less glucose

▲—▲ GA less glucose + 20 μM nitroQ at 0′

■—■ GA less glucose + 20 μM nitroQ at 2.00′

glucose added to a flask as above

◑—◑ at 2.00′

◭—◭ at 3.00′

◨—◨ at 4.00′

Arrows indicate the time of addition of glucose.

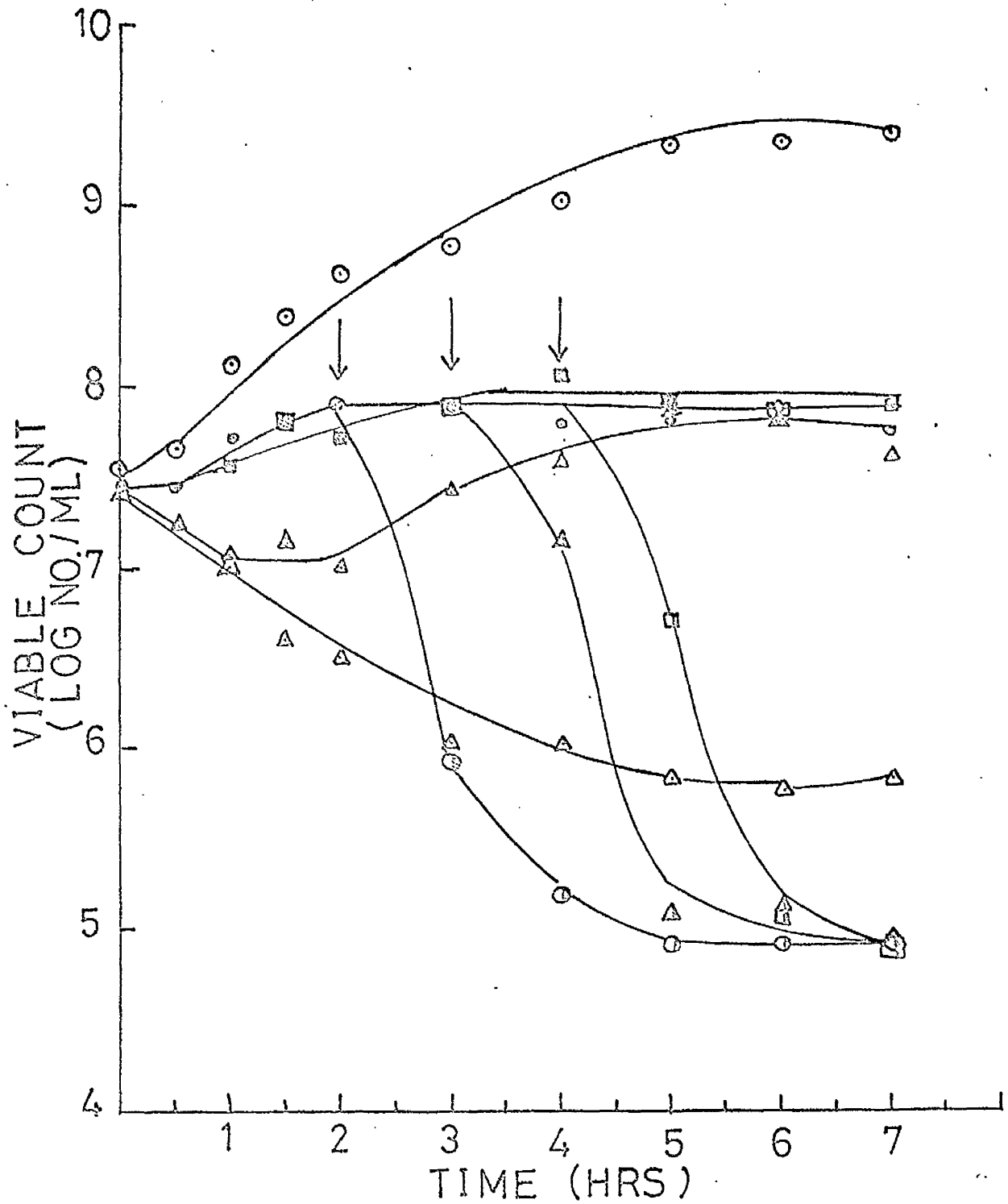


Fig. 23a

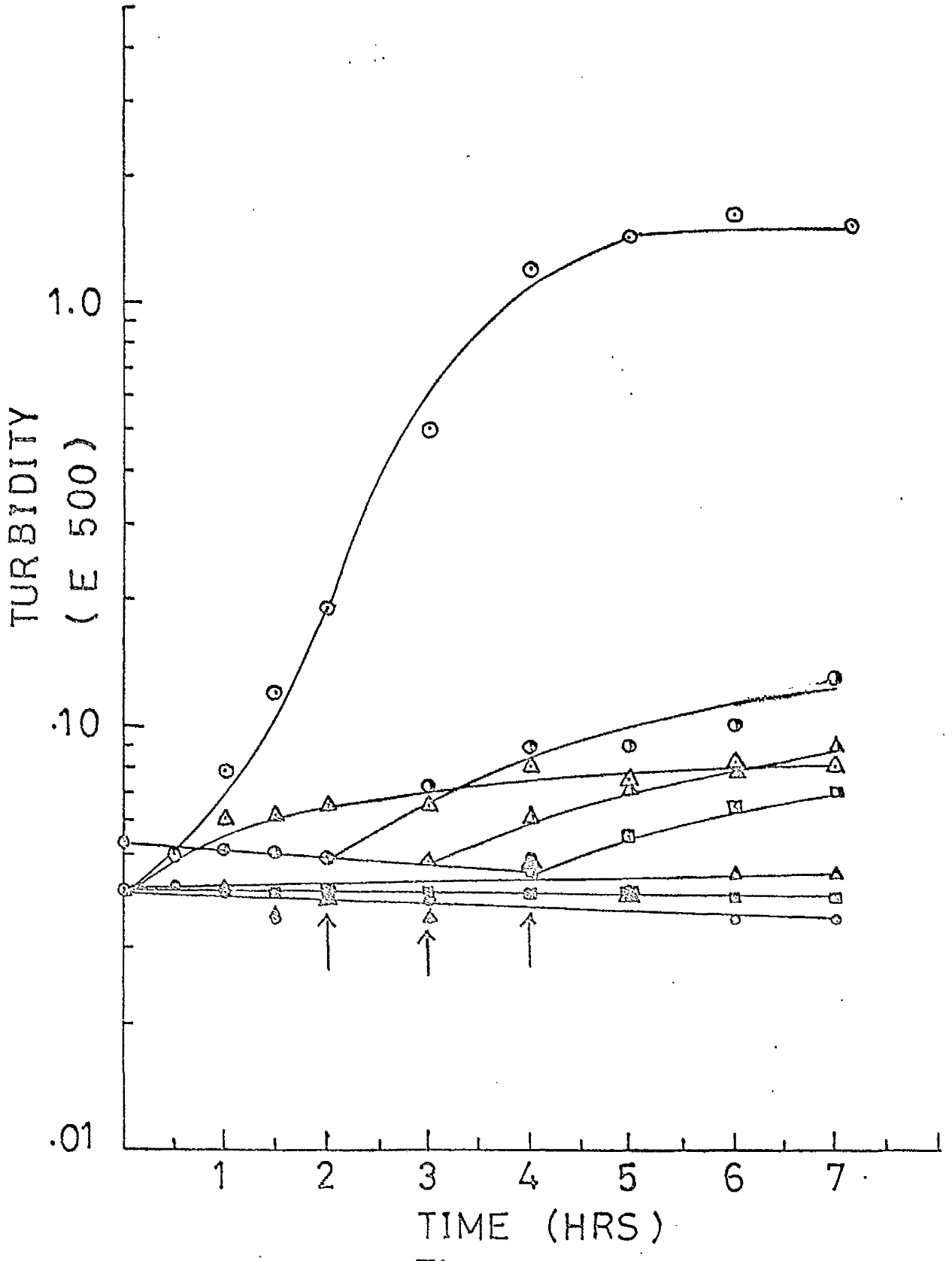


Fig. 23b

FIG. 24 : Effect of adding glucose to GA medium deficient in glucose on the action of carboxyQ on Escherichia coli 11229.

(a) Viability

(b) Turbidity

o—o Control in GA

Δ—Δ GA + 20 μM carboxyQ

●—● Control in GA less glucose

▲—▲ GA less glucose + 20 μM carboxyQ at 0′

■—■ GA less glucose + 20 μM carboxyQ at 2.00′ glucose added to a flask as above

◑—◑ at 2.00′

◭—◭ at 3.00′

◨—◨ at 4.00′

Arrows indicate the time of addition of glucose.

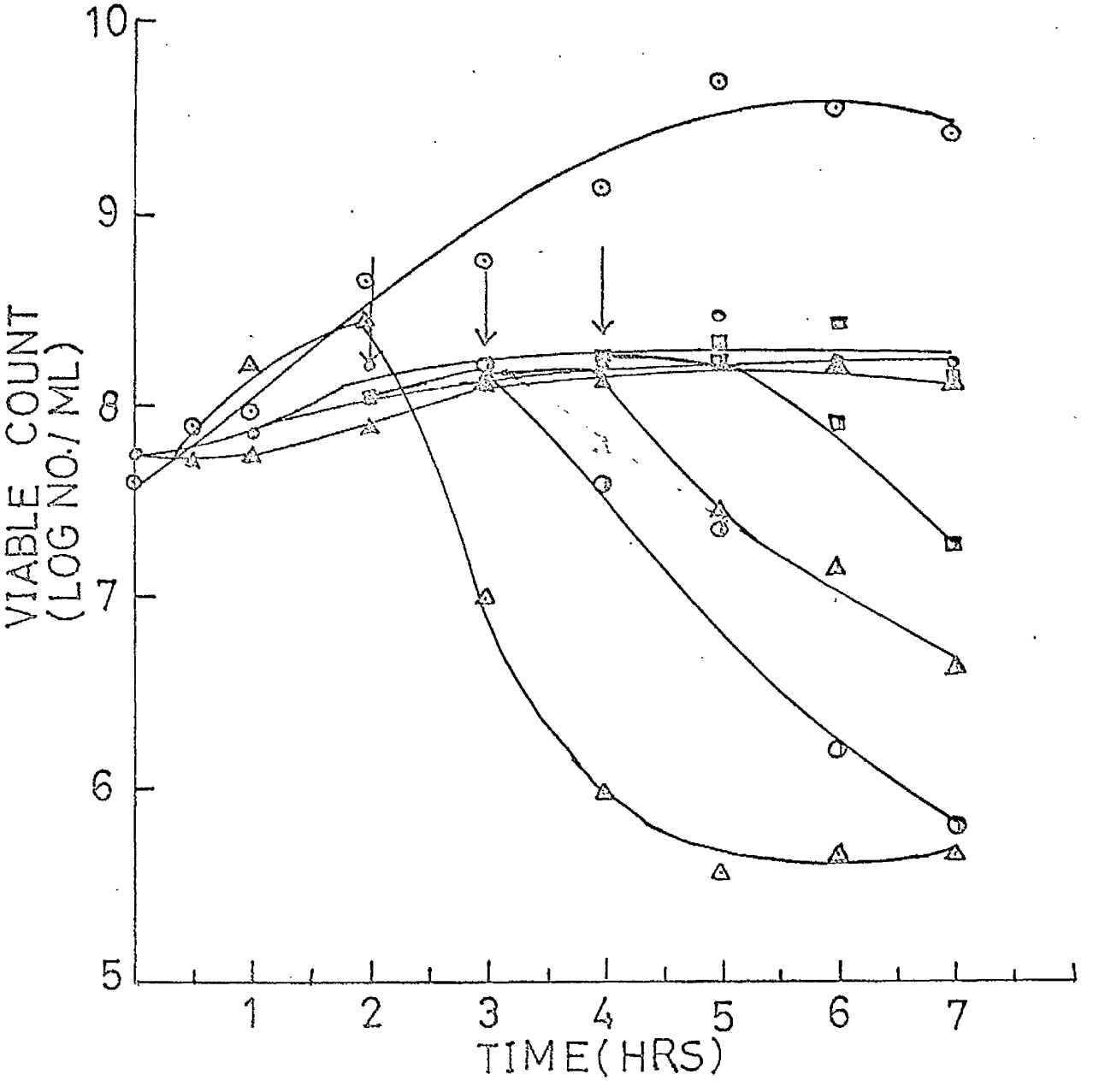


Fig. 24a

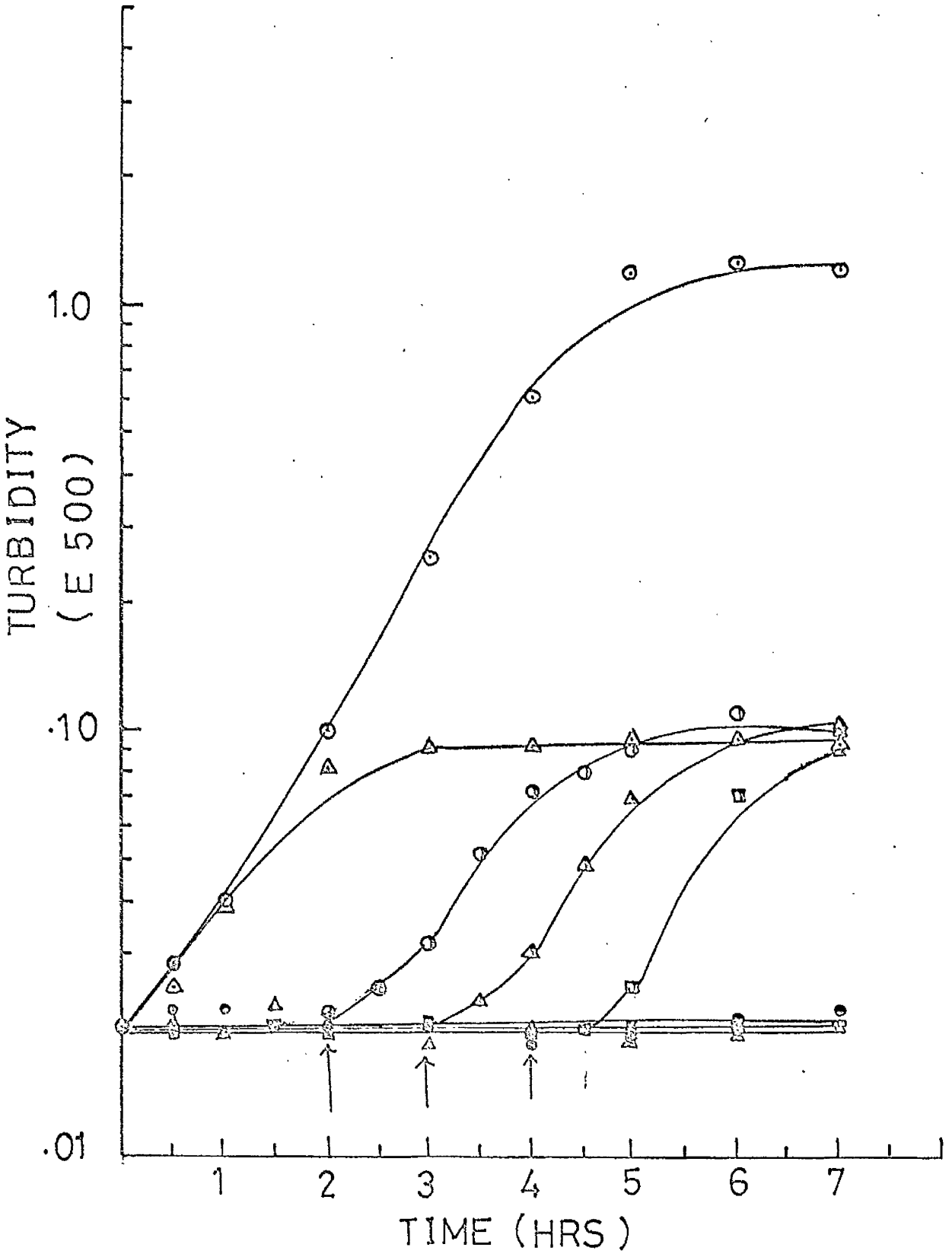


Fig. 24b

(c) Effect of glucose with and without NH_4^+ on the action nitroQ

Since nitroQ was found to be more active in absence of NH_4^+ (Figure 22a) it was interesting to see whether addition of glucose to media deficient in glucose and/or NH_4^+ would show any difference in the sensitivity of the cells. The results of such an experiment are shown in Figures 25a and 25b. The drug (20 μM) was added at two hours in all the cases. The curves a and b show the growth in whole GA medium without drug and when drug is added at two hours. The addition of NH_4^+ at zero minute or at two hours along with glucose and drug shows no difference in the sensitivity of cells to drug (curves c and d). Curves e and f show the survival of cells in GA medium deficient in both glucose and NH_4^+ with and without drug respectively. As shown the drug is without any effect in absence of glucose. Curves g and h show the effect of glucose in absence of NH_4^+ on the action of nitroQ. The cells are much more sensitive when glucose is added along with drug at two hours (curve h) than when it is present from start (curve g).

FIG. 25 : Effect of glucose with and without NH_4^+ on the action of nitroQ on Escherichia coli 11229.

(a) Viability

(b) Turbidity

Curve a ○—○ Control in GA medium

Curve b △—△ GA + 20 μM nitroQ at 2.00′

Curve e ●—● Control in basal medium (GA less glucose and NH_4^+)

Curve f ▲—▲ Basal medium + 20 μM nitroQ at 2.00′

Curve c □—□ Basal medium + NH_4^+ at 0′] + glucose and 20 μM nitroQ, at 2.00′

Curve d ■—■ Basal medium + NH_4^+ at 2.00′]

Curve g ◭—◭ Basal medium + glucose at 0′ | + nitroQ at 2.00′

Curve h ◪—◪ Basal medium + glucose at 2.00′ |

Arrow indicates the time of addition of nitroQ.

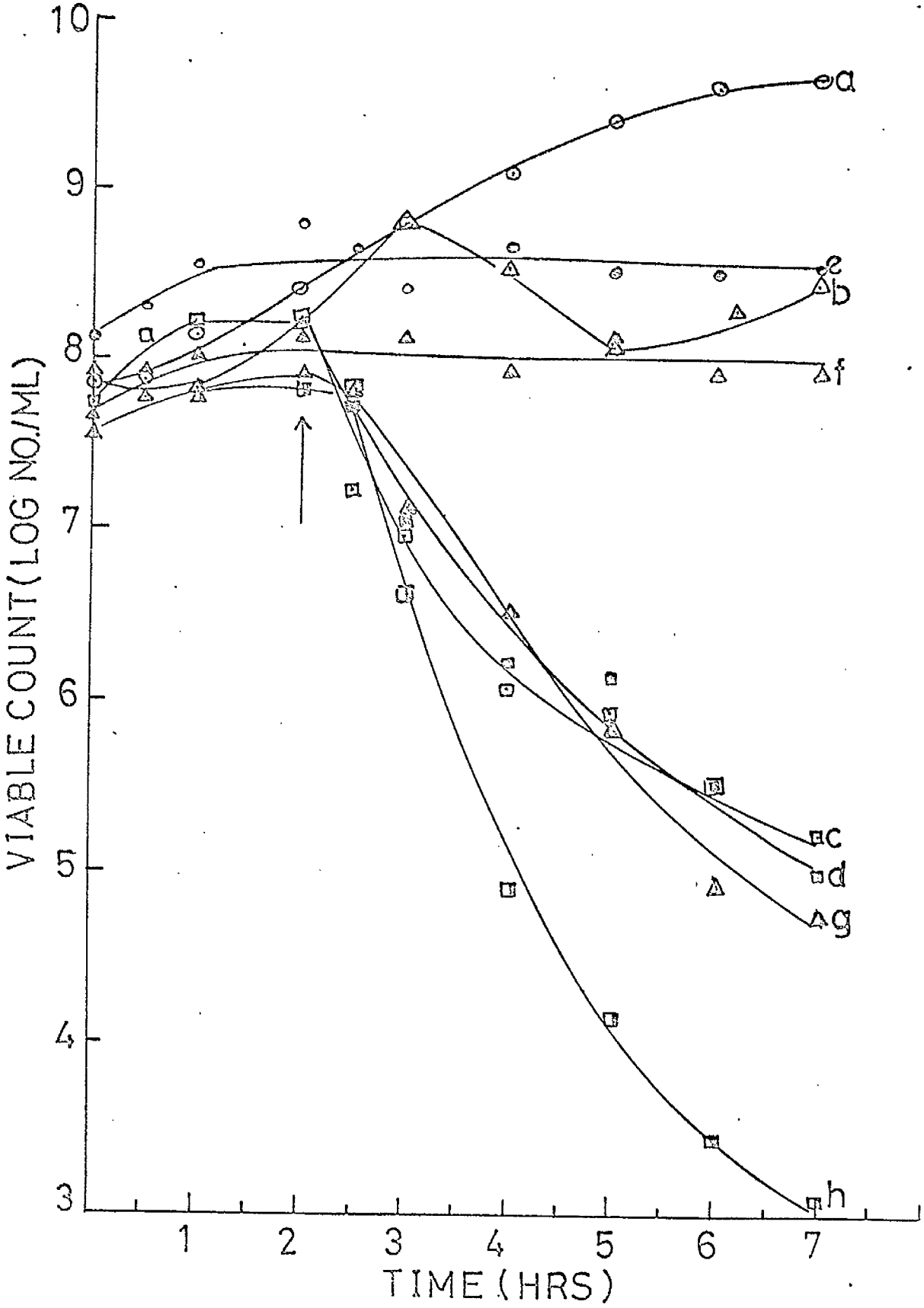


Fig. 25a

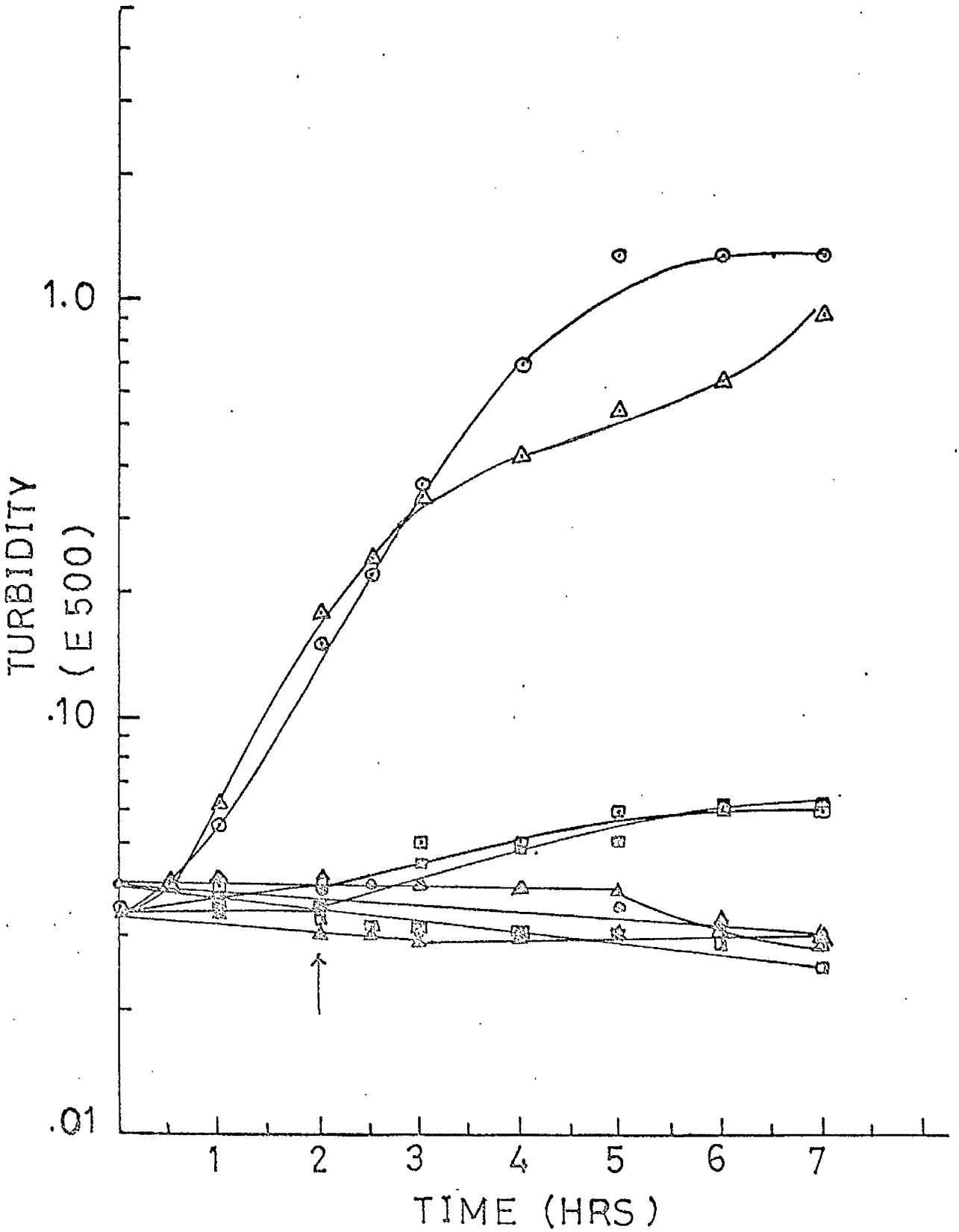


Fig. 25b

(d) Effect of limiting concentration of glucose on the action of nitroQ

Addition of glucose to an otherwise deficient medium with nitroQ causes a dramatic fall in the viable count. The concentration of glucose used (1.2%) is in excess of the requirement. It was found that .025% glucose supports about three or four generations after which further growth is limited due to exhaustion of glucose in the medium. Figures 26a and 26b show the effect .025% glucose with and without NH_4^+ on the action of 20 μM nitroQ. The rate and total loss of viability in presence of NH_4^+ is nearly of the same order of magnitude as in high concentration of glucose. Here again the cells are much more sensitive to the drug in absence of NH_4^+. This suggests that the low concentration of glucose is sufficient to allow the lethal activity of the drug as it is completely without any action in absence of glucose. This was further supported by an experiment in which readdition of .025% glucose to a culture which had stopped growing due to exhaustion of glucose did not cause any further loss of viability.

FIG. 26 : Effect of limiting concentration of glucose with and without NH_4^+ on the action of nitroQ on Escherichia coli 11229.

(a) Viability

(b) Turbidity

O—O	Control in low glucose (.025%) GA	+ NH_4^+
△—△	Low glucose GA + 20 µM nitroQ	+ NH_4^+
●—●	Control in low glucose (.025%) GA	- NH_4^+
▲—▲	Low glucose GA + 20 µM nitroQ	- NH_4^+

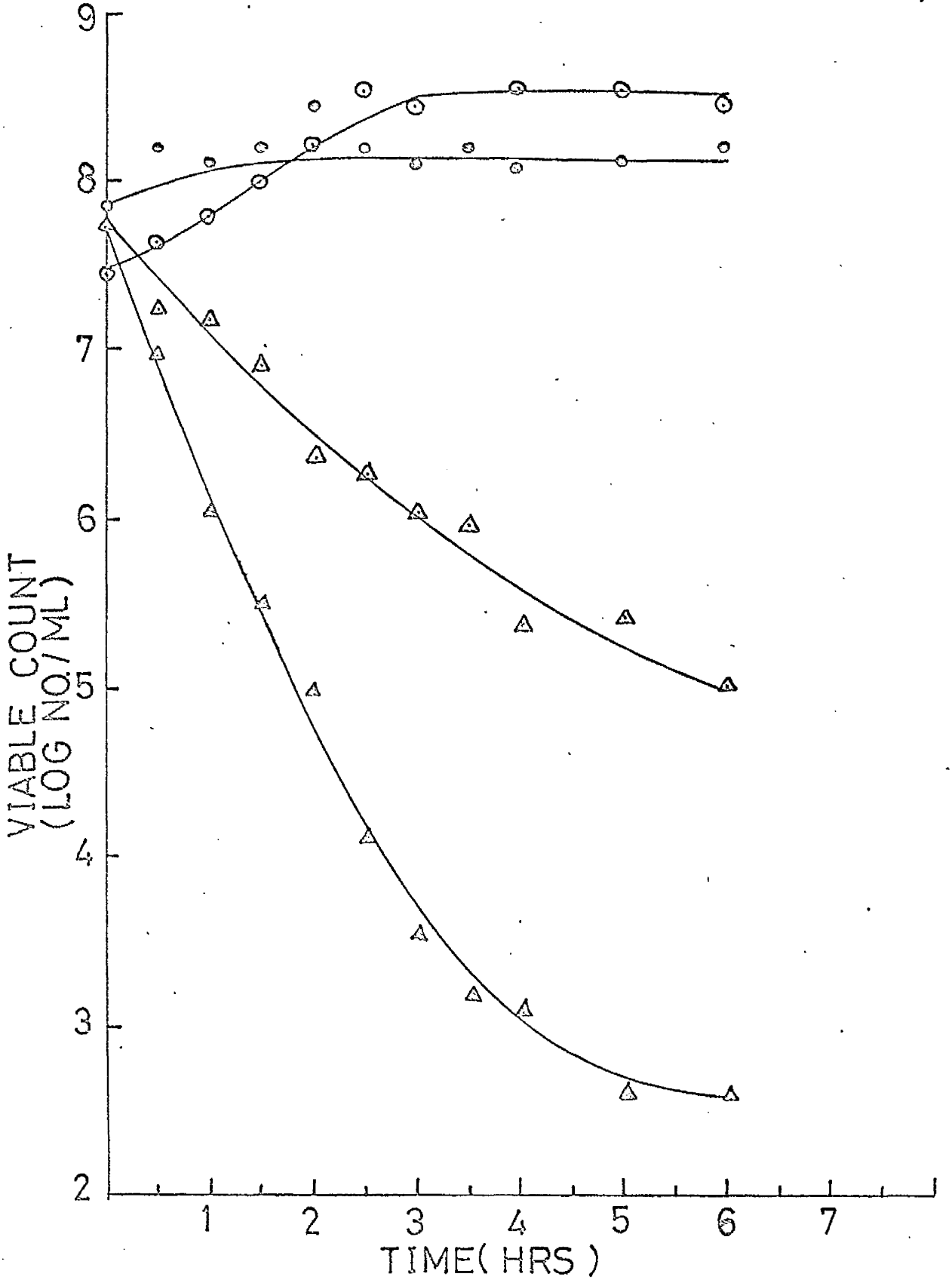


Fig.26a

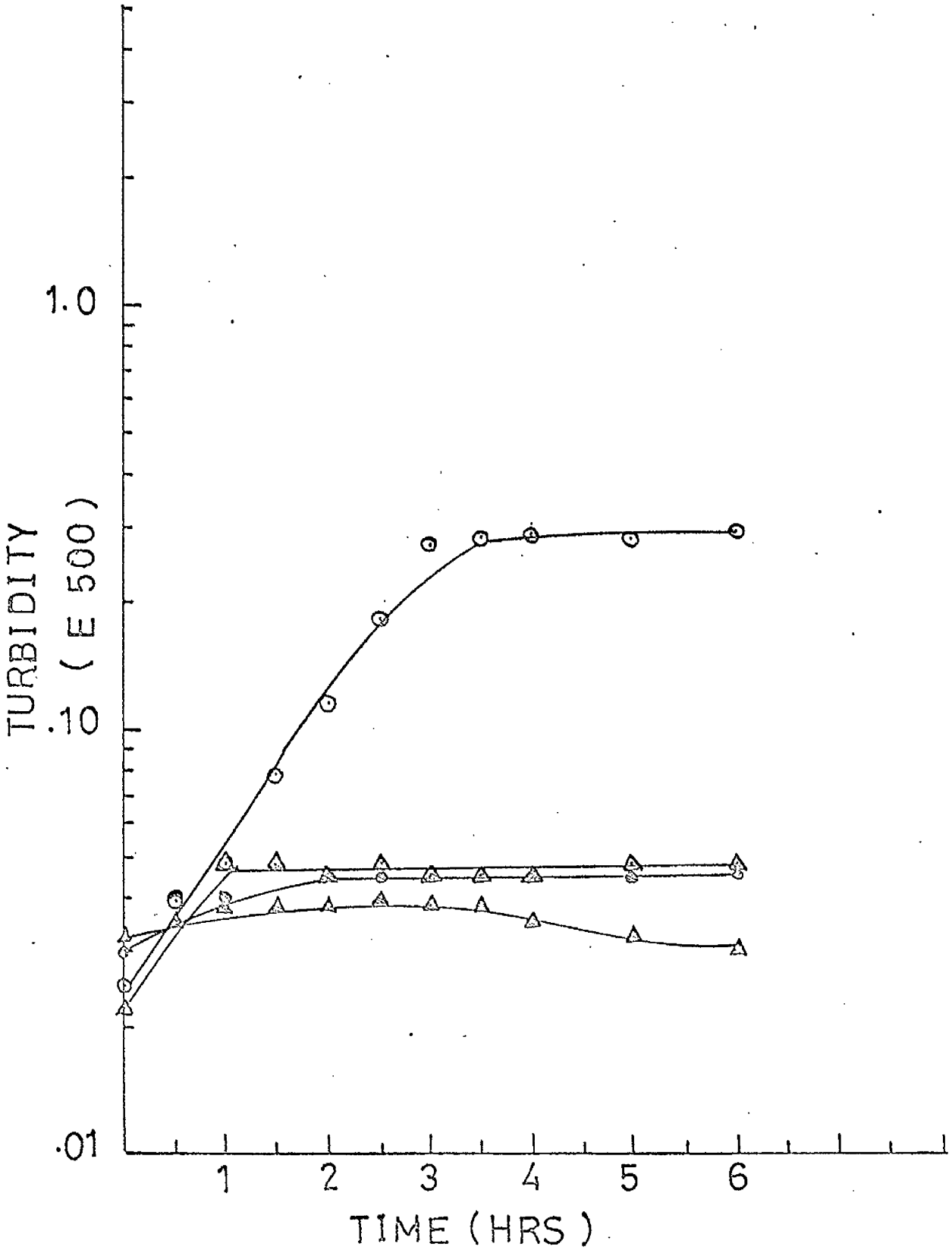


Fig. 26b

(e) Effect of limiting concentration of NH_4^+ on the action of nitroQ

The activity of nitroQ is much more pronounced in absence of NH_4^+. In order to see whether a lower concentration of NH_4^+ would give the same effect as the high concentration of NH_4^+, twice washed cells were inoculated into (a) a medium with .005% NH_4^+ instead of .12% usual concentration in presence or absence of 20 μM nitroQ; (b) a medium without NH_4^+ in presence or absence of 20 μM nitroQ. The results are shown in Figures 27a and 27b. Here again the small amount of NH_4^+ increased the final number of survivors almost hundredfold. This low concentration of NH_4^+ supported growth for only about three generations.

FIG. 27 : Effect of limiting concentration of NH_4^+ on the action of nitroQ on Escherichia coli 11229 in presence of excess glucose.

(a) Viability

●—● Control in low NH_4^+ (.005%) GA

▲—▲ Low NH_4^+ GA + 20 µM nitroQ

○—○ Control in GA less NH_4^+

△—△ GA less NH_4^+ + 20 µM nitroQ

(b) Turbidity

○—○ Control in low NH_4^+ (.005%) GA

△—△ Low NH_4^+ GA + 20 µM nitroQ

●—● Control in GA less NH_4^+

▲—▲ GA less NH_4^+ + 20 µM nitroQ

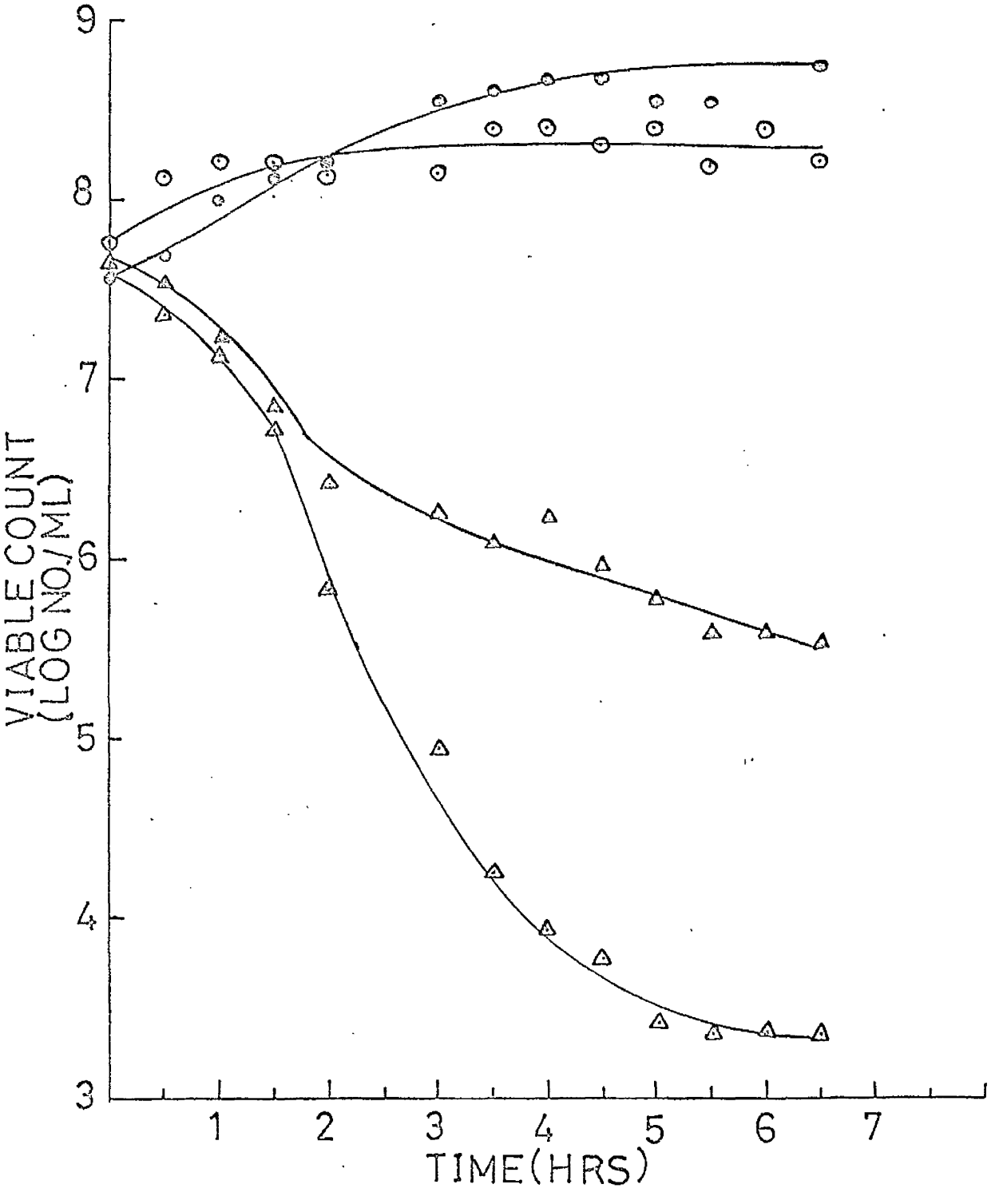


Fig. 27a

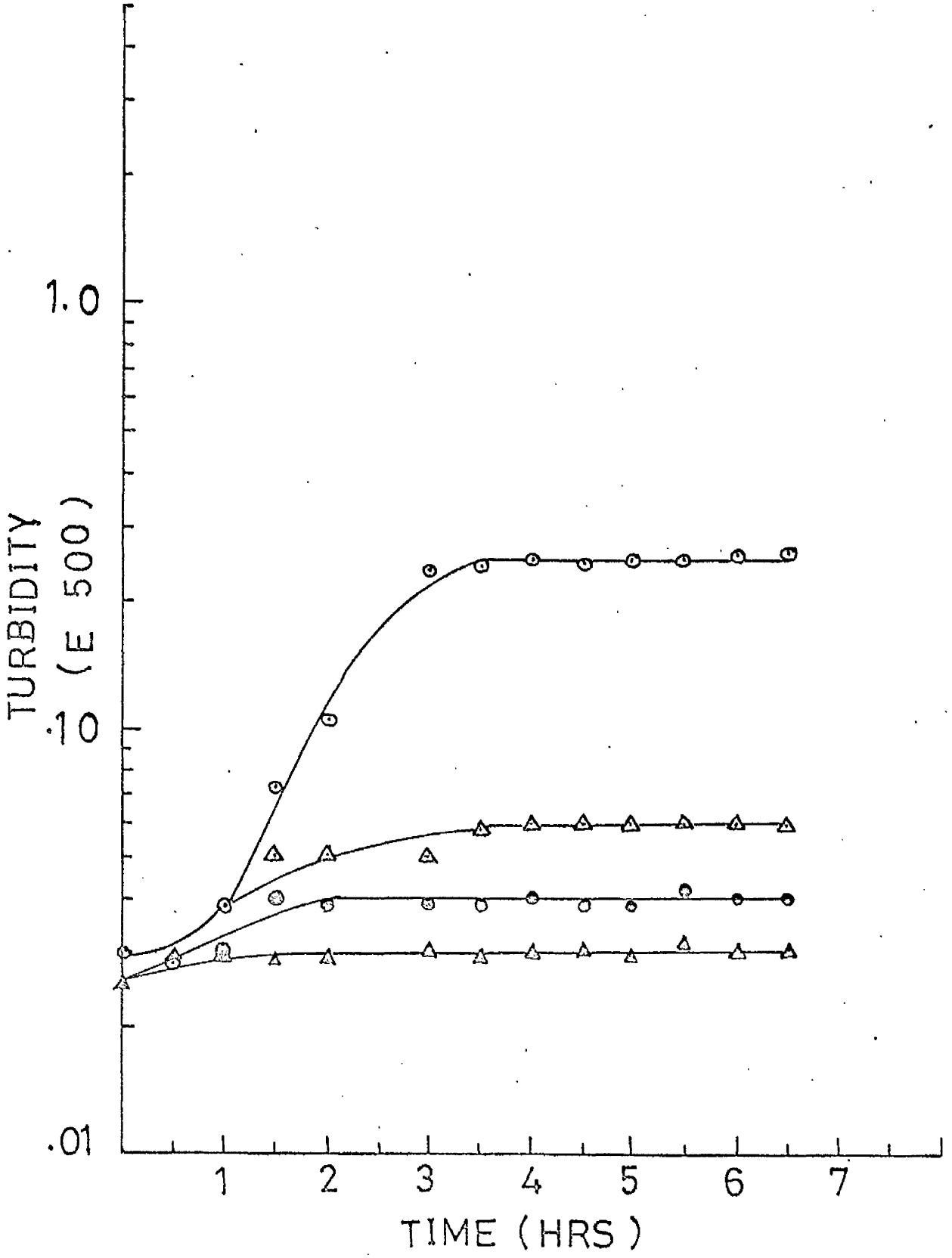


Fig. 27b

(f) Effect of nitroQ added at various times to cultures growing on limited glucose or NH_4^+

The activity of nitroQ reduces when it is added later than 30 minutes of growth in drug free medium. This loss of sensitivity could depend on the increasing density of the cultures or on changes in metabolic activities of the cells during logarithmic and stationary phases. Limiting the growth by low concentrations of either glucose or NH_4^+ which allowed only a few generations, restricted the total cell density. Under these conditions the growth is limited by exhaustion of either glucose or NH_4^+ from the medium. Figures 28a, 28b, 29a and 29b show the effect of 20 μM nitroQ when added at 0, 1, 2, 3, 4, 5 and 6 hours to cultures limited by glucose or NH_4^+ respectively. When growth is limited by glucose, addition of drug affects viability only up to three hours.

After this time glucose is exhausted from the medium and the drug has no effect thereafter. On the other hand when growth is limited by NH_4^+ in presence of excess glucose, some loss of viability is always observed even when growth has stopped. Here the reduction in the activity is presumably due to increasing number of resistant cells which appear with the increase in total population.

Effect of 20 μM carboxyQ on cells limited by NH_4^+ is shown in Figures 30 a and 30b. The carboxylic quinolone is active only up to two hours. The growth is limited by exhaustion of NH_4^+ from the medium at about three hours. CarboxyQ added at three hours or thereafter did not have any bactericidal effect unlike nitroQ which shows some activity even at six hours. This shows that carboxylic quinolones are active only when the cells are starting to grow or

FIG. 28 : Effect of nitroQ added at various times to cultures growing on limited glucose.

(a) Viability

(b) Turbidity

○—○ Control in low glucose (.025%) GA

20 μM nitroQ added to above at:

△—△ 0′

□—□ 1.00′

●—● 2.00′

▲—▲ 3.00′

Arrows indicate the time of addition of nitroQ. Viable counts and turbidity of the flasks to which nitroQ was added at 4, 5 and 6 hours were very similar to those of the control.

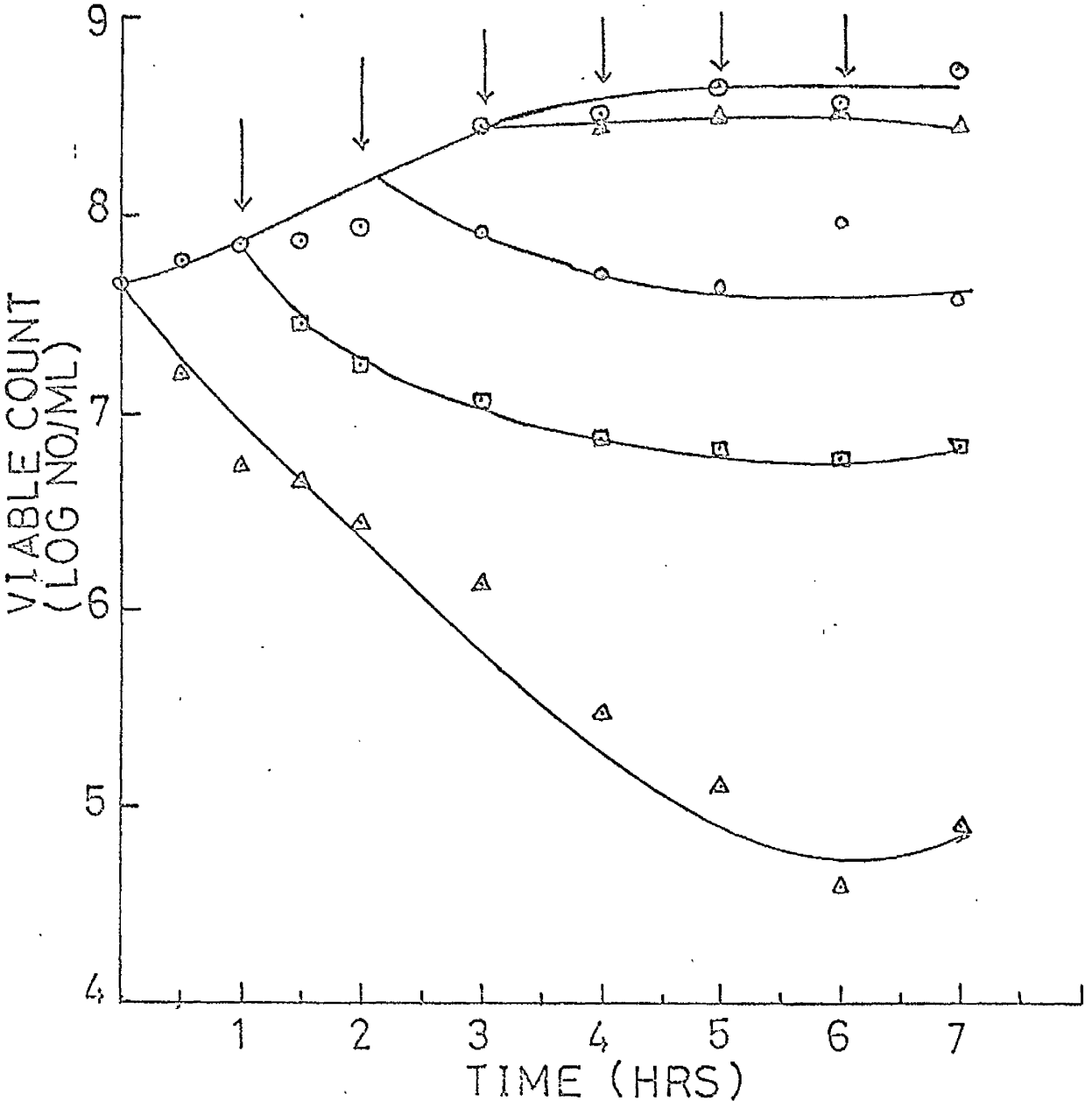


Fig. 28a

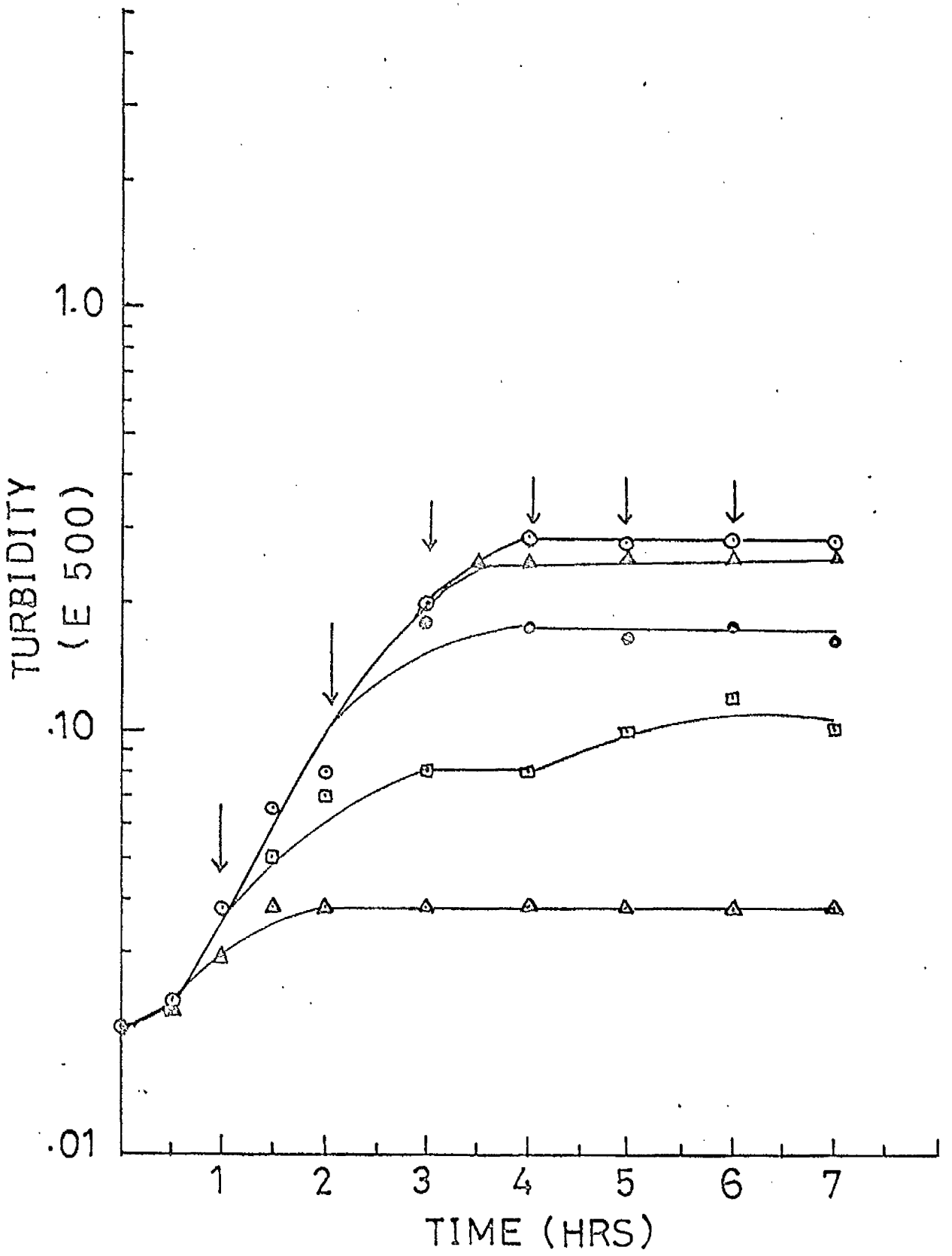


Fig. 28b

FIG. 29 : Effect of nitroQ added at various times to cultures growing on limited NH_4^+.

(a) Viability

(b) Turbidity

O—O Control in low NH_4^+ (.005%) GA.

20 µM nitroQ added to above at:

Δ—Δ 0′

□—□ 1.00′

●—● 2.00′

▲—▲ 3.00′

◑—◑ 4.00′

◭—◭ 5.00′

◨—◨ 6.00′

Arrows indicate the time of addition of nitroQ. Turbidity of the flasks to which nitroQ was added at 3, 4, 5 and 6 hours was very similar to that of control.

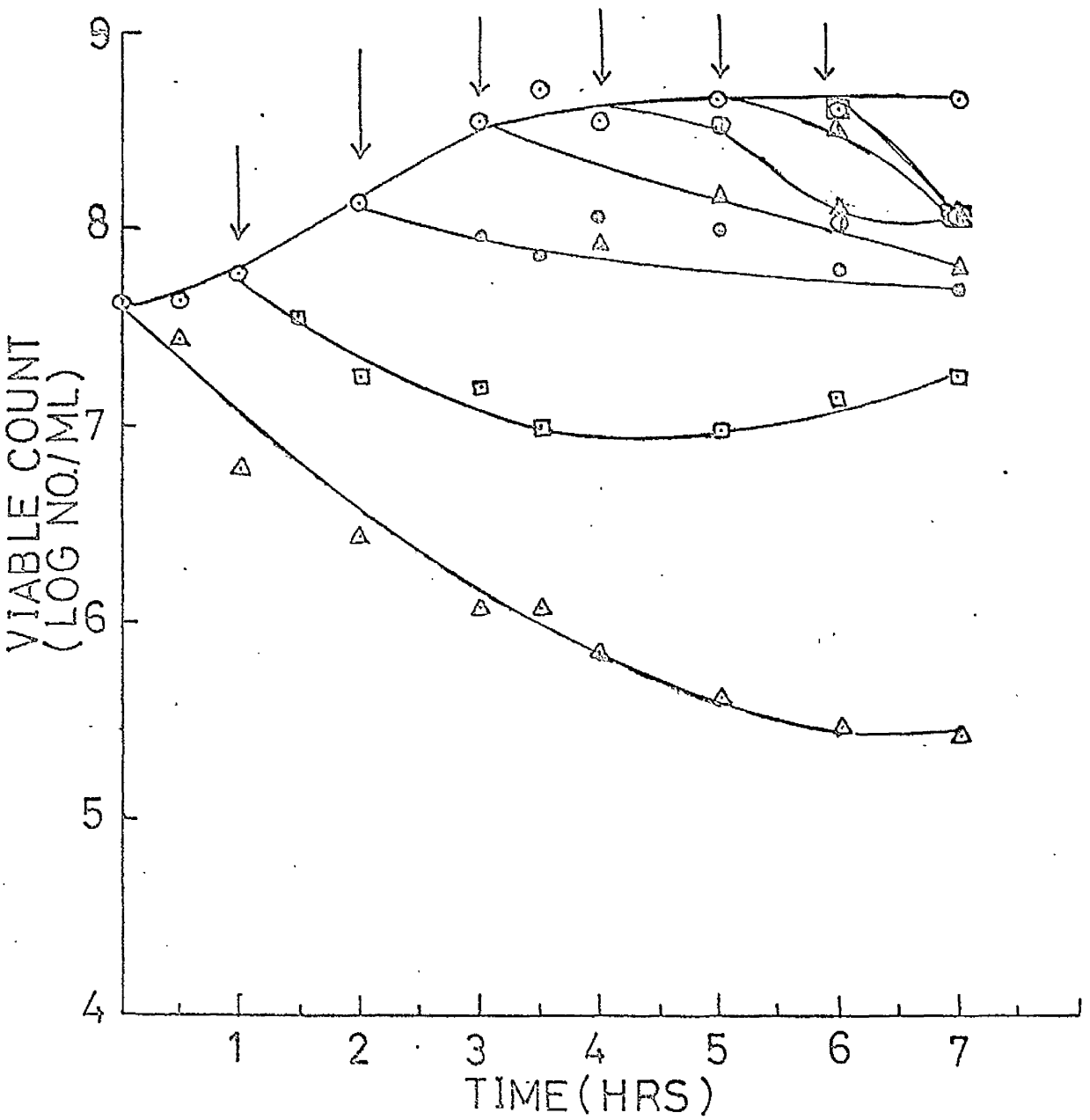


Fig. 29a

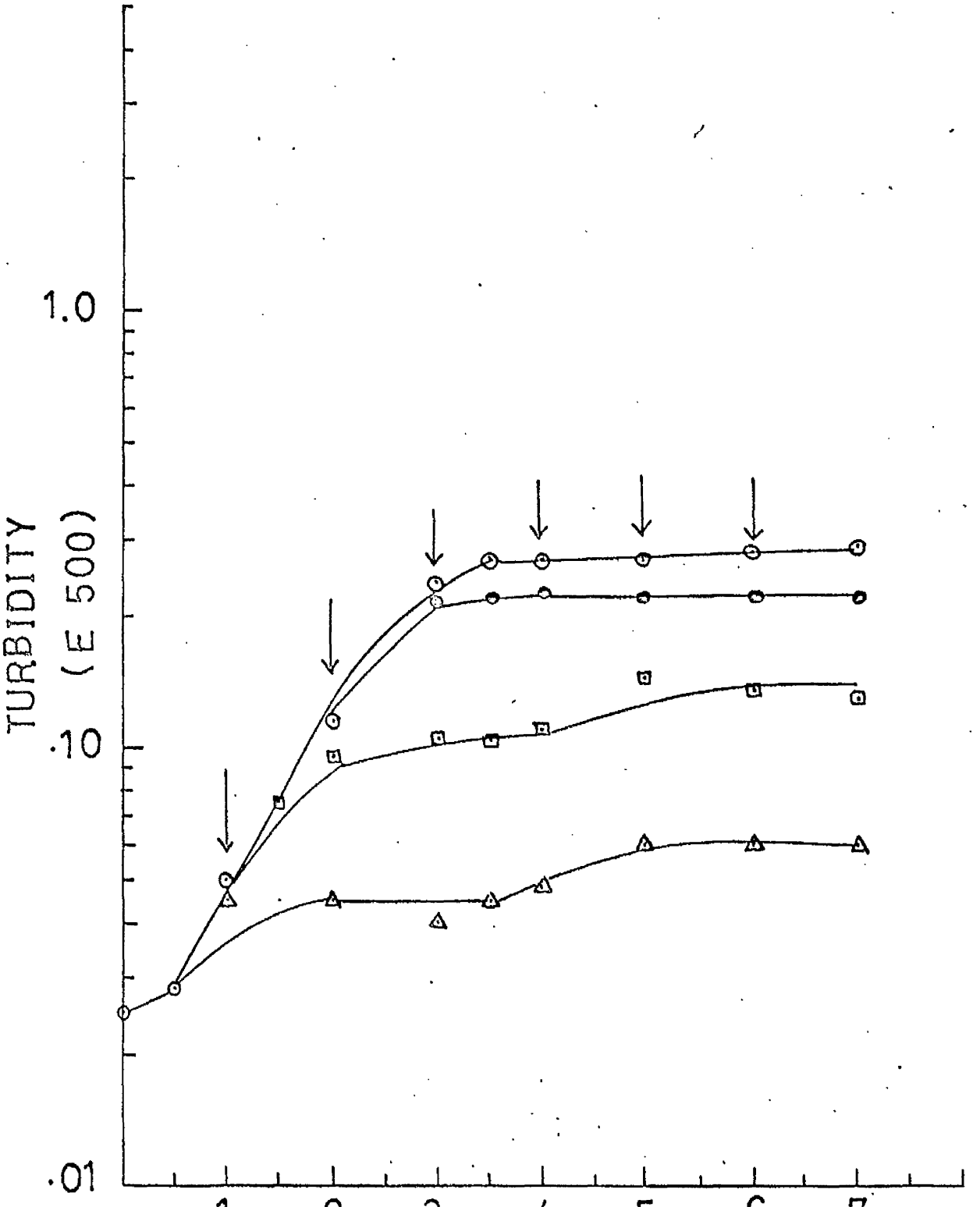


Fig. 29b

FIG. 30 : Effect of carboxyQ added at various times to cultures growing on limited NH_4^+.

(a) Viability

(b) Turbidity

o—o Control in low NH_4^+ (.005%) GA.

20 µM carboxyQ added to above at:

Δ—Δ 0′

□—□ 1.00′

●—● 2.00′

▲—▲ 3.00′

Arrows indicate the time of addition of nitroQ. Viability and turbidity of the flasks to which drug was added at 3, 4, 5 and 6 hours was very similar to those of control.

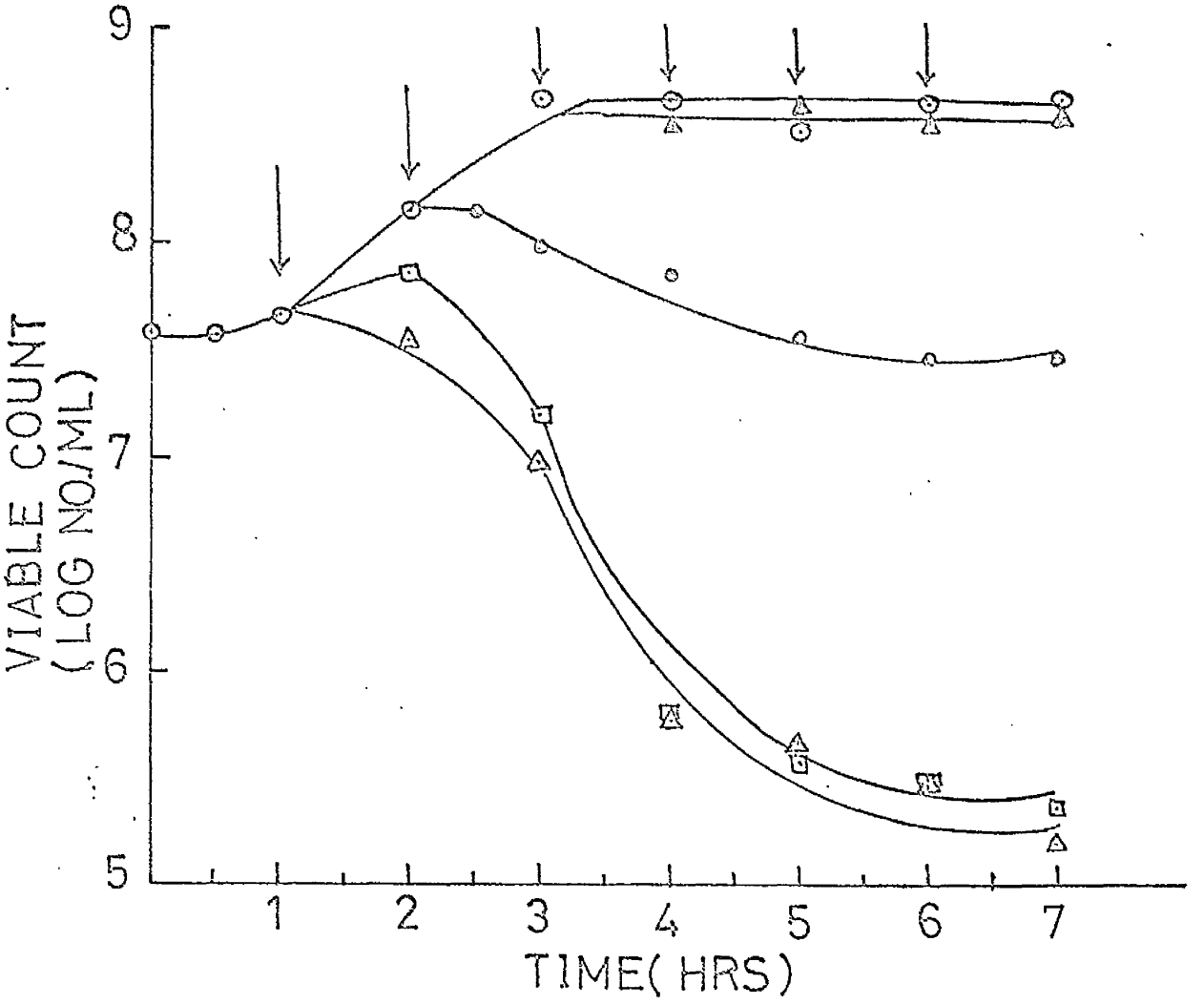


Fig. 30a

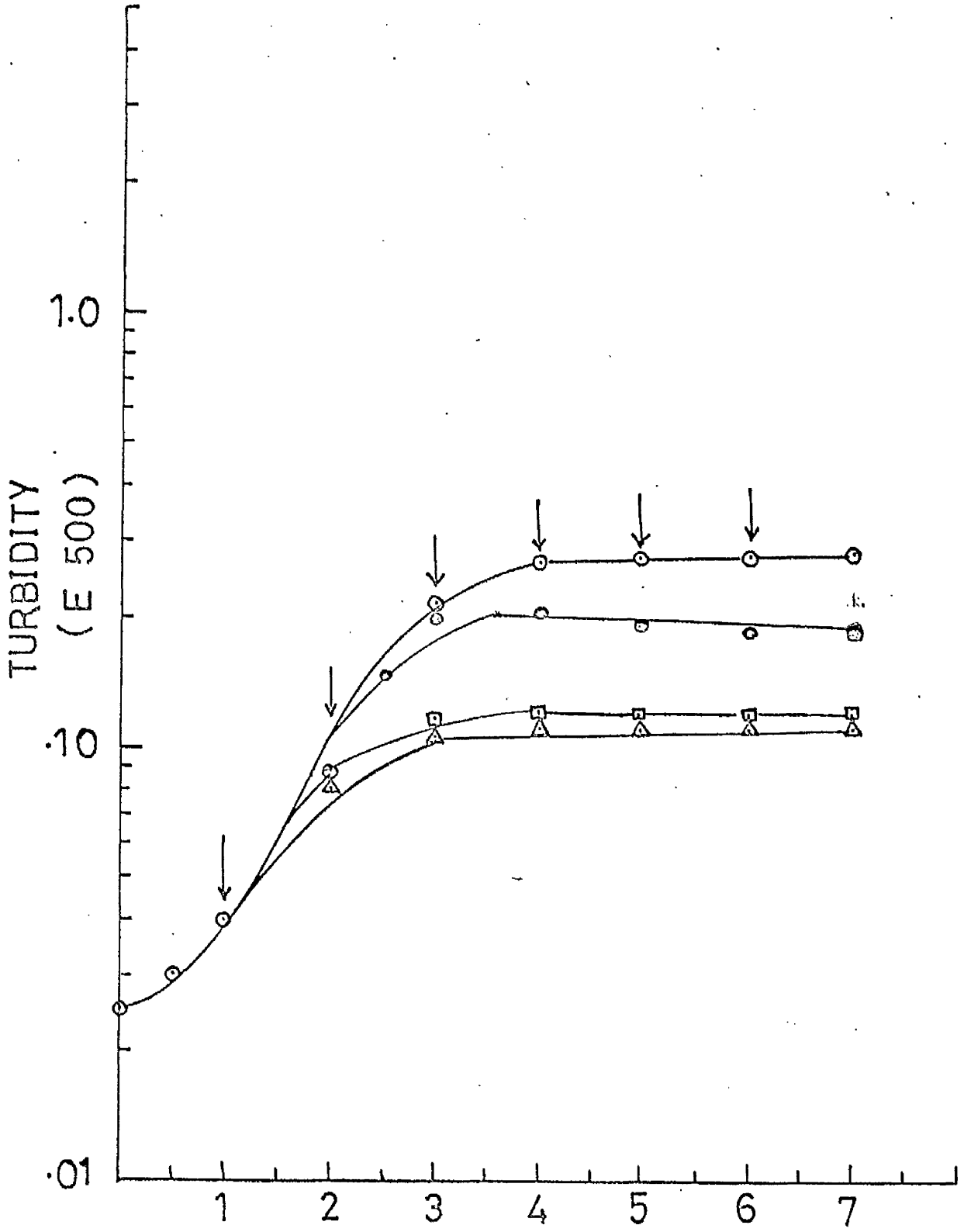


Fig. 30b

actually growing. As soon as growth is stopped, this drug fails to exert any lethal effect. Noncarboxylic quinolone activity is not affected by absence of growth but only by exhaustion of glucose from the growth medium.

(g) Effect of nitroQ with different carbon/energy sources

If washed cells are suspended in a medium without carbon/energy source with nitroQ, the drug does not exert any effect. However, as soon as glucose is added to such cells, there is an immediate fall in viable count. A washed cell suspension (which was previously grown in GA medium) was inoculated into (a) GA medium (b) GA medium and 20 μM nitroQ (c) GA medium without glucose (basal medium) + 20 μM nitroQ (d) basal medium + 1.2% L-glutamic acid + 20 μM nitroQ and (e) basal medium + 1.2% sodium succinate + 20 μM nitroQ.

The results are shown in Figs. 31a and 31b. The drug does not have any effect in absence of a carbon/energy source. There is a very slight delayed effect in the medium supplemented with L-glutamic acid which does not support the growth of E. coli but may act as a carbon source only. The drug is much more active in presence of succinate than glucose.

FIG. 31 : Effect of nitroQ with different carbon/ energy sources on Escherichia coli 11229.

(a) Viability

(b) Turbidity

○—○ Control in GA

△—△ GA less glucose (basal medium) + 20 µM nitroQ

□—□ GA medium (1.2% glucose) + 20 µM nitroQ

●—● Basal medium + 1.2% L-glutamic acid + 20 µM nitroQ

▲—▲ Basal medium + 1.2% Na-succinate + 20 µM nitroQ

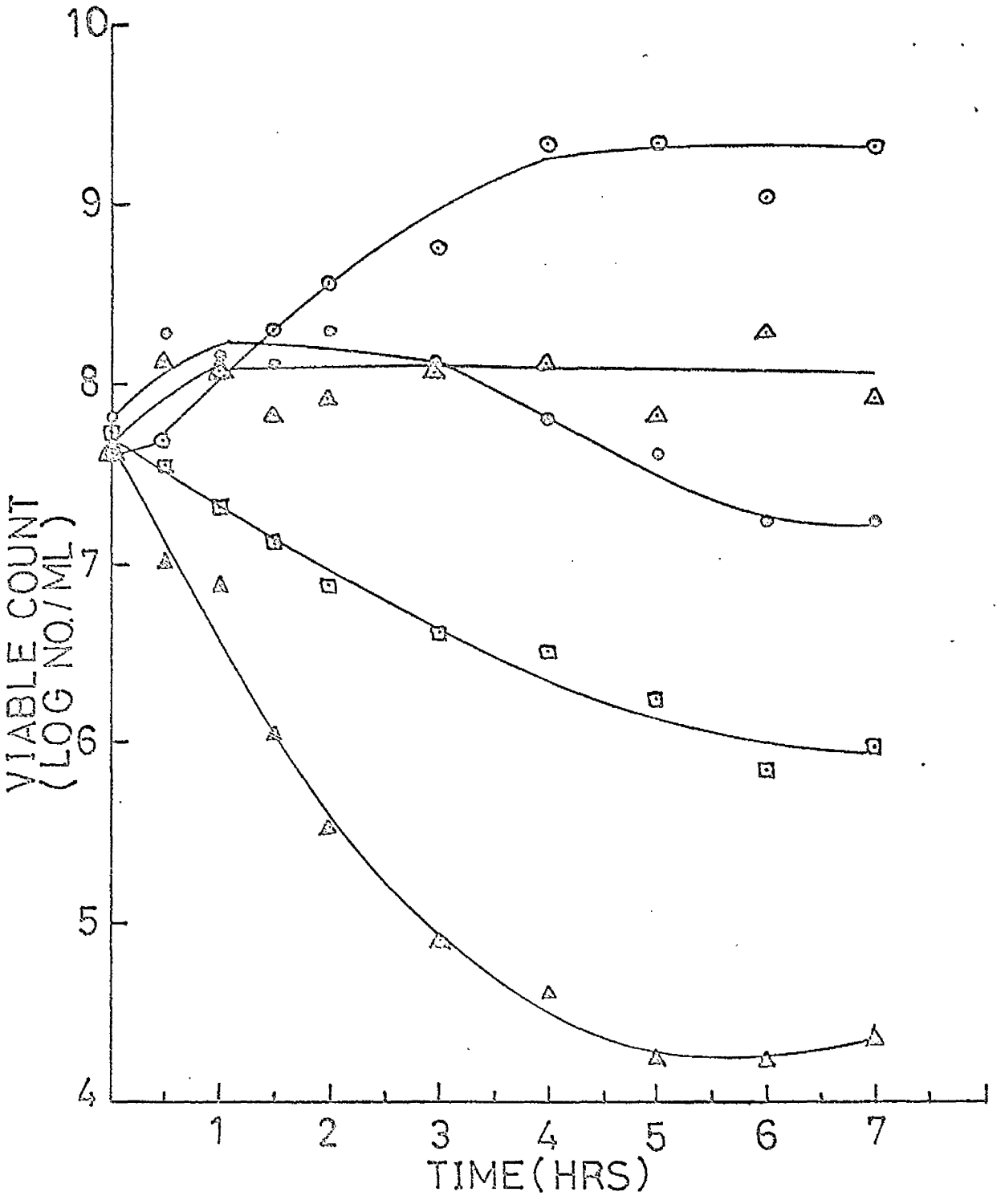


Fig. 31a

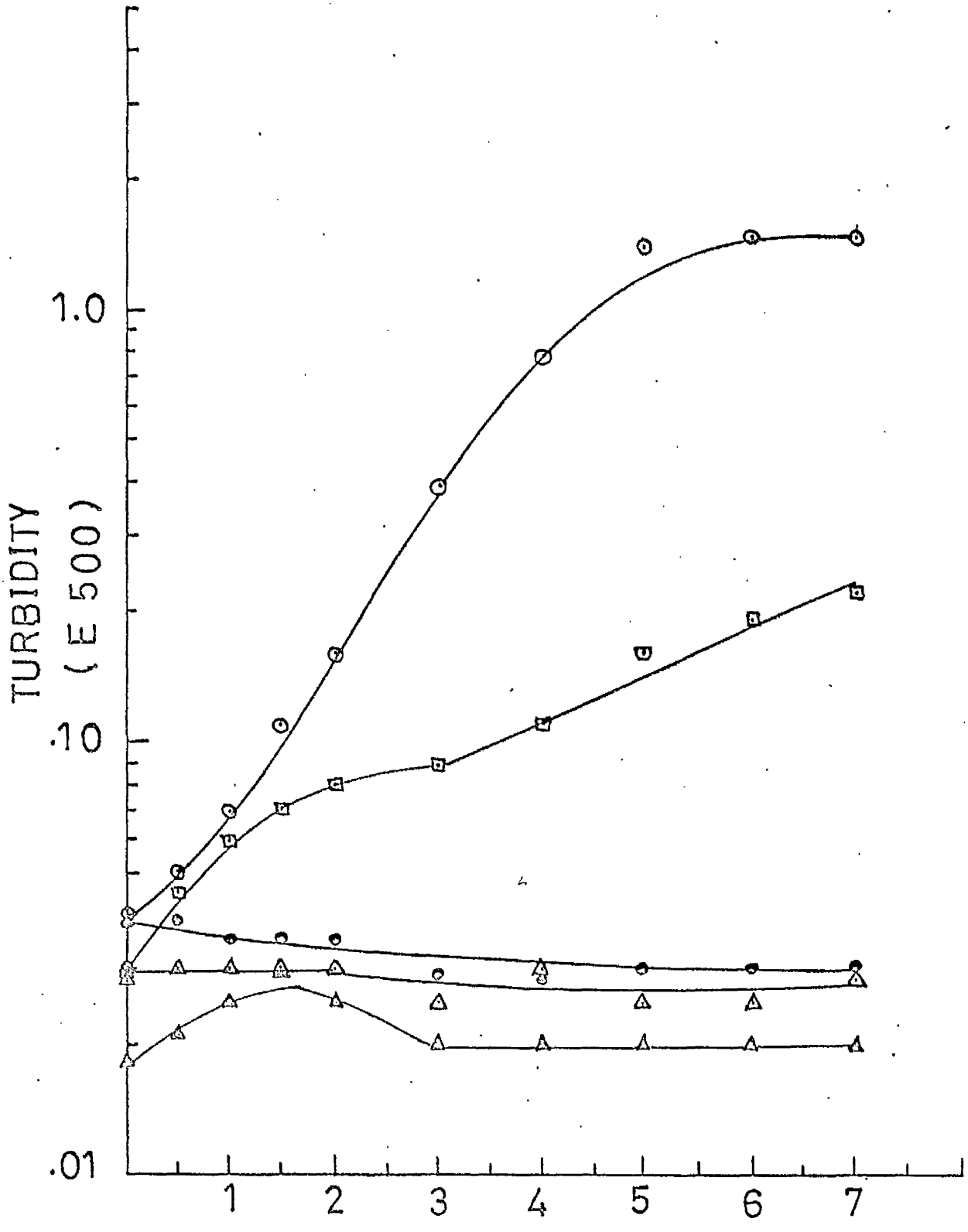


Fig. 31b

REVERSAL OF THE ACTION OF QUINOLONES

(a) Effect of added thymine and thymidine on the sensitivity of Escherichia coli 11229 to carboxyQ

Inhibition of DNA synthesis appears to be responsible for the bactericidal action of both the quinolones (see the section on macromolecular synthesis). Attempts were made to reverse the action of carboxyQ by supplying the precursors of DNA in the growth medium. At first experiments were carried out with E. coli 11229, a wild strain. Table 7 shows the result of a tube dilution test with various combinations of drug and thymine or thymidine concentrations. Inoculum was 1% and the result was recorded after overnight incubation at 37°. Growth is expressed as $\mathcal{E}350$. The drug gives 50% inhibition of growth at 10 μM concentration and no significant protection is afforded by either thymine or thymidine. E. coli 11229, being a wild strain, might not be able to utilize external thymine or thymidine for synthesis of DNA. Therefore a growth experiment was carried out with an inoculum which had been trained in GA medium with 5 μM thymine or thymidine. Figure 32 shows the result of such an experiment carried out in GA medium supplemented with 5 μM thymine with no drug, with 20 and 40 μM carboxyQ. No significant difference in the activity of the drug is observed in presence or absence of thymine. Very similar results were observed when thymine was replaced by thymidine.

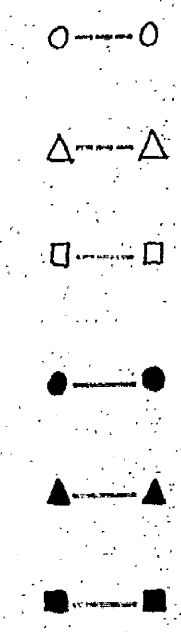
TABLE 7

Effect of thymidine and thymine on the growth of Escherichia coli. 11229 with increasing concentration of carboxyQ.

THYMIDINE CONCENTRATION µg/ml	CONCENTRATION OF CARBOXYQ (µM)					
	0	10	20	40	80	160
0	0.76	.368	.38	.185	.12	.065
1	0.74	.398	.435	.212	.16	.085
10	0.70	.333	.435	.175	.16	.085
100	0.77	.378	.415	.13	.15	.085
THYMINE CONCENTRATION µg/ml	**CONCENTRATION OF CARBOXYQ (µM)**					
	0	10	20	40	80	160
0	0.78	.338	.245	.19	.12	.06
1	0.73	.438	.245	.20	.17	.065
10	0.74	.428	.235	.205	.13	.075
100	0.71	.408	.235	.202	.10	.075

Test tubes with various drug and thymine and thymidine concentration were set up in GA medium. The inoculum was 1% and the tubes were incubated overnight. The result is recorded as $\mathcal{E}350$.

FIG. 32 : Effect of added thymine on the action of carboxyQ on Escherichia coli 11229 trained in presence of thymine.



o---o Control in GA

△---△ GA + 20 µM carboxyQ

□---□ GA + 40 µM carboxyQ

●---● Control in GA + 5 µM thymine

▲---▲ GA + 5 µM thymine + 20 µM carboxyQ

■---■ GA + 5 µM thymine + 40 µM carboxyQ

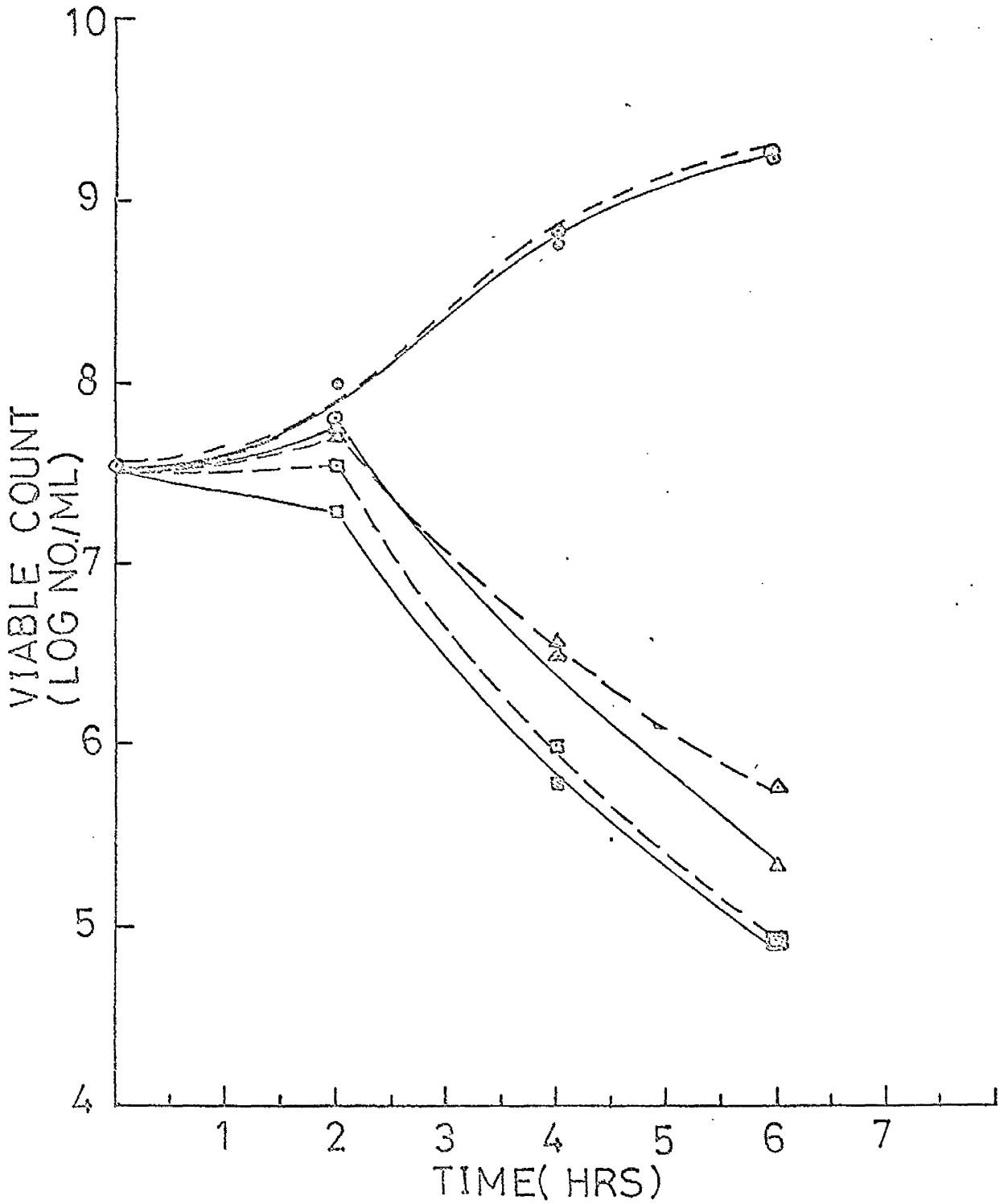


Fig. 32

(b) Effect of added thymine and deoxynucleosides on the sensitivity of Escherichia coli 8583 to carboxyQ

The precursors of DNA, deoxynucleosides are synthesized within the cell and finally assembled to form the DNA molecule in wild strains of bacteria. E. coli 8583, a thymine auxotroph, is unable to synthesize thymine within the cell due to a block in the biosynthetic pathway. Therefore, it can grow only in presence of an external source of thymine or thymidine which is taken inside the cell and utilised for biosynthesis of DNA. The biosynthetic pathway of thymine is exclusive to DNA as the other three nucleosides are synthesized by pathways common to RNA and DNA. Therefore, if a drug inhibits DNA synthesis but not RNA synthesis it must be blocking a pathway exclusive to DNA biosynthesis. In a thymine auxotroph this drug sensitive pathway may not be present and therefore addition of thymine should bring about reversal of inhibition.

In the following experiments attempts were made to bring about a reversal of action of quinolones by addition of various amounts of thymine alone and in combination with deoxyadenosine (dA), deoxycytosine (dC) and deoxyguanosine (dG).

E. coli 8583 is a thymineless mutant isolated from E. coli 8114 (E. coli 15) by ultraviolet treatment. This strain is unable to grow in a synthetic medium such as GA medium unless it is supplemented with thymine or thymidine. 5 µM thymine or thymidine is sufficient to support full growth in GA medium.

Table 8 shows the effect of 0, 5, 10, 20, 40, 80 and 160 μM thymine when tested against 0, 5, 10 and 20 μM carboxyQ. This experiment was carried out in 250 ml. conical flasks incubated in a shaking water bath. Viability was measured after three and six hours of incubation. In the series of flasks with no drug, the cells undergo thymineless death in a medium devoid of thymine and the amount of growth supported is more or less the same at all concentrations of thymine. In the series of flasks with drug, thymineless death is again observed. Whether lethal action is solely due to absence of thymine in the medium or is enhanced by presence of drug in the medium can not be definitely ascertained under these conditions. The sensitivity of the cells to drug does not alter significantly as the concentration of thymine is increased at all levels of drug except with 160 μM thymine where the number of survivors is consistently higher than lower levels of thymine.

All these cultures were further incubated for a total of 24 hours. All flasks except the ones without thymine and with 5, 10 and 20 μM drug showed full growth. The cells with no thymine and no drug had undergone thymineless death up to six hours but fully recovered during the next eighteen hours of incubation. The same phenomenon was observed with the cultures with drug and various levels of thymine. All of them underwent killing up to six hours but were fully grown after twenty-four hours. To test the possibility that these cultures represented cells that had become immune to thymineless death and resistant to drug respectively,

TABLE 8 :

A washed cell suspension of Escherichia coli 8583 was used to inoculate 50 ml. of GA medium supplemented with various concentrations of thymine and carboxyQ in 250 ml. conical flasks and incubated at 37° in a shaking water bath. Results are recorded as log number of viable cells/ml. at times indicated.

TABLE 8

Effect of increasing concentrations of Thymine on the antibacterial action of carboxyQ on *Escherichia coli* 8583

THYMINE Concn. µM	CARBOXYQ Concn. µM	TIME AFTER INOCULATION		
		Zero	3 Hours	6 Hours
0	0	6.44	4.93	2.96
5		6.44	7.01	8.09
10		6.44	6.87/7.01	8.22
20		6.34/6.44	7.01	8.32/8.42
40		6.34/6.44	6.87/7.01	8.32
80		6.34	6.87/7.01	8.42
160		6.34/6.44	7.01	8.42
0	5	6.44	4.74/4.83	2.96
5		6.44	5.84/5.94	3.75
10		6.34	5.94/6.04	3.75
20		6.34/6.44	5.94/6.04	3.85
40		6.34/6.44	5.94	3.85
80		6.44	5.94/6.04	3.85
160		6.34	6.04	4.26/4.36
0	10	6.34	4.83/4.93	2.84/2.96
5		6.44	5.47	3.05/3.15
10		6.44	4.74	3.05/3.15
20		6.44	4.56	3.05/3.15
40		6.34/6.44	5.09/5.23	3.31
80		6.34/6.44	4.86	3.15/3.25
160		6.44	4.62	3.31
0	20	6.34	3.99	2.44
5		6.34	3.85	2.96
10		6.44		3.05
20		6.34/6.44	4.16	2.96/3.05
40		6.44	4.06	3.05
80		6.34	4.16	3.05/3.15
160		6.34/6.44	5.09	3.25

they were used for further experiments. These cultures were transferred to nutrient broth and maintained as strains of E. coli 8583. Table 9 shows the results of an experiment performed with a number of these strains isolated from increasing concentrations of drug and 5 μM thymine. The growth conditions were the same as the previous experiment. The original E. coli 8583 strain showed a typical pattern of growth i.e., thymineless death in absence of thymine, normal growth in presence of thymine and high sensitivity to 160 μM carboxyQ. The strain which had become immune to thymineless death grew slightly better in absence of thymine than in its presence but it was highly sensitive to the drug. The strains (which had been isolated from cultures which had shown sensitivity to drug up to six hours but were fully grown after twenty-four hours incubation) resistant to increasing levels of drug showed a gradually increasing immunity to thymineless death and resistance to 160 μM drug. As a result the strain isolated from 30 μM carboxyQ and 5 μM thymine was fully immune to thymineless death and highly resistant to 160 μM carboxyQ. This experiment was performed in a medium supplemented with 5 μM thymine. A similar experiment with the same strains but in a medium with equimolar concentration of thymine and carboxyQ (160 μM) was performed. The results are shown in Table 10. Here again the same relationship of drug resistance and immunity to thymineless death was observed.

The addition of thymine alone did not bring about any appreciable reversal of drug action in the previous experiments. Therefore

TABLES 9 & 10 :

Growth conditions are the same as for Table 8. Results are again expressed as log number of viable cells/ml. at times indicated.

Strain Isolated From	Thymine Concn. µM	CarboxyQ Concn. µM	TIME AFTER INOCULATION				
			0 HR.	2 HRS.	4 HRS.	6 HRS.	24 HRS.
5 µM Thymine 10 µM CarboxyQ	0	0	6.52	4.16/4.26	3.31	2.84	8.79
	0	160	6.61	4.74	3.31	2.84	1.48
	5	0	6.61	6.84	7.55	8.22	8.39/8.49
	5	160	6.61	6.31	6.04	6.04	8.39/8.49
5 µM Thymine 20 µM CarboxyQ	0	0	6.87	6.40	6.87/7.01	7.55	8.79/8.89
	0	160	6.81	6.34	6.04	5.63	7.31
	5	0	6.71	7.01	7.72	8.18	8.65/8.79
	5	160	6.64	7.01	6.87	6.71	8.02
5 µM Thymine 30 µM CarboxyQ	0	0	7.01/7.11	7.72/7.82	8.32	9.09	8.79
	0	160	7.11	7.31/7.41	7.82	8.3	8.3
	5	0	7.11	7.72/7.82	8.39	9.19	8.79
	5	160	7.11	7.55	7.82	8.18/8.30	8.3

Thymine independence and drug resistance of

various strains of Escherichia coli 8583 with and without Thymine

Strain Isolated From	Thymine Concn. μM	Carboxy Q Concn. μM	TIME AFTER INOCULATION				
			0 HR.	2 HRS.	4 HRS.	6 HRS.	24 HRS.
5 μM Thymine No Drug (E. coli 8583)	0	0	5.77	3.45/3.55	2.62	2.20	3.31/3.38
	0	160	5.77	2.41	1.84	1.60	0
	5	0	5.77	6.04	7.01	7.31/7.41	8.49/8.59
	5	160	5.77	2.6	1.48	1.84	0
No Thymine No Drug	0	0	7.11	7.82	9.33	8.65/8.79	8.79
	0	160	7.11	6.64	5.47	4.62	1.8
	5	0	7.11	7.55/7.62	8.18	8.49	8.49/8.59
	5	160	7.25	6.87/7.01	5.94/6.04	4.74	2.45
5 μM Thymine 5 μM Carboxy Q	0	0	6.04	3.45	2.75	2.84/2.96	8.79
	0	160	6.14	4.16/4.26	3.15/3.25	3.05	1.60
	5	0	6.04	6.64	7.11	7.82	8.59
	5	160	6.04	5.94/6.04	5.33	4.74/4.83	8.12

TABLE 10

Thymine Independence and Drug Resistance of Various Strains of *Escherichia coli* 8583 with and without equimolar carboxyQ and thymine concentration

Strain Isolated From	Thymine Concn. µM	CarboxyQ Concn. µM	TIME AFTER INOCULATION			
			0 HR.	3 HRS.	6 HRS.	24 HRS.
5 µM Thymine No Drug (E. coli 8583)	0	0	6.87	4.06	2.75	7.01
	0	160	7.01	4.06	3.31	1.7
	160	0	6.71/6.81	7.55	8.79	9.33
	160	160	6.71	4.53	3.55	1.6
No Thymine No Drug	0	0	7.55	7.72/7.82	8.79	9.33/9.40
	0	160	7.55	5.33	4.53	1.8
	160	0	7.55/7.62	7.55	8.39/8.49	9.33
	160	160	7.72	5.94	5.03	2.84
5 µM Thymine 5 µM CarboxyQ	0	0	7.11/7.25	4.66	3.85	9.19
	0	160	6.71	4.74	4.16/4.26	2.36
	160	0	6.71	7.82	9.5	8.79
	160	160	6.61	6.71	6.4	9.6

Strain Isolated From	Thymine Concn. µM	CarboxyQ Concn. µM	TIME AFTER INOCULATION			
			0 HR.	3 HRS.	6 HRS.	24 HRS.
5 µM Thymine 10 µM CarboxyQ	0	0	6.52	4.74/4.83	4.62	9.33/9.40
	0	160	6.40	4.74	4.16/4.26	1.7
	160	0	6.52	7.41	8.39/8.49	9.6
	160	160	6.40	6.87	5.94/6.04	8.79
5 µM Thymine 20 µM CarboxyQ	0	0	7.55	8.02	9.5/9.6	9.9
	0	160	7.82	7.55	7.01/7.11	9.5
	160	0	7.25	7.72/7.82	8.79/8.89	9.5
	160	160	7.72	7.72/7.82	7.01	8.49
5 µM Thymine 30 µM CarboxyQ	0	0	7.55	8.39	9.33	9.9
	0	160	7.41/7.55	8.62	6.87/7.01	9.5/9.6
	160	0	7.72/7.82	8.49	8.89	9.7
	160	160	7.55	7.82/7.92	7.01	8.39

the other three deoxynucleosides were also added to the medium with and without 20 μM carboxyQ to observe the effect on growth and drug sensitivity of E. coli 8583. Growth conditions were the same as in previous experiments. Thymine (5 μM), was always present with dA, dC and dG (20 μM) singly or in all possible combinations with or without 20 μM carboxyQ. The results are shown in Table 11.

No significant difference was observed either in growth or drug sensitivity as judged by number of viable cells. This nonreversal of drug inhibition by added thymine and deoxynucleosides may be due to impermeability of E. coli 8583 to the added deoxynucleoside or due to improper molar ratios of thymine and deoxynucleosides.

In order to avoid the permeability barriers, the cells of E. coli 8583 were pretreated with EDTA by the method of Lieve (1965) which is claimed to make the cells of E. coli permeable as judged by a change from actinomycin insensitivity to sensitivity. The pretreatment with EDTA is essentially as follows. The cells are washed twice with 0.12MTris/Cl buffer, pH8. 5×10^{-3} M EDTA (final concentration) was added to one aliquot of this washed cell suspension and to the other a mixture of 5×10^{-3}M EDTA and 10^{-2}M $Mgcl_2$ (final concentration) was added. Both were incubated at 37° for two minutes after which 10^{-2}M $Mgcl_2$ (final concentration) was added to the first to stop the reaction. These EDTA treated and untreated suspensions were then used as inocula. The EDTA treatment causes a fall in the number of viable cells as compared to untreated cells.

Table 12 shows the effect of pretreatment with EDTA on the growth and sensitivity to carboxyQ (20 μM) with and without added

TABLE 11 :

Growth conditions are the same as for Table 8, except that an unwashed inoculum was used. Results are expressed as log number of viable cells/ml. at times indicated.

TABLE 11

Effect of added deoxynucleosides on growth of *Escherichia coli* 8583 with and without carboxyQ

CarboxyQ Concn.	Concn. of Nucleosides μM				TIME AFTER INOCULATION			
μM	T	dA	dC	dG	0 HR.	3 HRS.	6 HRS.	24 HRS.
0	5	-	-	-	7.01	7.62	8.49	8.49/8.59
20	5	-	-	-	6.61	4.26	2.96	8.65/8.79
0	5	20	-	-	6.87	7.72	8.39	8.39/8.49
20	5	20	-	-	6.81	4.74	3.15	8.39/8.49
0	5	-	20	-	6.87	7.62	8.49	8.49
20	5	-	20	-	7.01	4.36	3.25	9.5
0	5	-	-	20	6.71	7.72	8.59	8.79
20	5	-	-	20	6.61	4.26	3.05	9.5
0	5	20	20	-	6.61	7.82	8.49	9.5
20	5	20	20	-	6.61	4.56	3.25	8.89
0	5	20	-	20	6.71	7.82	8.6	8.89
20	5	20	-	20	6.87	4.83	3.15	8.65/8.79
0	5	-	20	20	6.71	7.82	8.49	8.65/8.79
20	5	-	20	20	6.61	4.26	3.05	8.65/8.79
0	5	20	20	20	6.71	7.82	8.65	8.59
20	5	20	20	20	6.61/6.71	4.62	3.25	8.49

TABLE 12 :

Escherichia coli 8583 cells were pretreated with EDTA by the method of Lieve (1965) as described in the text. Results are expressed as log number of viable cells/ml. at times indicated.

TABLE 12

Effect of pretreatment with EDTA on the growth and sensitivity to carboxyQ of Escherichia coli 8583 with and without added thymine, deoxyadenosine, deoxycytosine and deoxyguanosine

Pretreatment with EDTA	CarboxyQ Concn. µM	Thymine Concn. µM	Concentration of Nucleosides µM			TIME AFTER INOCULATION		
			dA	dC	dG	0 HR.	3.5 HRS.	7 HRS.
-	0	0	0	0	0	7.01	3.99	2.96/3.05
+	0	0	0	0	0	6.04/6.14	3.99	3.05/3.15
-	20	0	0	0	0	6.87	4.36/4.46	3.05/3.15
+	20	0	0	0	0	6.04/6.14	4.06/4.16	3.25
-	0	20	0	0	0	6.71	7.62	8.79
+	0	20	0	0	0	6.04/6.14	7.72/7.82	8.79
-	20	20	0	0	0	6.87/7.01	4.93/5.03	3.99
+	20	20	0	0	0	6.14	4.73/4.83	3.45
-	0	20	20	20	20	6.87/7.01	7.62/7.72	8.65/8.79
+	0	20	20	20	20	6.14	7.72/7.82	8.65/8.79
-	20	20	20	20	20	6.61/6.71	6.52	6.4/6.52
+	20	20	20	20	20	6.14	6.52	6.81

thymine, dA, dC and dG (20 μM). Both treated and untreated cells (a) undergo thymineless death to the same extent; (b) grow equally well in presence of thymine alone or with the other three deoxynucleosides; and (c) are equally sensitive to 20 μM carboxyQ in presence of 20 μM thymine alone; (d) are slightly protected against the lethal effect of drug when 20 μM each of dA, dC and dG are present along with 20 μM thymine. The EDTA treatment does not cause any significant difference in the growth and sensitivity to carboxyQ of E. coli 8583. Protection of cells against carboxyQ is manifested only when thymine and the other three deoxynucleosides are present in equimolar (20 μM each) concentrations. EDTA treatment does not alter the protective effect of equimolar concentrations of thymine, dA, dC and dG.

In the last experiment the addition of equimolar (20 μM) thymine and the other three deoxyribonucleosides gave some protection against 20 μM carboxyQ and this protection was not significantly altered in the EDTA treated cells. The duration of EDTA treatment was increased from two minutes to four and eight minutes to observe any further protection against 20 μM carboxyQ. This increase in EDTA treatment caused an appreciable loss of viable cells in the inocula. This loss of viability was proportional to the duration of the EDTA treatment.

Table 13 shows the effect of increased EDTA treatment on protection against 20 μM carboxyQ. Here again partial reversal of drug action was observed. There was no appreciable difference in the number of survivors at the end of six hours, between the untreated and EDTA treated cells.

TABLE 13 :

Escherichia coli 8583 cells were pretreated with EDTA for longer time periods as shown by the method of Lieve (1965) as described in the text. Results are expressed as log number of viable cells/ml. at times indicated.

TABLE 13

Effect of Increase in EDTA treatment on the survival and carboxyQ sensitivity of *Escherichia coli* 8583 in presence of thymine, deoxyadenosine, deoxycytosine and deoxyguanosine

Duration of EDTA treatment Min.	CarboxyQ Concn. µM	Thymine Concn. µM	Deoxynucleoside Concentration µM			TIME AFTER INOCULATION		
			dA	dC	dG	0 HR.	3 HRS.	6 HRS.
NIL	-	20	20	20	20	6.81	7.55/7.62	8.65/8.79
NIL	20	20	20	20	20	6.87/7.01	6.61	5.94
2	-	20	20	20	20	6.61/6.71	7.22	7.55
2	20	20	20	20	20	6.61	6.31	5.84
4	-	20	20	20	20	6.04	6.87/7.01	8.09
4	20	20	20	20	20	6.04	6.61/6.71	5.77
8	-	20	20	20	20	4.93	7.01	8.39/8.49
8	20	20	20	20	20	4.93	6.81	6.52/6.61

(c) Reversal of action of quinolones by removal

The bactericidal effect of quinolones can be reversed by merely washing and resuspending the cells in drug free medium. Cells of E. coli 11229 were treated with either carboxyQ or nitroQ for various durations. The cells were collected by centrifugation and washed once in warm drug free GA medium. Then they were resuspended in prewarmed GA medium and the incubation continued. The whole procedure took about an hour. Figures 33a and 33b show the effect of removal of carboxyQ at 1, 2 and 3 hours. When the drug was removed at 1 or 2 hours the cells started to grow immediately without a lag while a lag of about an hour was observed when drug was removed at 3 hours.

A similar experiment was performed with nitroQ treated cells of E. coli 11229. Figures 34a and 34b show the results of this experiment. Here the drug was removed at 30 minutes, 1 and 2 hours. Here again the cells recovered but after a time lapse.

FIG. 33 : Reversal of action of carboxyQ on *Escherichia coli* 11229 by removal.

(a) Viability

(b) Turbidity

O—O	Control in GA
Δ—Δ	GA + 20 μM carboxyQ not removed
□—□	GA + 20 μM carboxyQ removed at 1.00′
●—●	GA + 20 μM carboxyQ removed at 2.00′
▲—▲	GA + 20 μM carboxyQ removed at 3.00′

Arrows indicate the time of removal of carboxyQ. Broken lines represent the time spent in collecting and washing the cells before start of reincubation.

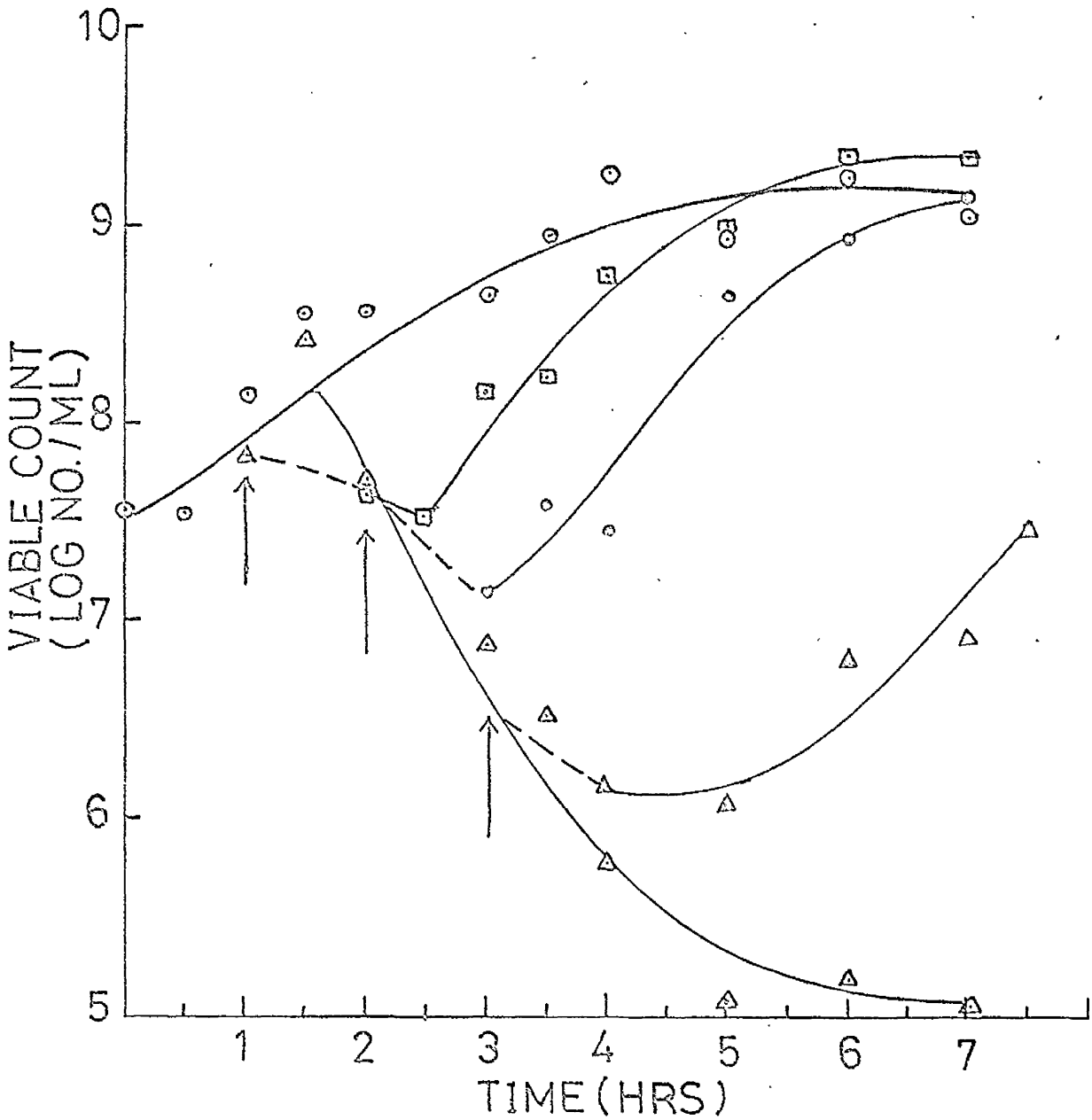


Fig. 33a

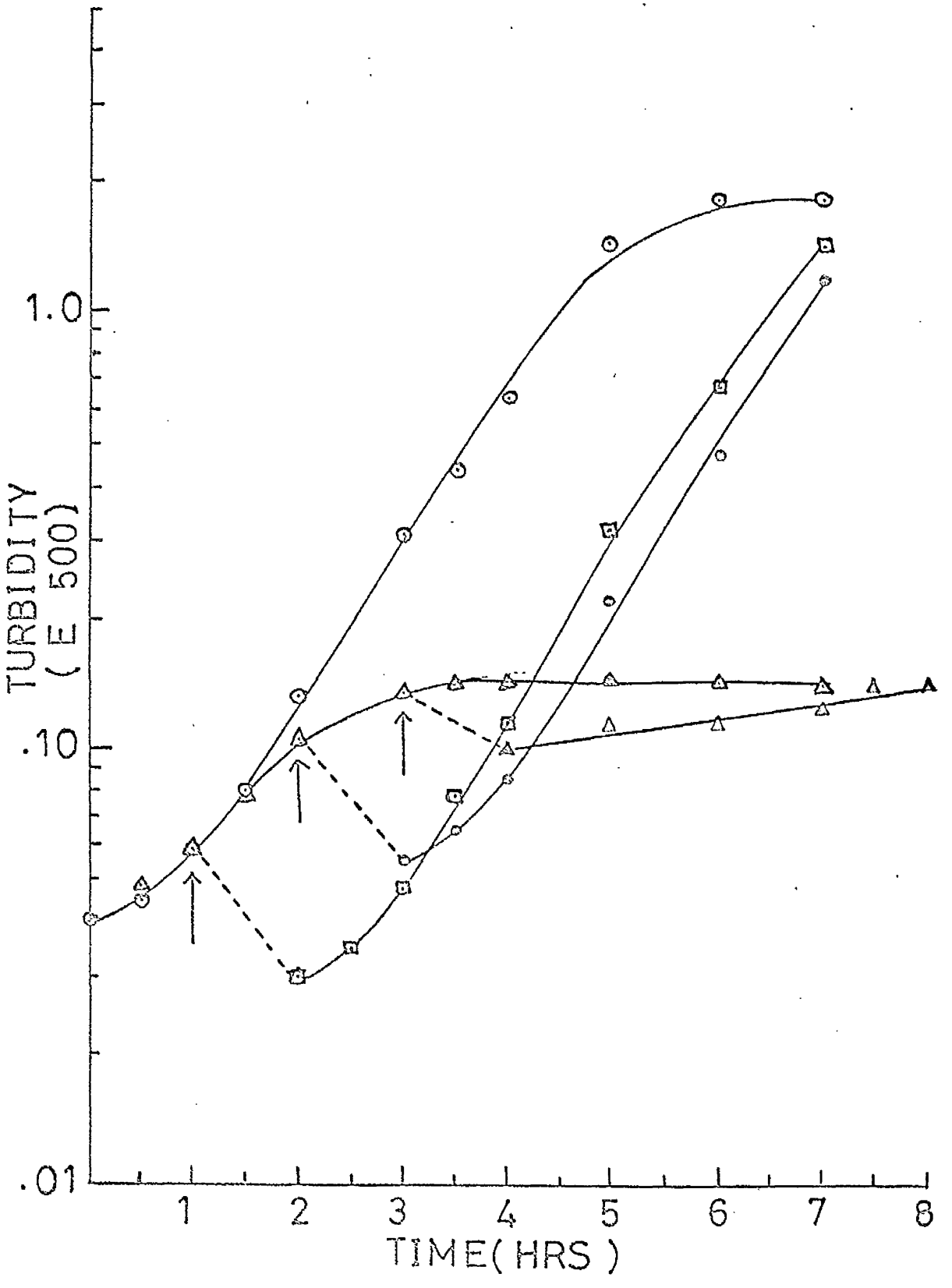


Fig. 33b

FIG. 34 : Reversal of action of nitroQ on *Escherichia coli* 11229 by removal.

(a) Viability

(b) Turbidity

o—o	Control in GA
△—△	GA + 20 μM nitroQ not removed
□—□	GA + 20 μM nitroQ removed at 30′
●—●	GA + 20 μM nitroQ removed at 1.00′
▲—▲	GA + 20 μM nitroQ removed at 2.00′

Arrows indicate the time of removal of nitroQ. Broken lines represent the time spent in collecting and washing the cells before start of reincubation.

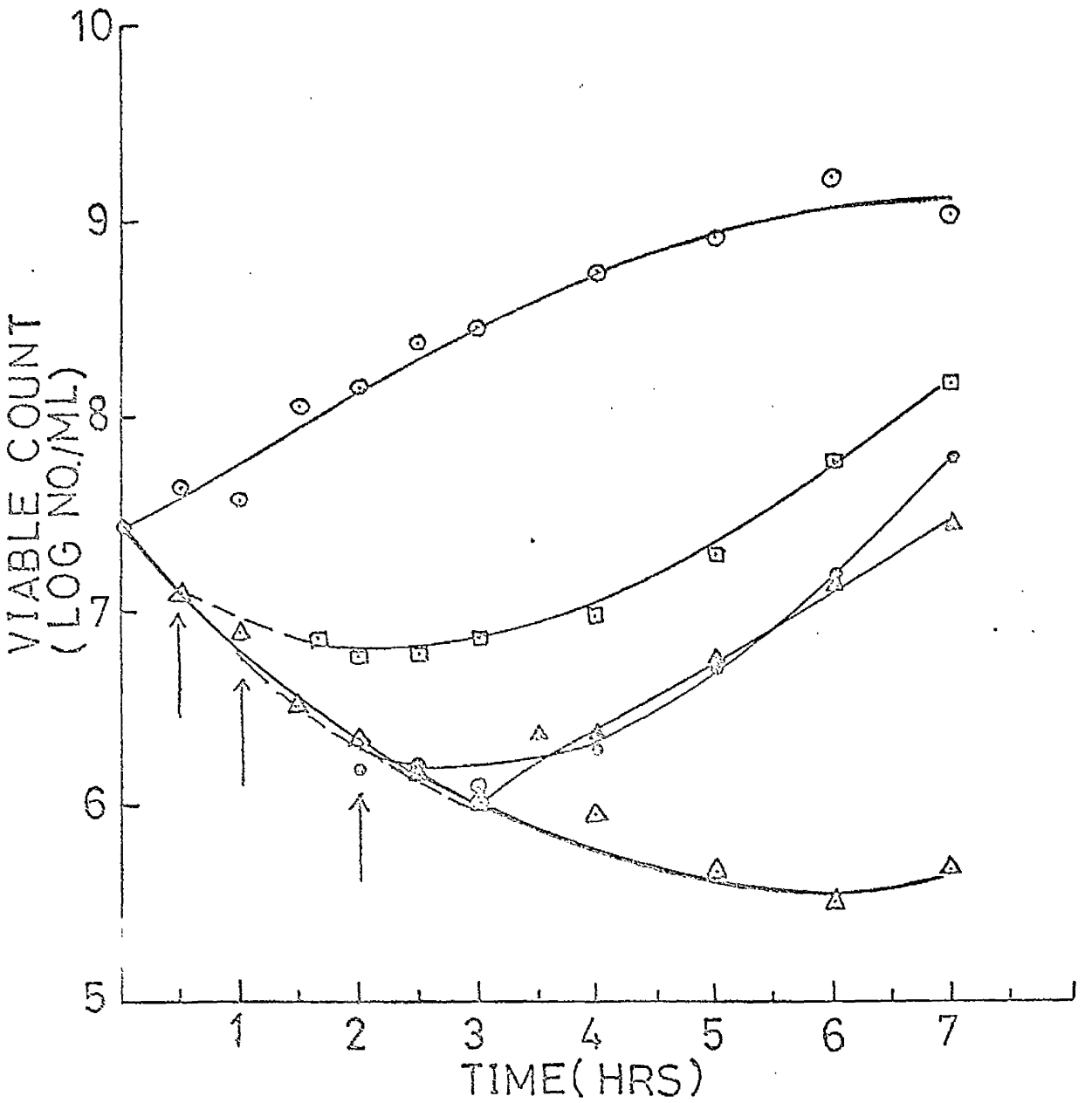


Fig. 34a

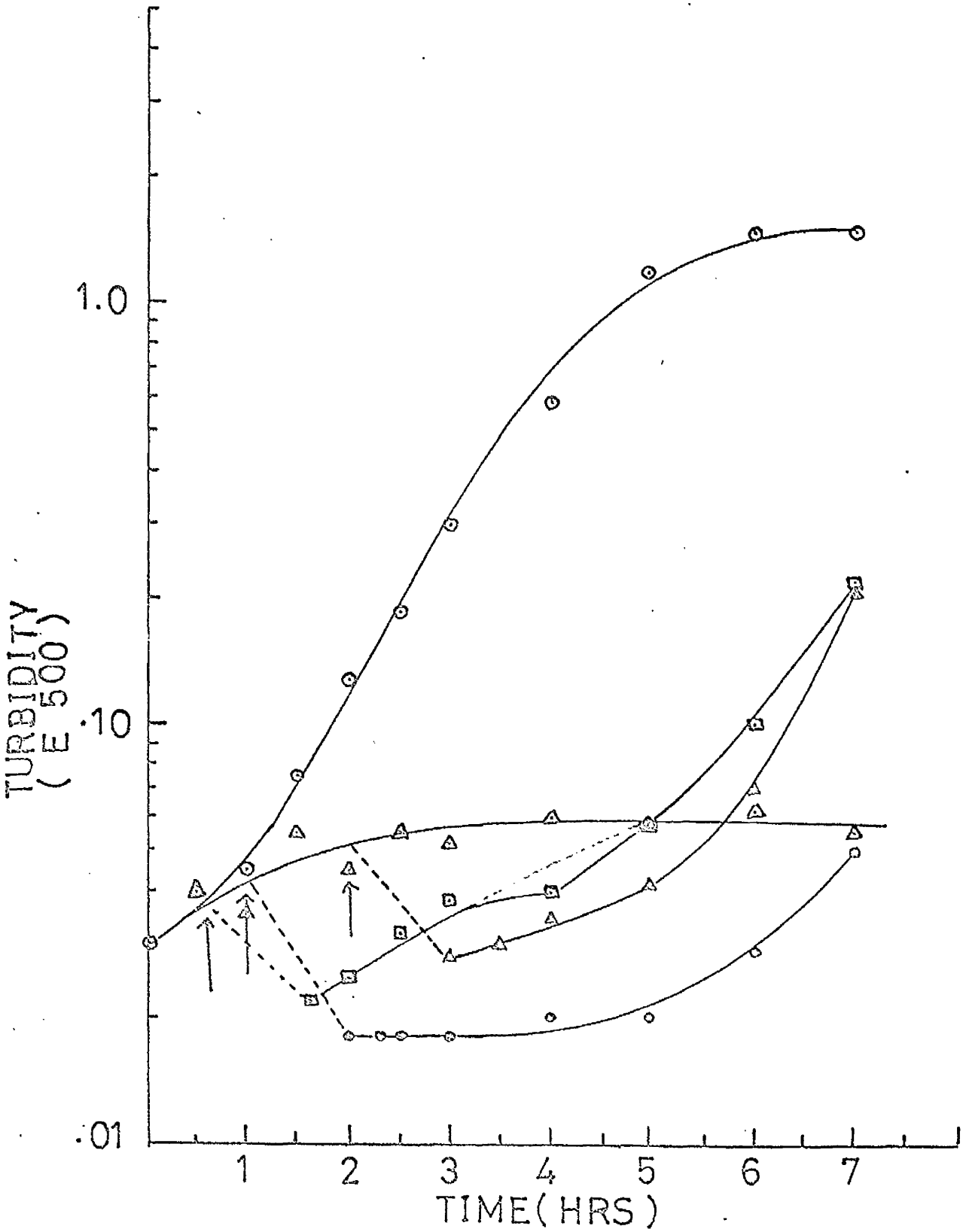
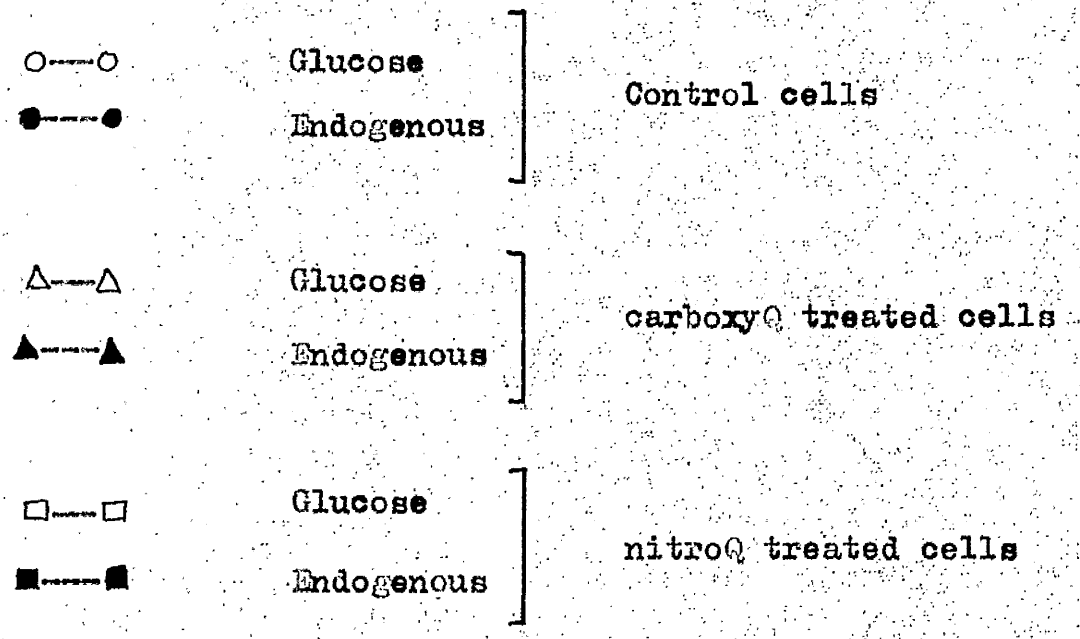


Fig. 34b

Effect of Quinolones on Respiration of Escherichia coli 11229

Figure 35 shows the rate of oxygen uptake endogenously and with glucose by resting cell suspensions of cultures grown without drug and with 20 μM each of carboxyQ and nitroQ. The cells were harvested at one hour intervals up to four hours and the results are expressed as Qo_2 (μl O_2 uptake/mgs protein/hour). The no drug control shows an immediate rise in Qo_2 with a peak at two hours. The cells grown in presence of 20 μM carboxyQ also show a slight rise up to an hour after which the rate of respiration falls rapidly in the next three hours. On the other hand the cells grown in presence of nitroQ show an immediate inhibition of rate of respiration up to three hours after which the Qo_2 rises slightly.

FIG. 35 : Effect of quinolones on the respiration rate of resting cells of Escherichia coli 11229 expressed as Q_{O_2} (µl O_2 uptake/mg protein/hour).



Each Warburg vessel received 5 mg of cells suspended in 100 mM phosphate buffer pH 7.0, 25 mM glucose (tipped in from side arm) and 0.2 ml of 20% KOH in the centre well in a total volume of 2 ml.

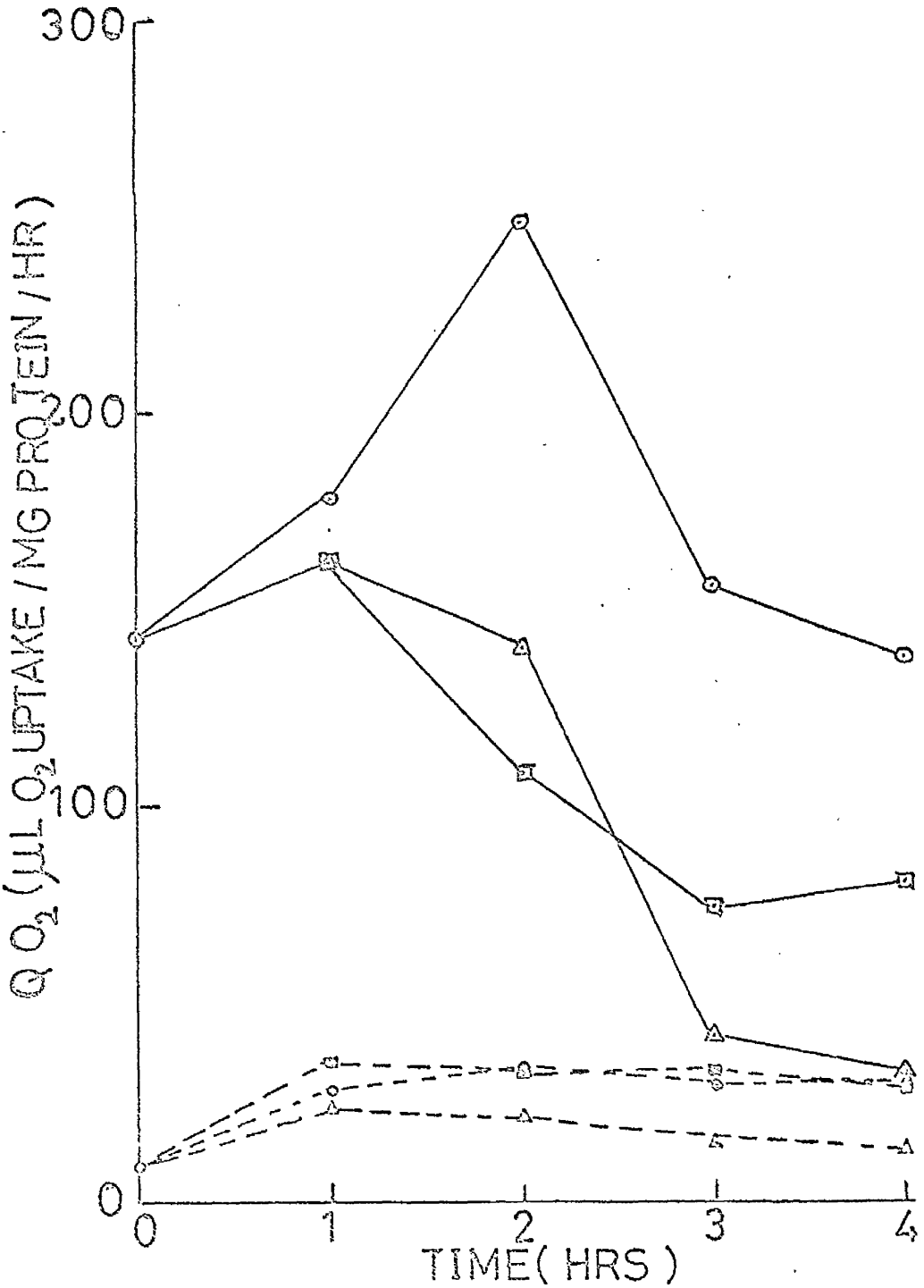


Fig. 35

Figure 36 shows the effect of quinolones on the respiration of growing cells of Escherichia coli 11229. The control shows steady oxygen uptake after a lag of about 20 minutes. The nitroQ (20 μM) inhibits the respiration completely. On the other hand the cells growing in presence of 20 μM carboxyQ show a much higher oxygen uptake as compared to the control.

FIG. 36 : Effect of quinolones on oxygen uptake of growing cells of Escherichia coli 11229.

O—O Control

Δ—Δ carboxyQ

□—□ nitroQ

Each Warburg vessel received 150 mmoles ammonium sulphate, 15 m moles magnesium sulphate, 25 m moles glucose, 10 m moles phosphate buffer and 0.2 ml 20% KOH in centre well. The control Warburg flask received no drug while 20 μM each of carboxyQ and nitroQ were present in the other two. The inoculum cells were 10% tipped in from the side arm.

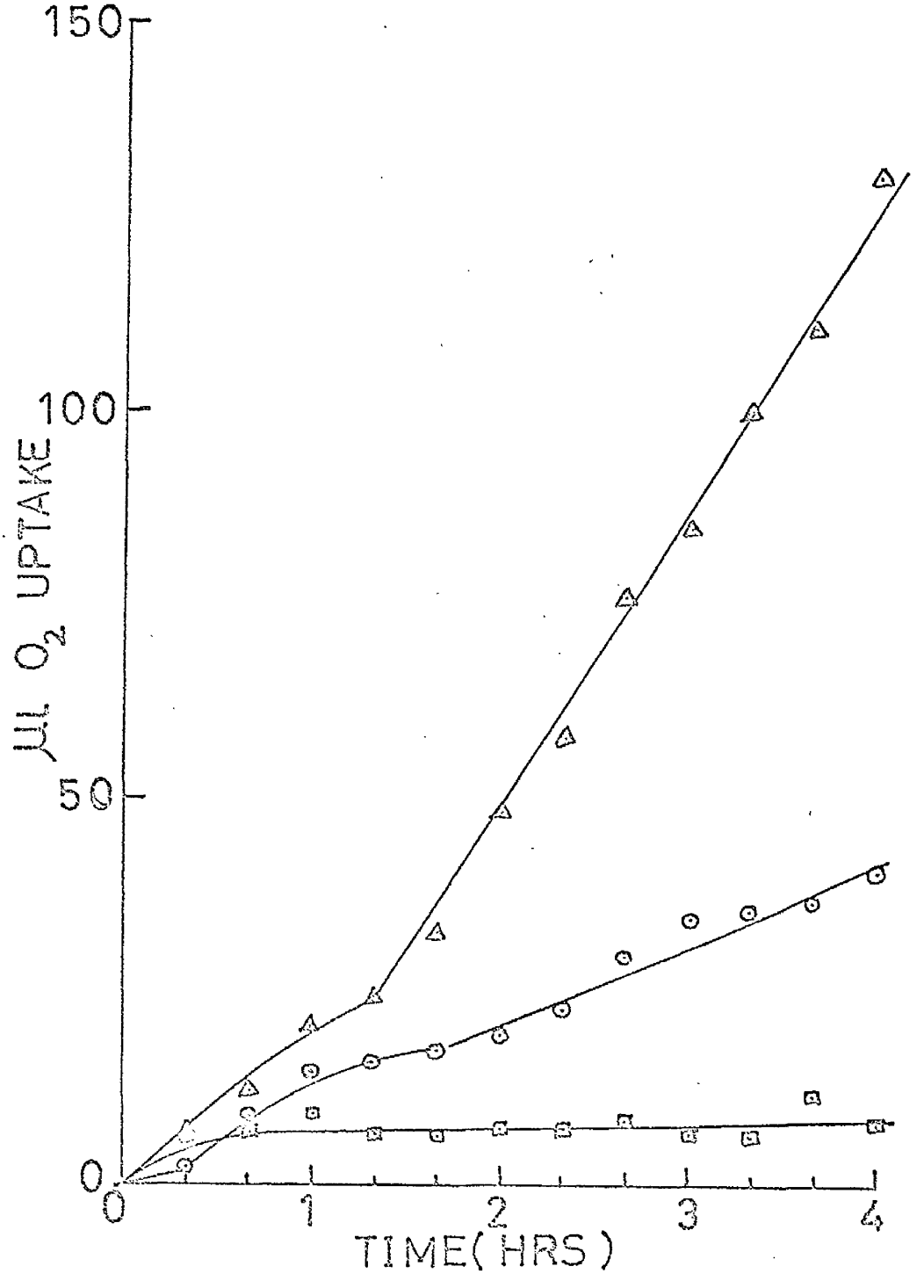


Fig. 36

Effect of quinolones on macromolecular synthesis in Escherichia coli 11229

Effect of both carboxyQ and nitroQ on the net synthesis of protein, RNA and DNA synthesis was studied. Figures 37 and 38 show the effect of carboxyQ and nitroQ respectively. The cells were harvested at the end of seven hours incubation and fractionated and assayed for protein RNA and DNA as described in the Methods. CarboxyQ has no effect at all on RNA synthesis even up to 20 μM concentration. Protein synthesis, however, is slightly inhibited by bactericidal concentrations. The most drastic effect is observed on DNA synthesis which is inhibited significantly even at 5 μM concentration which is only slightly bactericidal. Further increases up to 10 and 20 μM do not show any further appreciable inhibition of DNA synthesis.

NitroQ has an immediate lethal effect but the viable counts always show an increase after a period of time even with high drug concentrations. Figure 38 shows the effect of nitroQ on macromolecular synthesis after seven hours of incubation. The 5 and 10 μM concentrations are bactericidal only in the first hour after which they start to grow (see Figure 9a on page 75a). The 20 μM concentration is lethal up to six hours when it also shows a slight increase in viable count. Due to this regrowth, the net synthesis of protein, RNA and DNA is not appreciably different by no drug cells and with drug cells. However, here also DNA synthesis is the process most drastically inhibited. Due to the immediate lethal effect of nitroQ, enough cell material can not be obtained for

FIG. 37 : Effect of carboxyQ on macromolecular synthesis by Escherichia coli 11229.

The cells were grown for 7 hours with various concentrations of carboxyQ. They were washed and resuspended at a constant wet weight (20 mg./ml.). The suspensions were then fractionated as described in the Methods.

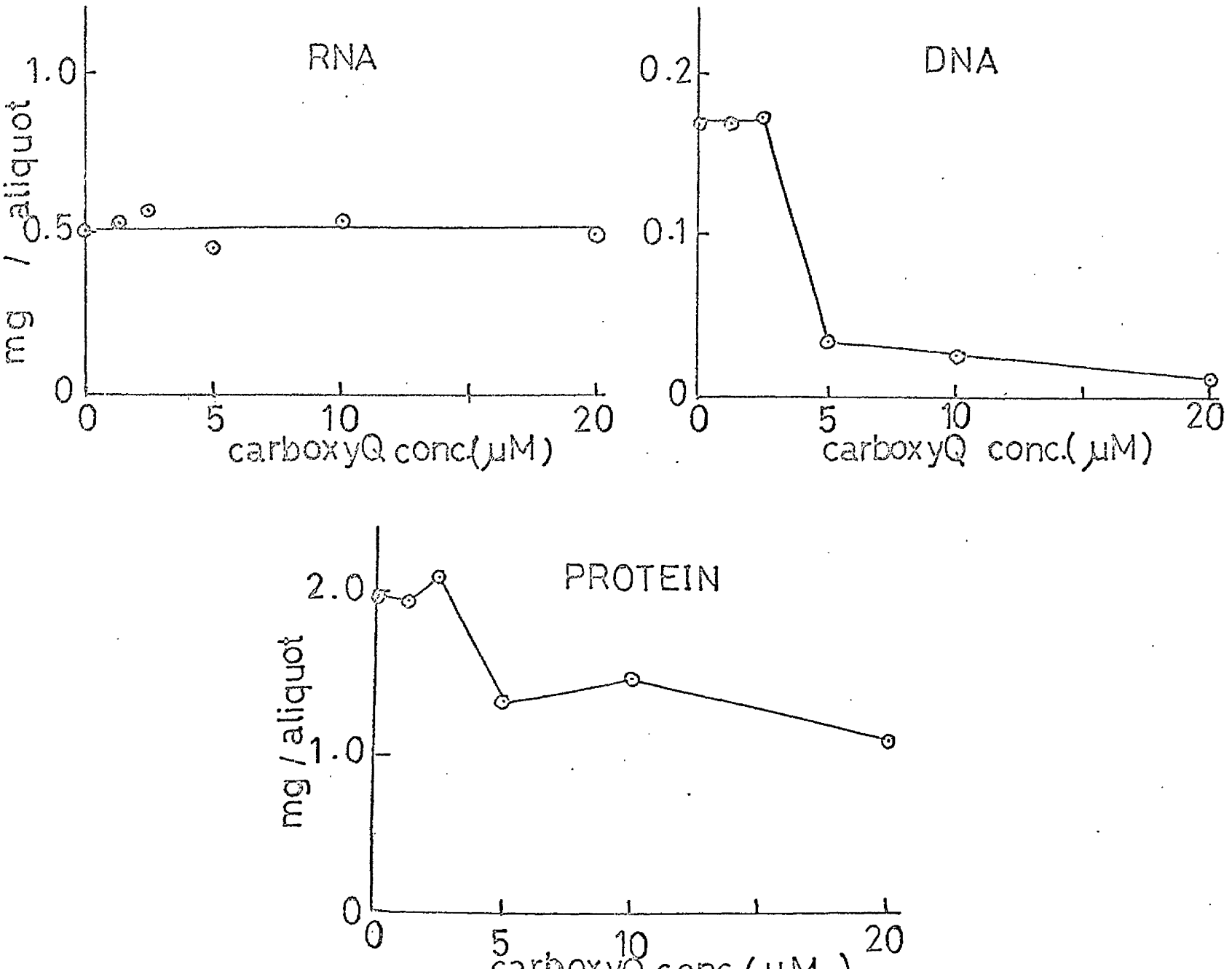

RNA
mg / aliquot
1.0
0.5
0
0
5
10
20
carboxyQ conc.(μM)
DNA
0.2
0.1
0
0
5
10
20
carboxyQ conc.(μM)
PROTEIN
mg / aliquot
2.0
1.0
0
0
5
10
20
carboxyQ conc.(μM)

Fig. 37

FIG. 38 : Effect of nitroQ on macromolecular synthesis by Escherichia coli 11229.

The conditions were the same as in Fig. 37.

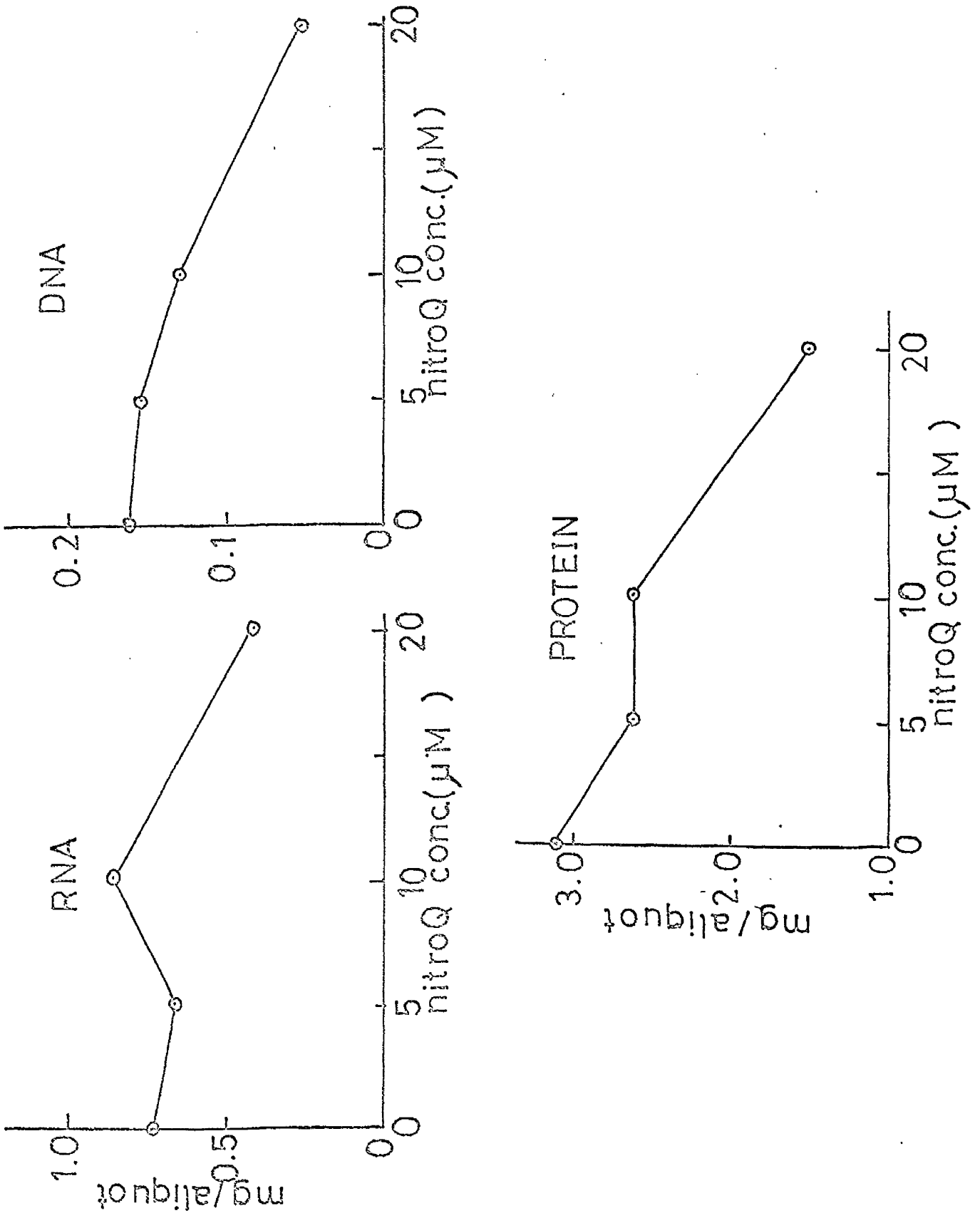
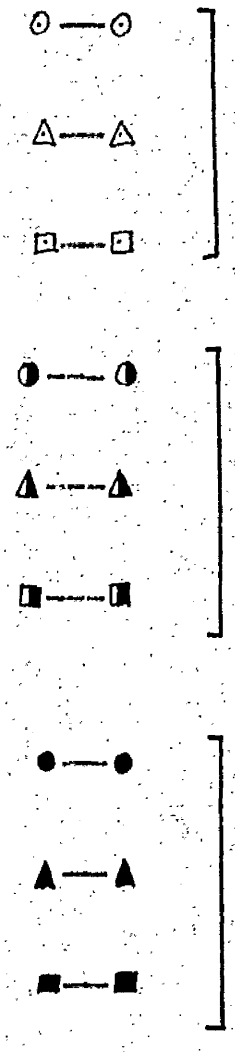

DNA
0.2
0.1
0
0
5
10
20
nitroQ conc.(μM)
RNA
1.0
0.5
0
mg/aliquot
PROTEIN
3.0
2.0
1.0

Fig.38

FIG. 39 : Resistance of Escherichia coli 11229 to nitroQ in absence and presence of nitroQ in the counting medium.



	Growth medium	Counting medium
⊙—⊙	GA	No drug
△—△		+ 10 µM nitroQ
⊡—⊡		+ 40 µM nitroQ
◑—◑	GA + 10 µM nitroQ	No drug
◭—◭		+ 10 µM nitroQ
◧—◧		+ 40 µM nitroQ
●—●	GA + 40 µM nitroQ	No drug
▲—▲		+ 10 µM nitroQ
■—■		+ 40 µM nitroQ

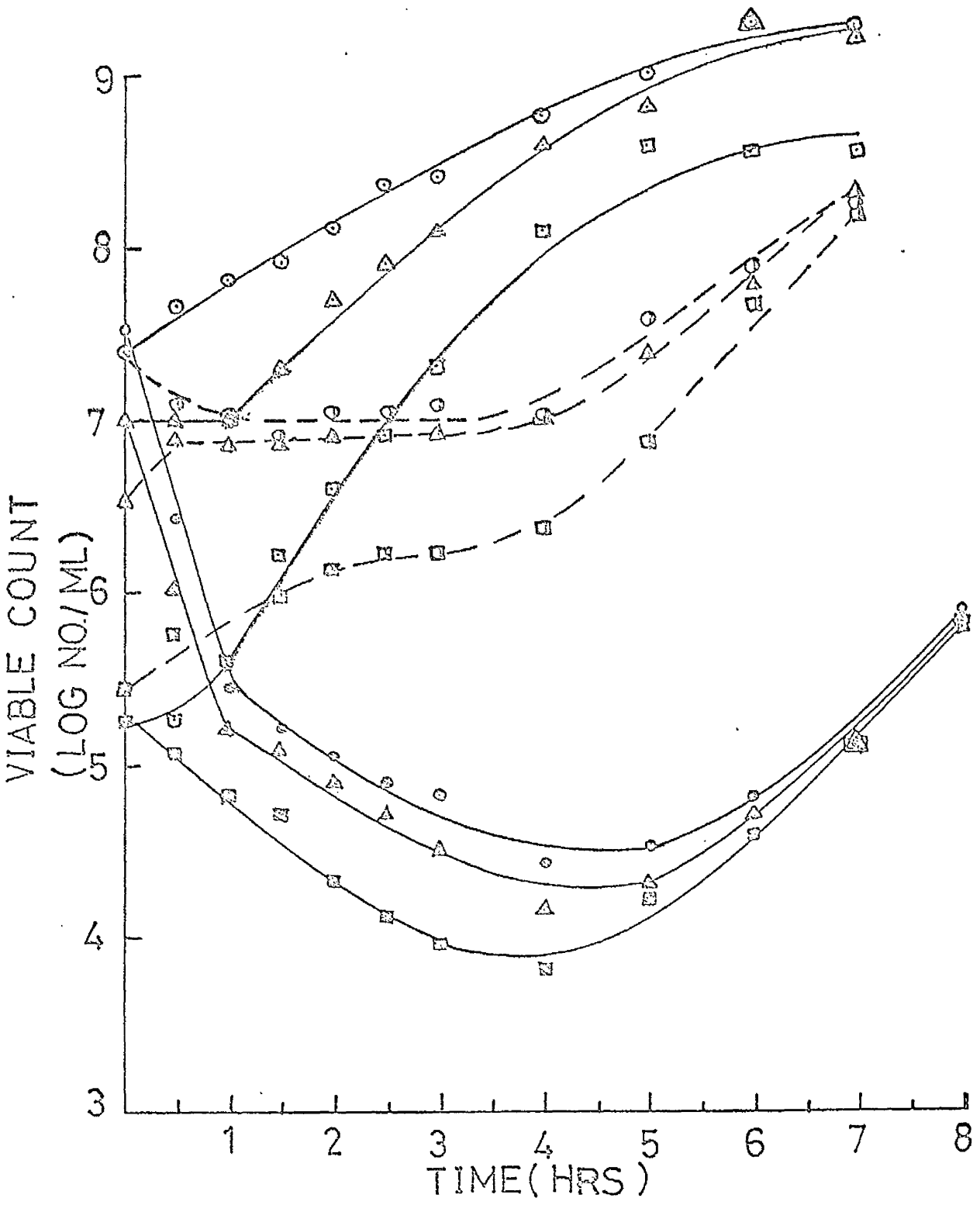


Fig. 39

biochemical analysis with higher drug concentrations. Increasing the inoculum size changes the sensitivity to nitroQ and thus the effect on macromolecular synthesis. Therefore it was not possible to obtain a sufficiently accurate picture of macromolecular synthesis during inhibition by nitroQ.

Tables 14 and 15 show the effect of quinolones on the inter-relationships of the macromolecules. RNA and DNA ratios increase with increasing drug concentrations particularly with bactericidal concentrations. RNA and protein remain more or less at a constant ratio at all the drug concentrations but DNA/protein ratios fall considerably by increasing the drug concentration.

TABLES 14 & 15 :

Escherichia coli 11229 was grown in GA medium with various concentrations of carboxyQ and nitroQ for 7 hours. RNA, DNA and proteins were estimated by procedures described in Methods. Parenthesis () indicate that the amounts were too low for accurate measurements.

T A B L E 14

Effect of carboxyQ on macromolecular synthesis by *Escherichia coli* 11229

	CONCENTRATION OF CARBOXYQ					
	0 μM	1.25 μM	2.5 μM	5 μM	10 μM	20 μM
RNA/DNA	3.09	3.20	3.34	12.5	22.8	43.6
RNA/Protein %	26	28	28	35	35	46
DNA/Protein %	8.4	8.7	8.2	2.8	1.5	.93
Protein/D.W %	36	39	41	35	( )	( )
RNA/D.W %	9.4	10.7	10.9	11.8	( )	( )
DNA/D.W %	3.03	3.36	3.27	.95	( )	( )

T A B L E 15

Effect of nitroQ on macromolecular synthesis by *Escherichia coli* 11229

	CONCENTRATION OF NITROQ			
	0 μM	5 μM	10 μM	20 μM
RNA/DNA	4.52	4.29	6.64	8.50
RNA/Protein %	29	28	40	33
DNA/Protein %	6	7	6	4
Protein/D.W %	74.9	58.7	94.1	33.8
RNA/D.W %	21.5	16.7	37.4	11.2
DNA/D.W %	4.8	3.9	5.6	1.3

RESISTANCE TO QUINOLONES

(a) Resistance to nitroQ

Cultures of *E. coli* exposed to nitroQ have a tendency to grow out after a period of treatment. This population could represent either a fraction of original population which is resistant to drug or merely slowgrowing cells. Fig. 39 shows the result of an experiment to test the presence of resistant cells in the original population. *E. coli* 11229 was grown in GA medium with no drug, and with 10 and 40 μM nitroQ. Samples from each of these flasks were introduced into counting medium with no drug and with 10 and 40 μM nitroQ. The rationale behind this approach was that if some cells in the samples are resistant to drug, they will be able to form colonies even in presence of drug in the counting medium. On the other hand, if all the cells are sensitive, no colonies will appear in the counting medium with drug. The results show that when cells are grown without drug and counted in medium with and without drug, about 50% of cells are resistant to 10 μM nitroQ and about 1% to 40 μM nitroQ even though they had never been exposed to drug. Approximately the same ratio of sensitive and resistant cells was observed when the cells were grown in presence of drug. In each case they were able to grow out, even in presence of drug in the counting medium, after a time depending on the concentration of drug in growth medium.

(b) Resistance to carboxyQ

Cultures grown in presence of carboxyQ do not show the growing out phenomenon as observed with nitroQ. However, they can be trained to become resistant after a number of subcultures in the media containing gradually increasing concentrations of carboxyQ.

E. coli 11229 was tested in the conventional tube dilution test for sensitivity against carboxyQ. The tubes were incubated overnight. Appreciable growth was allowed only in 5 μM concentration. These cells when tested, were found to be completely resistant to up to 80 μM concentration. The cells which had grown in presence of 80 μM were in turn resistant to 160 μM concentration, the highest concentration used in this study. These highly resistant cells were cloned and the single cell isolates were found to be resistant to carboxyQ to the same extent even after subcultures in nutrient broth and GA medium.

In the thymineless mutant, E. coli 8583, immunity to thymineless death was observed after an initial period of thymineless death. The cells tested in presence of increasing concentration of thymine and carboxyQ also showed growth, after being killed for the first six hours of incubation (Table 8).

The various strains isolated from the above experiment were cloned and tested for immunity to thymineless death and drug resistance (Table 9 and 10). The results show that the strain which had become thymine-independent was sensitive to carboxyQ to the same extent as the original E. coli 8583. However, the strains isolated from increasing concentrations of carboxyQ showed an increasing level of thymine independence proportional to the drug resistance level of each strain.

D I S C U S S I O N

Quinolones are antibacterial drugs active against Escherichia coli and Salmonella species (Holms, unpublished data). They exhibit both bacteriostatic and bactericidal actions against the three strains of E. coli viz., E. coli 11229/198, E. coli 8114 (15) and E. coli 8583 ($15T^-$) a thymineless strain derived from E. coli 8114, used for this study. The quinolones have been divided into two broad groups, the carboxylic and noncarboxylic, on the basis of the different patterns of growth inhibition observed with the two groups.

The overall sensitivity to quinolones varies with the strain although the different patterns of inhibition of growth exhibited by carboxyQ (N-ethyl-3-carboxyl-7-chloro-4-quinolone) and nitroQ (1, 2-dimethyl-6-nitro-4-quinolone) are maintained in each strain. As the concentration of drugs is increased there is a shift from bacteriostatic to bactericidal effect. The concentrations when this change takes place vary according to the sensitivity of the strain to each drug. With E. coli 11229 and 8114, this critical concentration is between 2.5 and 5 μM for carboxyQ (Figs. 7 and 10) and between 10 and 20 μM for nitroQ (Figs. 9 and 11). E. coli 8583, a thymineless auxotroph, is much more sensitive to carboxyQ than the two wild strains. There is a great reduction in the growth and multiplication rate even with 2.5 μM carboxyQ (Fig. 12) while there is little or no growth inhibition at this concentration with the other two strains. The sensitivity of E. coli 8583 to nitroQ is about the same order as for the other two strains (Fig. 13)

Both the quinolones induce the formation of long filamentous cells from the short coccobacillary forms of normal E. coli cells (Fig. 14). In E. coli 11229 there was no difference in the total cell mass with 2.5 μM carboxyQ as compared to the control, but the average cell size is increased in the early stages of growth resulting in a slight fall in number of colony forming units (Figs. 15a and 7). By the end of the experimental period the cell size falls to nearly the same as control and thus the final cell number is also nearly the same as control. At 5 μM concentration the average cell size is increased fivefold due to inhibition of cell division resulting in a decrease in colony forming units. At this concentration there is some reduction in the total mass as well. Similarly with nitroQ in E. coli 11229 there is little or no inhibition of total cell mass synthesis up to 10 μM but the average cell size is appreciably increased. The increase in size in presence of nitroQ is not as great as with carboxyQ (Figs. 9 and 15b). At bactericidal concentrations of both drugs, there is an actual decrease in colony forming ability accompanied by appreciable reduction in the total cell mass as measured turbidimetrically. The average cell size is also not increased to the same extent as with bacteriostatic concentrations.

The development of similar long forms has been observed under a variety of conditions. They have been induced in E. coli by thymine deficiency (Cohen & Barner, 1954); by 5 fluorodeoxyriboside (Cohen et al, 1958); by vitamin B_{12} deficiency (Beck, Hook & Barnett, 1962); by mitomycin C (Reich, Shatkin & Tatum, 1961); by phenethyl alcohol (Berrah & Konetzka, 1962); by nalidixic

acid (Goss, Deitz & Cook, 1964) and by hydroxyurea (Rosenkranz, Garro, Levy and Carr, 1966). Cell elongation has also been observed when E. coli cells are exposed to low doses of ultraviolet light or to X-rays (Hughes, 1956). Most of these conditions have been found to cause an inhibition of DNA synthesis without concomitant inhibition of protein and RNA synthesis, the socalled "unbalanced growth". In absence of DNA synthesis the cell is unable to divide but grows in size due to uninhibited protein and RNA synthesis. The appearance of enormously elongated cells in quinolone-treated cultures gave strong presumptive evidence that they also are causing "unbalanced growth" in E. coli. Our starting hypothesis was therefore that inhibition of growth and loss of viability could be attributed to preferential inhibition of DNA synthesis.

Filamentous cells are formed in presence of both bacteriostatic and bactericidal concentrations of quinolones (Figs. 14 and 15). The increase in size is most marked in lower than in higher concentrations in the case of carboxyQ (Fig. 15a). Suzuki, Pangborn & Kilgore (1967) have reported similar relationship between mitomycinC concentration and filament formation. They have shown, by electron microscopy, characteristic differences in the arrangement of nuclei in the filaments formed in presence of low and high concentrations of mitomycin C. DNA synthesis is inhibited at the bactericidal levels of mitomycin C but not at bacteriostatic levels. These workers suggest that the formation of filaments under bacteriostatic concentration is due to an unknown mechanism of division inhibition

while that under bactericidal concentrations is due to inhibition of DNA synthesis. It is quite possible that similar situation exists in the case of carboxyQ but not nitroQ where the increase in average cell size is not very marked and is proportional to the concentration of drug (Fig. 15b). This suggests that the filaments formed in presence of nitroQ probably arise due to inhibition of DNA synthesis while the two mechanisms described by Suzuki, Pangborn & Kilgore (1967) may be operating in the case of carboxyQ.

Conditions required for lethality

CarboxyQ and nitroQ exhibit different killing patterns. Many kinds of experiment show that their activity depends on the physiological state of the cell and not on the drug to cell density ratio. When nitroQ is added at various times during the lag and early logarithmic phases, the drug is equally active during lag phase but its activity diminishes with the onset of active growth (Fig. 18). When experiments were carried out with high inocula carboxyQ is without effect during lag phase but exerts its lethal action as soon as the controls start to actively multiply (Fig. 16). On the other hand, nitroQ is active immediately after inoculation. Its lethal action does not coincide with the onset of growth in the control but actually is reduced when the control starts growing. (Fig. 17).

CarboxyQ requires growth for its lethal action. When growth is stopped by addition of chloramphenicol, which inhibits protein synthesis, the activity of carboxyQ is greatly reduced. The action of nitroQ is not reduced by chloramphenicol but is in fact slightly enhanced, indicating a nonrequirement of protein synthesis and hence growth (Fig. 19).

The lethal activity of carboxyQ is proportional to the rate of growth. *E. coli* 11229, when grown under anaerobic conditions, shows a long lag and about 50% reduction in growth rate. CarboxyQ did not have any activity during the 3 hr. lag. The lethal activity again coincided with the onset of growth in the control culture and the rate of killing was greatly reduced when compared

to that under aerobic conditions. The activity of nitroQ was unaffected by the conditions of aeration (Fig. 20).

CarboxyQ is without any activity against starving cells and also when added along with the cells to media deficient in either carbon/energy source or nitrogen source. NitroQ is inactive against cells starved of energy source. It shows slight activity when added along with the cells to medium deficient in energy source presumably due to endogenous sources of energy. However, it is highly active against cells suspended in a medium devoid of a nitrogen source (Figs. 21 and 22). These facts suggest that carboxyQ loses its antibacterial properties when growth is prevented either by lack of energy/carbon source or by lack of nitrogen source. NitroQ does not require growing cells but does require a source of energy in order to kill the cells. Addition of glucose to an otherwise complete medium with quinolones results in an immediate lethal action of nitroQ while carboxyQ is active only after a period of induction in the full medium. Preincubation with the drugs does not change the pattern (Figs. 23 and 24). This indicates that carboxyQ has an absolute requirement for conditions allowing growth and the induction period required is not for adsorption or transport of drug to its sensitive site. The sensitive site of this drug is closely associated with growth and metabolism. In absence of growth this sensitive site or metabolic step does not present itself so that carboxyQ is unable to exert any effect on the viability of cells. NitroQ does not require growth conditions and therefore is capable of exerting inhibitory effect even on nongrowing cells.

The activity of nitroQ is higher in a medium deficient in nitrogen source. The number of survivors is about a hundredfold greater in presence of nitrogen source. The protection afforded by nitrogen is the same whether the cells have been preincubated with or without nitrogen source before the addition of glucose and nitroQ (Fig. 25). There are two possible explanations for this protection afforded by nitrogen source:

(a) The addition of nitrogen completes the medium and allows the growth of cells resistant to the drug. Therefore, a favourable balance towards survival is maintained between sensitive cells losing the ability to form colonies and resistant cells starting to grow.

(b) The addition of nitrogen causes some unknown repair mechanism to operate (e.g. synthesis of enzymes which repair damage caused by the drug or turnover of proteins for an alternative pathway for the sensitive step).

The former possibility can be ruled out by the observation that incubation of cells in absence of nitrogen source but in presence of glucose before addition of drug results in an increase in the number of survivors of the same order as in presence of nitrogen source (curve g in Fig. 25a). Under these conditions no resistant cell can grow out in absence of nitrogen source but there is definite protection against drug action. This would support the latter explanation viz., turnover of proteins protecting against drug action.

The amount of glucose required for the lethal activity of nitroQ is very small. Concentration as low as .025% instead of

1.2% in GA medium is sufficient to allow the bactericidal action of nitroQ (Fig. 26).

The lethal effect of nitroQ is dependent on the amount of glucose in the medium. NitroQ loses its activity when growth is limited by glucose but not when growth is limited by NH_4^+ in presence of excess glucose (Figs. 28 and 29). On the other hand, activity of carboxyQ is affected by cessation of growth even when excess glucose is present (Fig. 30). Therefore it is the exhaustion of energy/carbon source that reduces the activity of nitroQ but carboxyQ is inactive whenever growth ceases for any cause.

NitroQ requires an energy source and not necessarily a carbon source for its activity. L-glutamic acid, which is not an energy source for E. coli but may act as a carbon source, does not allow lethal action of nitroQ whereas glucose and succinate both allow bactericidal action of nitroQ (Fig. 31). NitroQ is more active with succinate as the energy source than with glucose. This can be explained by the fact that succinate allows growth of E. coli at a lower rate than glucose. NitroQ shows highest activity in presence of glucose without nitrogen source or in other words, in presence of an energy source under conditions which do not allow growth. Therefore succinate which only allows a slow growth rate increases the activity of nitroQ.

Effect of quinolones on respiration

Cells harvested from quinolone treated cultures exhibit an inhibition of rate of oxygen uptake on glucose as compared to the untreated control cells (Fig. 35). However, carboxyQ stimulates oxygen uptake of cells growing in the Warburg flask in presence of carboxyQ (Fig. 36). These conflicting results might be explicable on the grounds that when oxygen uptake is studied directly, the cells have not been damaged by harvesting procedure. The cells which show a stimulation of respiration under the influence of carboxyQ being abnormal, may be injured by the harvesting procedure. NitroQ inhibits respiration under both conditions.

Effect of quinolones on macromolecular synthesis

When *E. coli* cells are treated with moderate concentrations of quinolones, increase in cell mass continues for some time even while the viable count is falling at an appreciable rate (Figs. 7 and 9). The appearance of long filaments in drug treated cultures also suggested that increase in cell mass is taking place without cell division due to "unbalanced growth". Therefore, net synthesis of protein, RNA and DNA was measured. The results showed that while RNA and protein syntheses are not significantly altered, DNA synthesis is inhibited appreciably at bactericidal concentrations (Figs. 37 and 38).

Dean and Hinshelwood (1966) have described the variations in cell composition during a growth cycle. According to them average mass per cell increases in the early stages of growth but it falls back to the initial value in the stationary phase. RNA/DNA ratios also show a similar pattern but the highest ratios are observed earlier in growth cycle than the maximum mass per cell. DNA per unit mass and protein/RNA ratios remain more or less constant throughout the growth cycle. Quinolone treated cultures show greatly increased RNA/DNA ratios in comparison with control cultures in the stationary phase (Tables 14 and 15). RNA/protein ratios are unaffected at the lower drug concentration but slightly increased at higher concentrations. DNA/protein or DNA/dry-weight ratios are unaffected at bacteriostatic concentrations but are greatly decreased at bactericidal concentrations. Macromolecular syntheses by *E. coli* 11229 in absence and presence of carboxyQ were

studied kinetically over a growth cycle (Tewson, Holms & Mahmood, unpublished data). It was found that RNA/protein ratios rise during initial stages of growth but gradually fall back to the initial values in the control and with moderate bactericidal concentrations of drug. Only at very high concentration (160 uM) of drug was protein and RNA synthesis affected. DNA/protein ratios showed an increase in the early stages of growth and remained constant for the rest of the experimental period in the control. At bacteriostatic concentrations no change was observed in the DNA/protein ratios throughout the experimental period. Bactericidal concentrations, however, caused a drastic fall in the DNA/protein ratios in the beginning, after which the fall became much slower. The most dramatic effect was observed with RNA/DNA ratios in presence of drug. The control showed a rise up to 1 hr. and then a gradual fall to the initial value in the stationary phase. Bacteriostatic concentrations showed a similar pattern but the level reached was higher than in the control. At bactericidal concentration, the RNA/DNA ratios continued to rise up to 3 hr. and were maintained at that high level during the rest of the experimental period. These results strongly suggest that DNA synthesis is preferentially inhibited with respect to RNA and protein synthesis.

The correlation of the bactericidal concentrations to inhibition of DNA synthesis suggests that it is the main biochemical event responsible for the loss in viability. This observation along with the formation of filamentous cells in the drug treated

culture indicates that the antibacterial action of quinolones is due to so-called "unbalanced growth" resulting from preferential inhibition of DNA synthesis without concomitant inhibition of protein and RNA synthesis.

The above hypothesis was given further support by ^{14}C labelled thymine incorporation studies (Eason, Fewson & Holms, unpublished data). It was found that 20 μM carboxyQ causes progressive inhibition of incorporation of ^{14}C labelled thymine into DNA of E. coli 8114. About 99% inhibition of ^{14}C thymine into DNA is observed in drug treated cells as compared to the control.

Partial success was also achieved in attempts to reverse the action of carboxyQ in E. coli 8583 by addition of thymine and the three deoxynucleosides, dA, dC and dG in the growth medium (Tables 11 and 12). This result gives more support to the hypothesis that inhibition of DNA synthesis is responsible for the lethal action of quinolones. Similar results have also been obtained in the case of hydroxyurea treated cells (Adams & Lindsay, 1967; Young et al, 1967). These workers have also concluded that the reversal of drug action by addition of deoxynucleosides is due to partial restoration of DNA synthesis and hence hydroxyurea is an inhibitor of DNA synthesis.

Most known antimicrobial drugs which inhibit DNA synthesis do so either by altering the physical properties of DNA molecule or by inhibition of enzymes involved in DNA synthesis. Experiments were carried out to study the effect of carboxyQ on the structure and biosynthesis of DNA in E. coli 8114 (Mason, Fewson & Holms, unpublished data). These studies were carried out along three lines:

1) Effect on physical interaction of the drug with DNA molecule.

2) Effect on metabolism of thymine containing compounds.

3) Direct interaction of drug with the enzymes concerned with synthesis of precursors of DNA.

The following possibilities for physical interaction of carboxyQ and DNA were examined:

1) Incorporation of drug into polydeoxyribonucleotide chain by replacing a normal base or by intercalation.

2) Cross linking of complementary DNA strands.

3) Binding on the surface of DNA molecule so as to block movement of DNA polymerase.

4) Production of DNA with "nicks" e.g. by blocking synthesis of TMP.

5) By producing some kind of degraded DNA.

No incorporation of carboxyQ into DNA molecule could be observed as there was no difference in base composition or thermal denaturation (Tms) points of DNA from drugtreated cells and from control cells. Cross-linking of DNA which causes immediate renaturation after heat denaturation of DNA molecule (e.g. due to mitomycin) could not be demonstrated. No evidence could be obtained for either binding of drug on the DNA molecule or production of DNA containing nicks. DNA from drug treated colls was also not degraded as both control and drug treated DNA have identical S-values of about 20S.

Effect on the metabolism of thymine and thymine containing compounds was also studied (Eason, Fewson & Holms, unpublished data). It was assumed that RNA synthesis and therefore ribonucleoside synthesis was not inhibited by quinolones. Any selective inhibition of DNA synthesis must result from inhibition of biosynthesis or incorporation of thymine containing compounds (Fig. 40).

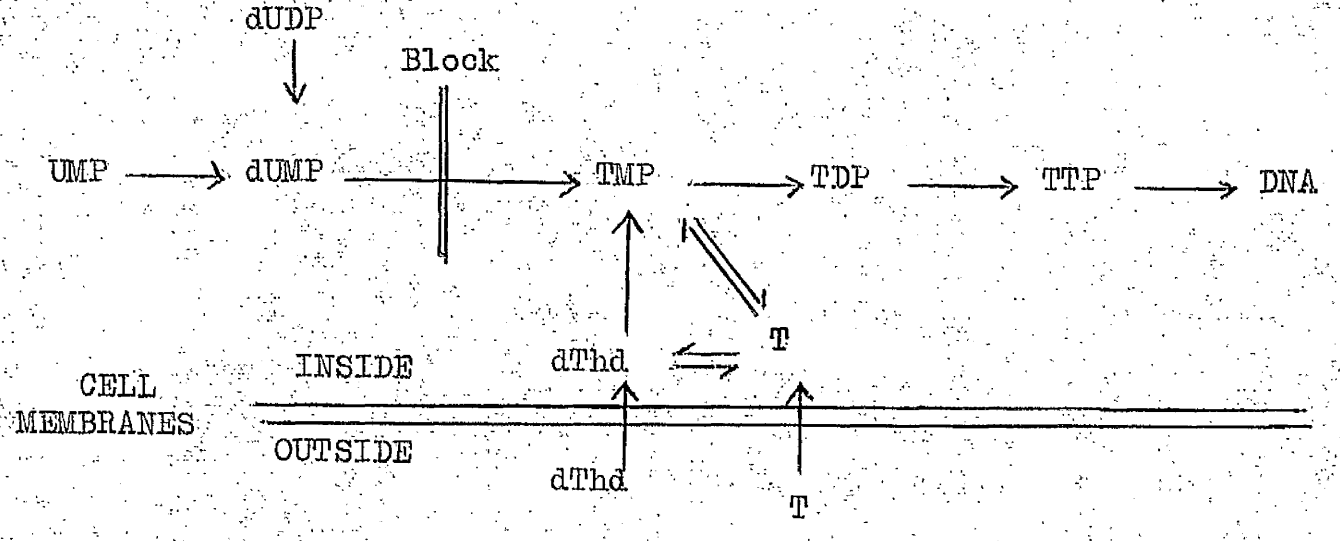


FIG. 40 : Pathway for synthesis and incorporation of thymine and thymidine into DNA.

(dThd = deoxythymidine)

As already mentioned, carboxyQ causes 99% inhibition of incorporation of ^{14}C-labelled thymine and thymidine in E. coli 8114. Drug could limit permeability of the cell membrane to ^{14}C-labelled thymidine or thymine. A number of attempts were made to measure the pools of these compounds. However no efficient method of measuring these pools was available in literature at that time. Recently Cannon & Breitman (1967) working with the same strain of E. coli have shown that 50% of the thymine incorporated into DNA was derived from exogenous dTMP. In addition, cells grown in presence of 10^{-4}M dTMP have a 50% increase of the total thymine deoxynucleotide pool. In the light of their results, it can be concluded that cells of E. coli 8114 are permeable to exogenous sources of thymine and carboxyQ could be probably responsible for the inhibition of incorporation of ^{14}C thymine and thymidine.

CarboxyQ could also stimulate degradation of thymidine into thymine and thus impose severe limitation of DNA synthesis. Crude extracts of control and drug treated bacteria were tested for increased production of thymine after addition of drug and for differences in specific activities. No evidence for stimulation of thymidine breakdown could be obtained (Eason, Fewson & Holms, unpublished data).

Fig. 41 depicts the pathway for DNA biosynthesis. Selective inhibition of DNA synthesis may result from inhibition of enzymes exclusively involved in DNA synthesis. These enzymes are:

1) Thymidine (dThd) and TMP kinase
2) DNA polymerase (DNA nucleotidyltransferase)
3) Ribonucleotide reductase.
4) Thymidylate synthetase.

These enzymes were isolated from E. coli and were studied for inhibition by carboxyQ (Eason & Holms, unpublished data). Thymidine and TMP kinase catalyse the phosphorylation of dThd and TMP to TTP which is an immediate precursor of DNA. Studies in vitro gave evidence that carboxyQ did not inhibit these enzymes.

DNA polymerase catalyses the synthesis of new deoxyribonucleotide chains in presence of a DNA primer and four deoxyribonucleoside triphosphates (dATP, dGTP, dCTP and dTTP). Many antimicrobial drugs are known to affect DNA synthesis by forming complexes with DNA molecule and thus impairing the ability of the DNA primer to act as template in the reaction catalysed by DNA polymerase. Mitomycins and acridines prevent the separation of the complementary DNA strands by cross-linking and intercalation respectively. Actinomycin also causes the inhibition of separation of the two DNA strands at high concentrations and thus inhibit replication by DNA polymerase (Reich, 1964). DNA polymerase was isolated from E.coli but carboxyQ failed to inhibit the enzyme.

Ribonucleotide reductase is the enzyme involved in the conversion of ribonucleotides to the corresponding deoxyribonucleo-

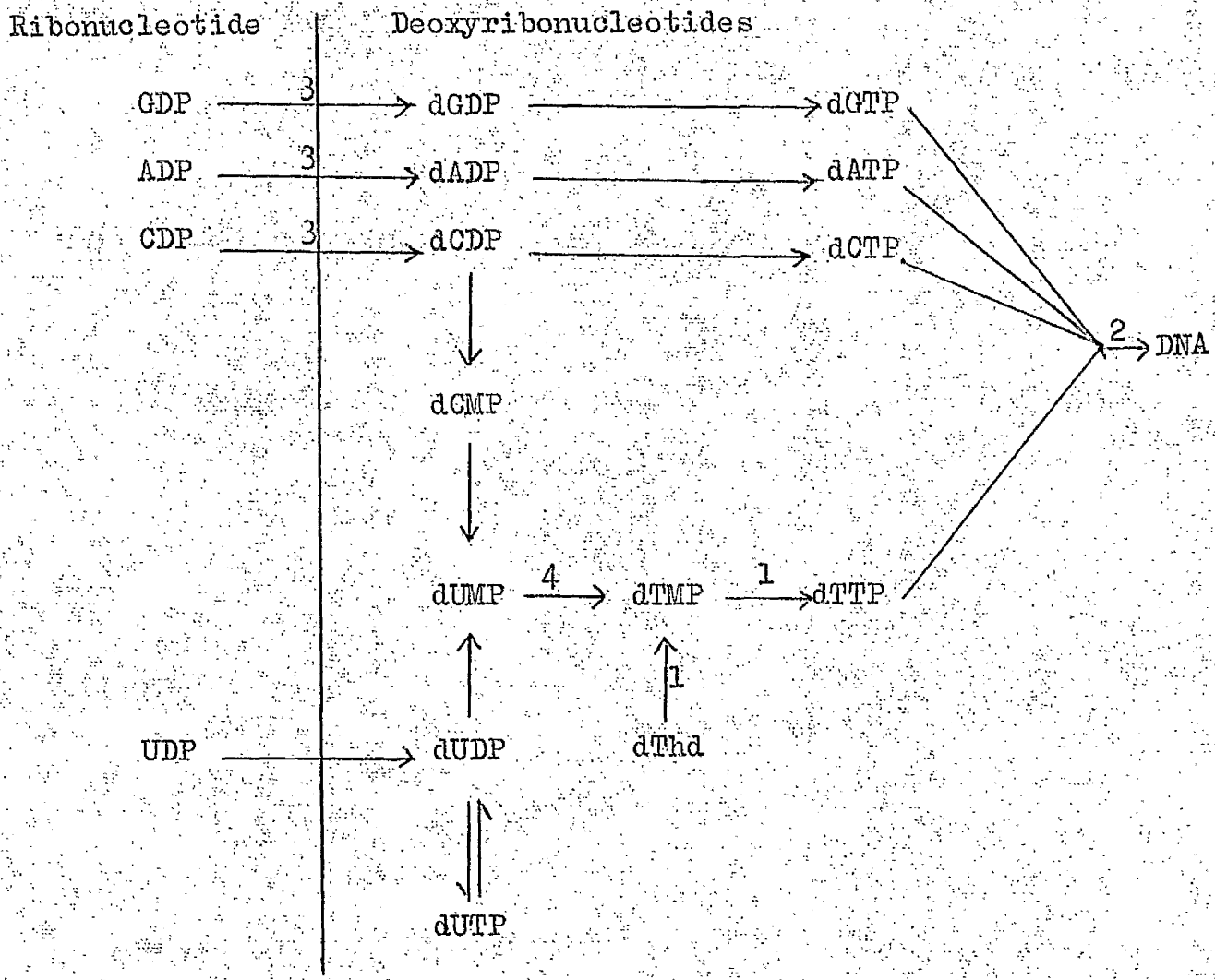


FIG. 41 : Pathway for DNA Biosynthesis

Numbers refer to the enzyme catalysing the various reactions.

1) Thymidine (dThd)(E.C.2.7.1.21) and TMP Kinase (E.C.2.7.4.C)*

2) DNA polymerase (E.C.2.7.7.7.)

3) Ribonucleotide reductase.

4) Thymidylate synthetase.

* Provisional number.

tides. Hydroxyurea, a potent inhibitor of DNA synthesis in a variety of cell systems has been found to interfere with ribonucleotide reduction in mammalian cells in vivo (Adams, Abrams & Lieberman, 1966; Young & Hodas, 1964). Partial reversal of hydroxyurea action by addition of deoxyribonucleosides has also been reported (Adams & Lindsay, 1967; Young et al, 1967). No completely satisfactory method for isolation of ribonucleotide reductase from E. coli is available. The enzyme was purified by the available method and the low activity obtained was unaffected by carboxyQ. However, in vivo studies for reversal of carboxyQ action by addition of deoxynucleosides had partial success. Therefore, the quinolones might also be interfering with DNA synthesis in E. coli analagously to hydroxyurea inhibition of DNA synthesis in mammalian cells.

The synthesis of dTMP from dUMP is catalysed by thymidylate synthetase which converts dUMP to dTMP in presence of N^5- N^{10} methylene tetrahydrofolate which is itself converted to dihydrofolate. Tetrahydrofolate (FH_4) is regenerated from dihydrofolate (FH_2) by a reaction catalysed by an enzyme dihydrofolate reductase (Fig. 42).

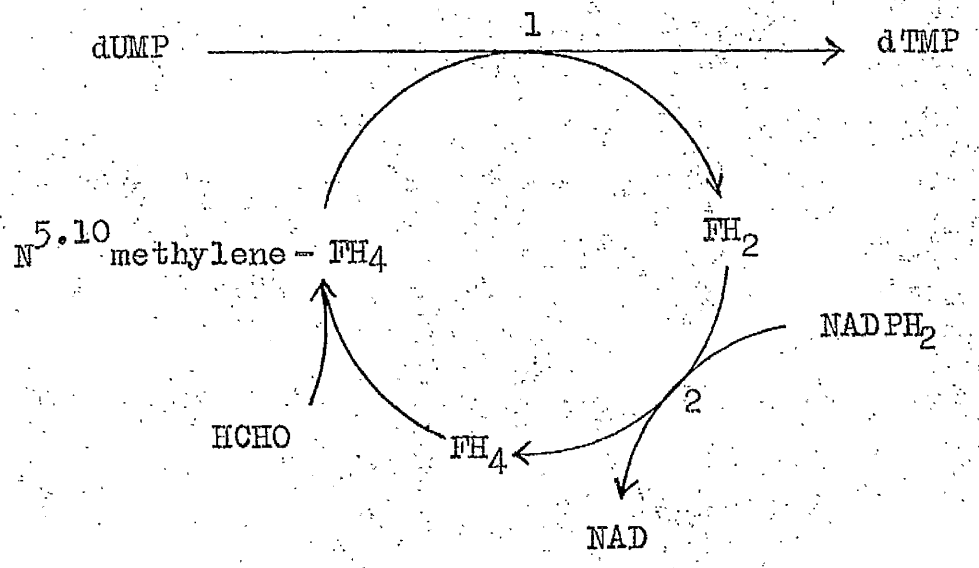


FIG. 42 : Synthesis of dTMP

1) Thymidylate synthetase.

2) Dihydrofolate reductase (E.C.1.5.1.3.)

Inhibition of either of these enzymes may cause inhibition of DNA synthesis through lack of dTMP. CarboxyQ was found to inhibit both these enzymes but the inhibition was not very high (Eason & Holms, unpublished data). The Ki value obtained for dihydrofolate reductase from E. coli 11229 and carboxyQ is 4.41×10^{-4} M while the Km for combination of FH_2 with its enzyme is 4.16×10^{-6} M. Aminopterin, a known inhibitor of dihydrofolate reductase, has a Ki of 1.46×10^{-9} M. This suggests that the enzyme has a higher affinity for its substrate than for carboxyQ and that carboxyQ is a much less efficient inhibitor than aminopterin. The activity of dihydrofolate reductase was not increased in extracts of E. coli 11229 trained for resistance against carboxyQ (Smillie & Eason, unpublished data). Therefore, inhibition of this enzyme may not be significantly responsible for the lethal effect of carboxyQ on the grounds

that one of the mechanisms for acquiring resistance to a drug inhibiting an enzyme reaction is an increase in the activity of the sensitive enzyme in the resistant cells (Pollock, 1960).

Reversal of action of quinolones

In the study of antimicrobial drugs, one of the basic approaches to the problem is an attempt to find some substance which, when added to the test system in which the growth or activity of an organism is inhibited, will antagonize the action of the drug. In view of the inhibition of DNA synthesis as the main lethal effect of quinolones, attempts were made to reverse the action of these drugs by addition of precursors of DNA (thymine or thymidine, dA, dC and dG) to the growth medium.

Addition of thymine or thymidine to CA medium inoculated with E. coli 11229 directly or after previous training in presence of thymine or thymidine failed to give any protection against the bactericidal action of quinolones (Table 7 and Fig. 32). This could result either from impermeability to external thymine and thymidine or from the absence of any metabolic pathway for utilization of exogenous source of thymine and thymidine in this wild strain. Therefore, further attempts to reverse drug action were made only with the thymine auxotroph, E. coli 8583.

Full growth of E. coli 8583 is supported in CA medium with 5 µM thymine. Increasing this concentration to 160 µM did not give any significant protection against the antibacterial action of carboxyQ (Table 8). It has been shown that the limited utilization of exogenous thymine by E. coli is related to the availability of deoxyribosyl donors rather than on specific permeability restrictions (Breitman & Bradford, 1964; Kammen, 1967). This could explain the partial reversal observed in presence of proper proportions of

thymine and the three deoxynucleosides, dA, dC and dG (Table 11). EDTA treatment to increase the permeability did not increase the protection afforded by thymine and deoxyribonucleosides (Tables 12 and 13). This result also gives further support to the above suggestion that limited utilization of exogenous thymine is not solely the result of permeability restrictions but is related to the availability of deoxyribosyl donors. The facts that thymine and the three deoxynucleosides are the exclusive immediate precursors of DNA and that they bring about partial reversal of antibacterial action of carboxyQ strongly suggest that inhibition of DNA synthesis is the main biological event responsible for the lethal action of quinolones.

The bactericidal action of quinolones can be reversed by merely washing and resuspending the cells in drug free medium (Figs. 33 & 34). This result suggests that quinolones do not bind strongly to their sensitive site as their lethal effect can be so easily washed out. When carboxyQ is removed at 1 or 2 hr. the washed cells immediately start to grow at the same rate as the control. The increase in mass is affected only when the drug was allowed to act up to 3 hr. This suggests that carboxyQ does not affect the growth up to 2 hr. but exerts its effect only when the control has achieved the highest growth rate in the mid-logarithmic phase. NitroQ, on the other hand, has an immediate lethal effect. Even when the drug is removed after 30 min. of treatment, the cells do not regain the same growth rate as the control for considerable time.

Resistance to Quinolones

NitroQ exhibit the unique property of an immediate lethal effect as compared to carboxyQ which always allows a period of induction before exerting any effect. However, the ability of cells to grow out after an initial killing period even in presence of high drug concentration diminishes the usefulness of the immediate lethal effect of nitroQ. This regrowth is due to the presence of a small number of resistant cells in the inoculum itself as seen in the experiment where the counting was done in a medium with added nitroQ (Fig. 39). The number of cells resistant to nitroQ is inversely proportional to the concentration. Thus about 50% of the cells were found to be resistant to 10 μM and about 1% to 40 μM nitroQ. This resistance is acquired without any previous contact with the nitroQ. If the size of inoculum is increased, the number of cells resistant to drug would also increase proportionally. Thus the reduced drug action with higher inocula (Fig. 17) can be explained on this basis.

Cultures treated with bactericidal concentrations of carboxyQ do not exhibit this growing out phenomenon. However, resistance can be acquired by growth in presence of gradually increasing concentrations of carboxyQ. This suggests that the cells become resistant to carboxyQ in a stepwise process after they had been grown in contact with drug.

Cross-resistance experiments with carboxylic and noncarboxylic quinolones (Holms, unpublished data) also confirm the hypotheses that the two groups differ in the manner by which resistance is acquired. The obvious difference between the two groups is that

the carboxyl compounds are ionised at physiological pH while non-carboxylic ones are not. A compound has been synthesised which has both carboxyl and a nitro group (N-ethyl-3-carboxylic-6-nitro-4-quinolone). All three compounds when tested for cross resistance showed the following relationships.

Challenged with	*Escherichia coli* trained to:		
	3-carboxy-quinolones	3-carboxy-6-nitro-quinolones	"non-ionised"-6-nitro-quinolones
3-carboxyl quinolones	Complete Resistance	Complete Resistance	No Resistance
3-carboxy-6-nitro-quinolones	Complete Resistance	Complete Resistance	Slight Resistance
"non-ionised"-6-nitro-quinolones	No Resistance	Slight Resistance	Complete Resistance

The lack of cross resistance between carboxylic and noncarboxylic quinolones indicates a different mechanism for acquiring resistance. The slight cross-resistance by 3-carboxy-6-nitro-quinolones to 6-nitro-quinolones indicates that resistance to carboxylic quinolones is dominant over the resistance to nitroquinolones.

An interesting relationship of thymine-independence and drug resistance was observed during work with the thymineless mutant, *E. coli* 8583. It was found that if *E. coli* 8583 is incubated in absence of thymine, thymineless death takes place for up to 6 hr. when approximately 99.9% of the inoculated cells are dead (Table 8). Further incubation of the flask overnight allowed these immune cells to grow to the same extent as the control with added thymine. The

only case where no further growth took place was in absence of thymine and in presence of carboxyQ. This suggests that drug had an additive effect on thymineless death. The cells were also able to recover in presence of thymine and various concentrations of carboxyQ. These thymine-independent and drug-resistant strains were isolated and again challanged with various concentrations of carboxyQ with and without thymine (Tables 9 & 10). The strain which had become thymine independent, was still sensitive to drug to the same extent in presence or absence of thymine. Presumably this was due to the selection of cells immune to thymineless death but sensitive to drug having had no contact with the drug. The strains which had grown in presence of increasing concentration of drug and thymine, when challenged with 160 μM of carboxyQ with and without thymine showed a gradually increasing resistance to drug. These strains, at the same time became gradually independent of thymine to such an extent that the strain isolated from 30 μM carboxyQ and 5 μM thymine was completely immune to thymineless death as well as resistant to 160 μM carboxyQ.

The development of immunity to thymineless death is directly and closely related to biosynthesis of DNA. In other words, the cells are undergoing some metabolic change so that they are no longer susceptible to thymineless death and can actually grow in absence of thymine. The inter-relationship of thymine independence and drug resistance suggests that the latter is also closely associated with DNA metabolism, and the metabolic step sensitive to thymine starvation and the drug is one and the same.

The results of *in vivo* and *in vitro* studies on the inhibition of *E. coli* by quinolones indicate that these drugs inhibit DNA synthesis. The following observations support this hypothesis:

1) The appearance of enormously long filamentous cells in drug treated cultures has been reported for other agents which inhibit DNA synthesis preferentially with respect to RNA and protein synthesis. The process is called "unbalanced growth".

2) The concentration of quinolones required to produce the biochemical effect of inhibition of DNA synthesis corresponds fairly well with the bactericidal concentrations.

   Incorporation of radioactive thymine and thymidine is strongly inhibited in drug treated cultures.

3) Some protection against the lethal action of quinolones is observed when the culture medium is supplemented with thymine and the three deoxyribonucleosides, dA, dC and dG.

The exact mechanism by which quinolones inhibit DNA synthesis is not very clear in the light of the present work. This problem was studied in comparison with the other known inhibitors of DNA synthesis. Thus the physical properties of treated DNA were studied by available methods to find any alterations in the DNA molecule which could account for inhibition of DNA synthesis and growth. No evidence could be obtained for cross-linking (like

mitomycins), intercalation (proflavins and related drugs) and binding to the DNA molecule in such a manner as to interfere with its functions as a template for de novo synthesis (actinomycins). Quinolones do not cause any degradation of DNA as measured by sedimentation properties of DNA from drug treated and control cells.

The in vitro study of enzymes involved in the biosynthesis of DNA shows that most of them are not inhibited in cell free systems. The only exceptions are thymidylate synthetase and dihydrofolate reductase which are moderately sensitive to the drug. This inhibition alone can not clearly be responsible for the highly antibacterial action of quinolones.

CarboxyQ has a structure very similar to nalidixic acid (1-ethyl-1, 4-dihydro-7-methyl-4-oxo-1, 8-naphthyridine-3-carboxylic acid) which has been shown to inhibit growth of E. coli by selective inhibition of DNA synthesis (Goss, Deitz & Cook, 1964, 1965). Nalidixic acid is not only structurally similar to carboxyQ but has very similar biological effects on E. coli. These workers have also used the same strain of E. coli viz., E. coli 11229 as used in the study of quinolones. Both carboxyQ and nalidixic acid (NA) give rise to elongated filamentous cells and are active only against growing cells. The addition of chloramphenicol causes a reduction in the bactericidal action of both carboxyQ and NA. Both drugs are not bound strongly to their sensitive sites as transfer to drug free media allows normal growth of treated cells. Both drugs inhibit the incorporation of radioactive precursors into DNA. Nalidixic acid does not affect the synthesis of the "initiator" of

DNA replication. Nalidixic acid, however, causes degradation of nucleic acids, DNA degradation being more pronounced than RNA degradation.

The action of nalidixic acid and quinolones differs in some respects. Quinolones do not appear to cause degradation of DNA while the lethal activity of nalidixic acid is attributed to the destruction of genetic material. Moreover, nalidixic acid has no effect on respiration of cells while quinolones affect the respiration of both growing and resting cells of E. coli. The inability to find any changes either in the physical properties of DNA or the activity of enzymes involved in DNA biosynthesis is rather strange when the net synthesis of DNA is selectively inhibited. Perhaps the inhibition of DNA synthesis is not the primary event as has been suggested for the selective and reversible inhibition of DNA synthesis by phenethylalcohol (PEA) by Silver & Wendt (1967). According to these workers PEA causes a rapid and reversible breakdown in the permeability barriers of bacterial cells. The processes inhibited by PEA include bacterial conjugation, sporulation and germination of spores. All of these processes as well as synthesis of DNA are believed to take place by a direct structural coupling of the process to the membrane. Therefore, the primary effect of PEA is on the membrane and the inhibition of DNA synthesis and cellular functions are secondary consequences of the alteration in the membrane structure. It may be possible that quinolones also act on the membrane primarily as is indicated by their effect on respiration, a process closely associated with proper functioning of membranes. Moreover, the action

of quinolones is completely reversed by merely washing and re-suspending in drug free medium which suggests that the quinolones do not bind strongly to their sensitive site. It might be possible that, the absorption of quinolones alters the integrity of the membrane, when the drug is removed the membrane returns to its original configuration and the cells recover as has been suggested for PEA.

The above suggestions are based on circumstantial evidence only. In absence of direct experimental data, nothing can be stated definitely about the primary mode of action of quinolones except that bactericidal action of quinolones is closely related to inhibition of DNA synthesis. Further work is needed to establish whether inhibition of DNA synthesis is the primary event or a secondary effect of inhibition of some other cell function.

Comparison of carboxyQ and NitroQ

The study of quinolones has revealed the unique property of noncarboxylic quinolones to affect nongrowing cells. Comparison with carboxylic quinolones shows that although the two groups differ greatly in the conditions required for lethality, the overall mechanism of growth inhibition is the same. Both groups cause the inhibition of DNA synthesis as shown by appearance of filamentous cells and increased RNA/DNA ratios in the drug treated cells. The antibacterial action of nitroQ is so rapid that only a very small proportion of the cells survive to actually grow in presence of drug to provide evidence of "unbalanced growth". The presence of resistant cells in the inoculum, which are capable of normal growth in presence of drug, further masks the action of drug on the sensitive cells. The immediate lethal effect and the appearance of resistant cells tends to reduce the effect of nitroQ on macromolecular synthesis when measured by routine analytical methods. However, the increased RNA/DNA ratios and greatly reduced DNA/dry weight ratios together with the appearance of filamentous forms of E. coli strongly suggest that DNA synthesis is the process mainly affected analagously to carboxyQ.

The action of both carboxyQ and nitroQ can be reversed by washing and resuspending in drug free medium indicating that the nature of binding of the drugs to the sensitive site may be similar.

CarboxyQ, like most of the known antimicrobial agents, requires growing cells for its antibacterial effect. This was

well established by the following facts. This drug is unable to exert any effect in media which do not support growth. Its bactericidal action can be reduced if growth is prevented e.g. by addition of chloramphenicol which inhibits protein synthesis and hence growth. The activity of nitroQ, on the other hand, does not depend on conditions allowing growth but is in fact enhanced in absence of growth as shown by the higher activity of nitroQ in absence of nitrogen source. This ability to affect non growing cells more rapidly explains the immediate and higher bactericidal action of nitroQ during lag phase before the onset of growth in the control. CarboxyQ allows a period of induction during which both cell mass and cell number are increased before exerting any effect. These differences form the basis for division of quinolones into carboxylic and noncarboxylic groups.

The antibacterial action of carboxyQ is proportional to the rate of growth as its action is greatly reduced under anaerobic conditions. No such relationship of growth rate and action of nitroQ is observed giving further support to the hypothesis that the activity of nitroQ does not depend on growth. NitroQ, however, does require a source of energy for its bactericidal action while carboxyQ is inactive under similar conditions. These two groups also differ in their effect on the rate of respiration by growing cells. CarboxyQ stimulates the rate of respiration and thus might be acting as an uncoupler of oxidative phosphorylation besides its main effect on DNA synthesis. NitroQ inhibits the rate of respiration under the same conditions. The inhibition of rate of respiration by nitroQ under both conditions may be

attributed to its immediate lethal effect.

The study of cross-resistance between carboxylic and non-carboxylic quinolones shows that these two groups differ in the mechanism for acquiring resistance. This does not necessarily mean that carboxyQ and nitroQ have a different mode of action as well. The same phenomenon has been observed with other closely related drugs e.g., benzyl penicillin and methicillin (Knox, 1962). Both of them have the same mode of action viz., inhibition of cell wall synthesis. Staphylococcus spp. have been found to develop resistance to benzyl penicillin by synthesizing penicillinase while resistance to methicillin is acquired by developing impermeability to methicillin so that cross-resistance to benzylpenicillin and methicillin is not observed. Therefore the fact that the two groups of quinolones do not show cross-resistance does not suggest that they have different modes of action.

The remarkable property of noncarboxylic quinolones to affect both growing and nongrowing cells is unique among all the known antimicrobial drugs. This might prove very useful clinically under conditions where the parasite lies dormant in the host and is not susceptible to any known antimicrobial agents which only affect growing cells. (There are a few exceptions such as surface-active agents which have an immediate bactericidal action. They have been mostly used for surface application or for intestinal infection being too toxic for parenteral use (Newton, 1958)). The presence of resistant cells without a pre-exposure to the drug diminishes its usefulness in chemotherapy. However, combined

therapy with carboxylic quinolones which do not show cross-resistance or with other drug might prove profitable analogously to isoniazid and streptomycin in the chemotherapy of tuberculosis.

C O N C L U S I O N S

The present study of antibacterial action of quinolones justifies the separation of quinolones into carboxylic and non-carboxylic types on the basis of their ability to affect growing and nongrowing cells respectively. The carboxylic quinolone has been shown to have an absolute requirement for actively growing cells. The noncarboxylic quinolone can affect nongrowing cells more efficiently than growing cells but requires a metabolisable energy source as a prerequisite for antibacterial action.

In spite of the difference in the conditions required for antibacterial action, both carboxyQ and nitroQ appear to have a similar mode of action. Both can inhibit cell division with appearance of long filamentous cells. Selective inhibition of DNA synthesis seems to be responsible for their antibacterial action but the exact mechanism is not clear. The relationship between carboxyQ activity and DNA metabolism is further observed in the thymine auxotroph which loses its requirement for thymine at the same time as it becomes drug resistant. However, the effect on respiration indicates that inhibition of DNA synthesis is not the sole effect.

The action of quinolones is reversible either by removing the drug or by addition of DNA precursors in the case of thymine auxotroph and carboxyQ. Resistance to carboxyQ and nitroQ depends on different mechanisms which does not necessarily indicate a different mode of action.

The bactericidal action of quinolones is presumably due to

inhibition of DNA synthesis although this may not be the primary effect. As is well known, growth is such an integerated and complex phenomenon that the observed effect might be remotely connected to the real and primary effect in a hitherto unknown manner. In the light of present knowledge, DNA synthesis may be inhibited by a change in membrane structure (Silver & Wendt, 1967) or merely by inhibition of the synthesis of the socalled "initiator" protein (Maaløe & Hanawalt, 1961; Lark, 1966).

The present work on quinolones suggests that a better understanding of the antibacterial action of quinolones could be achieved by approaching the problem from several directions. The effect on synthesis and integrity of cell membrane could be examined. The effect of quinolones on respiration suggests that in view of the relationship of cell membrane to energy metabolism, study of effect of quinolones on energy metabolism in general and oxidative phosphorylation in particular would be worthwhile. Moreover, the membrane has also been suggested to be the site of initiation of DNA synthesis. This aspect of membrane function also needs attention.

The work on the effect of carboxyQ on enzymes in cellfree system did not prove fruitful partly due to the fact that the methods available at present for purification of these enzymes are not completely satisfactory. The study of DNA biosynthesis is a rapidly advancing field and perhaps it would be better to return to the study of the effect of quinolones on enzymes in cellfree systems when we have a more detailed knowledge of the synthetic

routes and better methods for the purification of their enzymes.

It should be possible to synthesize both quinolones with ^{14}C in either the N-alkyl groups or in the ring structure. Availability of such labelled quinolones would permit a study of their primary sensitive site. They would also permit a comparison of the fixation of carboxyQ and nitroQ by bacteria in various physiological conditions. The specific labelling in N-alkyl or ring structure could relate any possible metabolism of quinolones to their antibacterial activity. Finally radiolabelled quinolones might illuminate the differences observed in the mechanisms for acquiring resistance to carboxyQ and nitroQ.

SUMMARY

Quinolones exhibit both bacteriostatic and bactericidal actions against three strains of Escherichia coli (ATCC 11229/198, NCIB 8114 and 8583). A carboxylic quinolone (N-ethyl-3-carboxyl-7-chloro-4-quinolone, "carboxyQ") is characterised by an absolute requirement for growing cells to exert its antibacterial action. It does not affect susceptible cells during lag phase when the cells are not actively dividing. Its effect on growth and viability is noticed only after the control enters the logarithmic phase of growth and its action diminishes when the control approaches stationary phase. Moreover, its action is completely antagonized under conditions where growth is prevented by withdrawal of an essential nutrient. If the essential nutrient is added to cells pre-incubated in deficient medium with carboxyQ, a period of induction is still required before any antibacterial effect of carboxyQ is observed. Inhibition of growth, e.g. by addition of chloramphenicol, reduces the antibacterial action appreciably. Its activity is also proportional to the rate of growth.

A noncarboxylic quinolone (1, 2-dimethyl-6-nitro-4-quinolone, "nitroQ") has been found to be more active against nongrowing cells. It exerts an immediate action on susceptible cells in the lag phase and its activity is reduced when growth occurs. Its activity is not affected by prevention of growth either by withdrawal of nitrogen source or by addition of chloramphenicol. Activity of nitroQ is not proportional to the rate of growth. However, the activity of nitroQ has been found to depend on concurrent energy metabolism.

The antibacterial action of quinolones is accompanied by appearance of long filamentous cells as seen microscopically and by measurement of the increase in average cell size. At bactericidal concentrations, DNA synthesis is preferentially inhibited as compared with RNA and protein synthesis. These results indicate "unbalanced growth" analogous to a variety of conditions which affect DNA synthesis. The close inter-relationship of quinolone activity and DNA synthesis is further supported by the observation that the thymine auxotroph, E. coli 8583, becomes independent of thymine at the same time as it becomes resistant to carboxyQ. However, DNA synthesis is not the sole process affected by quinolones. Both carboxyQ and nitroQ affect the respiration of E. coli 11229 at bactericidal concentrations.

The action of quinolones can be completely reversed by removal of drug which suggests that quinolones are not firmly bound to their sensitive site. Partial reversal can also be achieved by addition of precursors of DNA in the thymine auxotroph indicating that inhibition of DNA synthesis is the main if not the sole and primary effect.

Resistance to carboxyQ and nitroQ is developed by different mechanisms which adds weight to the division into carboxylic and noncarboxylic quinolones but does not necessarily reflect any fundamental difference in the mode of action.

REFERENCES

Adams, R.L.P., Abrams, R. & Lieberman, I. (1966)

J. Biol. Chem. 241, 903.

Adams, R.L.P. & Lindsay, G. (1967)

J. Biol. Chem. 242, 1314.

Albert, A. (1960)

In "Selective Toxicity" 2nd Ed. London : Metheun.

Ashwell, G. (1957)

In "Methods in Enzymology" vol. 3, p. 87. ed. by Colowick, S.P. & Kaplan, N.O. New York : Academic Press Inc.

Beck, W.S., Hook S. & Barnett, B.H. (1962)

Biochim. biophys. Acta 55, 455.

Berrah, G. & Konetzka, W.A. (1962)

J. Bact. 83, 738.

Breitman, T.R. & Bradford, R.M. (1964)

Biochem. biophys. Res. Commun. 17, 786.

Breitman, T.R. & Cannon, W.D. Jr. (1967)

Biochemistry 6, 810.

Brock, T.D. (1962)

Biochem. biophys. Res. Commun. 9, 184.

Brock, T.D. (1966)

In "Biochemical Studies of Antimicrobial Drug", Symp. Soc. gen. Microbiol., vol. 16, p. 131. Ed. by Newton, B.A. & Reynolds, P.E. Cambridge : Cambridge University Press.

Brock, T.D. & Brock, M.L. (1959)

Arch. Biochem. Biophys. 85, 176.

Brodie, A.F. & Gray, C.T. (1956)

J. Biol. Chem. 219, 853.

Buchanan, J.G. (1951)

Nature, Lond. 168, 1091.

Burton, K. (1956)

Biochem. J. 62, 315.

Butler, J.A.V., Godson, G.N. & Hunter, G.D. (1961)

UNESCO Symp. on "Protein Biosynthesis", p. 349.

Cairns, J. (1963)

J. Mol. Biol. 6, 208.

Cannon, W.D. Jr. & Breitman, T.R. (1967)

Biochemistry 6, 810.

Cannon, M., Krug, R. & Gilbert, W. (1963)

J. Mol. Biol. 7, 360.

Cavalieri, L.F. & Rosenberg, B.H. (1963)

In "Progress in Nucleic Acid Research" vol. 2, p. 2. Ed. by

Davidson, J.N. & Cohn, W.E. New York : Academic Press Inc.

Chamberlin, M. & Berg, P. (1962)

Proc. nat. Acad. Sci., Wash. 48, 81.

Chance, B. & Williams, G.R. (1956)

Advanc. Enzymol. 17, 65.

Cheer, S. & Tchen, T.T. (1962)

Biochem. biophys. Res. Commun. 9, 271.

Cohen, G.N. & Monod, J. (1957)

Bact. Rev. 21, 169.

Cohen, S.S. (1957)

In "The Chemical Basis of Heredity: A symposium" p. 651 Ed. by

McElroy, W.D. & Glass, B. Baltimore : The John Hopkins Press.

Cohen, S.S. & Barner, H.D. (1954)

Proc. nat. Acad. Sci., Wash. 40, 885.

Cohen, S.S., Flaks, J.G., Barner, H.D., Loeb, M.R. & Lichtenstein, J.

Proc. nat. Acad. Sci., Wash. 44, 1004. (1958)

Cook, T.M., Deitz, W.H. & Goss, W.A. (1966)

J. Bact. 91, 774.

Cook, T.M., Goss, W.A. & Deitz, W.H. (1966)

J. Bact. 91, 780.

Creaser, E.H. (1955)

J. gen. Microbiol. 12, 288.

Davies, J.E. (1964)

Proc. nat. Acad. Sci., Wash. 51, 659.

Davis, B.D. & Feingold, D.S. (1962)

In "The Bacteria", vol. 4, p. 343. Ed. by Gunsalus I.C.

& Stanier, R.Y. New York : Academic Press Inc.

Dean, A.C.R. & Hinshelwood, Sir C. (1966)

In "Growth, Function and Regulation in Bacterial Cells"

1st ed. p. 86. Oxford : Clarendon Press.

Deitz, W.H., Bailey, J.H. & Froelich, E.J. (1964)

Antimicrobial Agents and Chemotherapy - 1963, 583.

Deitz, W.H., Cook, T.M., & Goss, W.A. (1966)

J. Bact. 91, 768.

Demel, R.A., van Deenen, L.L.M. & Kinsky, S.C. (1965)

J. Biol. Chem. 240, 2749.

DeMoss, J.A. & Novelli, J.D. (1956)

Biochim. biophys. Acta 22, 49.

Dubin, D.T. & Davis, B.D. (1961)
Biochem. biophys. Acta. 52, 400.

Dubin, D.T. & Davis, B.D. (1962)
Biochim. biophys. Acta 55, 793.

Dubin, D.T., Hancock, R. & Davis, B.D. (1963)
Biochim. biophys. Acta 74, 476.

Eagle, H. & Saz, A.K. (1955)
Ann. Rev. Microbiol. 9, 173.

Eason, R., Fewson, C.A. & Holms, W.H.
unpublished data.

Eason, R. & Holms, W.H.
unpublished data.

Fewson, C.A. (1967)
J. gen. Microbiol. 46, 255.

Fewson, C.A., Holms, W.H. & Mahmood, F.
unpublished data.

Fildes, P. (1940)
Brit. J. Exp. Path. 21, 67 and Lancet 1, 755.

Flaks, J.G., Cox, E.C. & White, J.R. (1962)
Biochem. biophys. Res. Commun. 7, 385.

Franklin, D. (1966)
In "Biochemical Studies of Antimicrobial Drugs", Symp. Soc. gen. Microbiol., vol. 16, p. 192. Ed. by Newton, B.A. & Reynolds, P.E. Cambridge : Cambridge University Press.

Frieson, J.D. & Maaløe, O. (1965)
Biochim. biophys. Acta 95, 436.

Gale, E.F. (1963)

Pharmacol. Rev. 15, 481.

Gale, E.F. (1966)

In "Biochemical Studies of Antimicrobial Drugs", Symp. Soc. gen. Microbiol., vol. 16, p. 1. Ed. by Newton, B.A. & Reynolds, P.E. Cambridge : Cambridge University Press.

Gale, E.F. & Folkes, J.P. (1953)

Biochem. J. 53, 493.

Gale, E.F. & Folkes, J.P. (1955a)

Biochem. J. 59, 661.

Gale, E.F. & Folkes, J.P. (1955b)

Biochem. J. 59, 721.

Gale, E.F. & Taylor, E.S. (1947)

J. gen. Microbiol. 1, 77.

Goldberg, I.H. (1965)

Am. J. Med. 39, 722.

Goldberg, I.H., Rabinowitz, M. & Reich, E. (1962)

Proc. nat. Acad. Sci., Wash. 48, 2094.

Goldberg, I.H. & Reich, E. (1964)

Fed. Proc. 23, 958.

Gorini, L. & Kataja, E. (1964)

Proc. nat. Acad. Sci., Wash. 51, 995.

Goss, W.A., Deitz, W.H. & Cook, T.M. (1964)

J. Bact. 88, 1112.

Goss, W.A., Deitz, W.H. & Cook, T.M. (1965)

J. Bact. 89, 1068.

Grant, P.T. (1966)

In "Biochemical Studies of Antimicrobial Drugs", Symp. Soc. gen. Microbiol., Vol. 16, p. 281. Ed. by Newton, B.A. & Reynolds, P.E. Cambridge : Cambridge University Press.

Hahn, F.E. & Wolfe, A.D. (1961)

Biochem. biophys. Res. Commun. 6, 464.

Hamilton, L.D., Fuller, W. & Reich, E. (1963)

Nature, Lond. 198, 538.

Hancock, R. (1962)

J. gen. Microbiol. 28, 503.

Hancock, R. (1964)

J. Bact. 88, 633.

Hancock, R. & Fitz-James, P.C. (1964)

J. Bact. 87, 1044.

Hobby, G.L. & Lenert, T.F. (1958)

Antibiotics and Chemotherapy 8, 219.

Holms, W.H.

unpublished data.

Hotchkiss, R.D. (1944)

Advanc. Enzymol. 4, 153.

Hotchkiss, R.D. (1946)

Ann. N.Y. Acad. Sci. 46, 479.

Hughes, W.H. (1956)

In "Bacterial Anatomy" Symp. Soc. gen. Microbiol., Vol. 6, p. 341. Ed. by Spooner, E.T.C. & Stocker, B.A.D. Cambridge : Cambridge University Press.

Hurwitz, J., Furth, J.J., Anders, M., Ortiz, P.J. & August, J.T.
J. Chem. Phys. 58, 934 and (1961)
Cold Spring Harbor Symp. Quant. Biol. 26, 91.

Hurwitz, J., Furth, J.J., Mallamy, M. & Alexander, M. (1962)
Proc. nat. Acad. Sci., Wash. 48, 1222.

Ingram, V.M. (1965)
In "The Biosynthesis of Macromolecules", 1st Ed.,
New York : W.A. Benjamin, Inc.

Iyer, V.N. & Szybalski, W. (1963)
Proc. nat. Acad. Sci., Wash. 50, 355.

Jacob, F., Brenner, S. & Cuzin, F. (1963)
Cold Spring Harbor Symp. Quant. Biol. 28, 329.

Jordan, D.C. (1965)
Can. J. Microbiol. 11, 390.

Julian, G.R. (1965)
J. Mol. Biol. 12, 9.

Kahan, E., Kahan, F.M. & Hurwitz, J. (1963)
J. Biol. Chem. 238, 2491.

Kammen, H.O. (1967)
Biochim. Biophys. Acta 134, 301.

Kepes, A. (1963)
Biochim. biophys. Acta 76, 293.

Kersten, H. (1962)
Biochim. biophys. Acta 55, 558.

Kersten, H., Kersten, W., Leopold, G. & Schneiders, B. (1964)
Biochim. biophys. Acta 80, 521.

Kirk, J.M. (1960)

Biochim. biophys. Acta 42, 167.

Knox, R. (1962)

In "Resistance of Bacteria to the Penicillins" Ciba Foundation Study Group No. 13. p. 76. Ed. by de Reuck, A.V.S. & Cameron, M.P. London : J. & A. Churchill Ltd.

Konetzka, W.A. & Berrah, G. (1962)

Biochem. biophys. Res. Commun. 8, 407.

Kurland, C.G. & Maaløe, O. (1962)

J. Mol. Biol. 4, 193.

Lacks, S. & Gros, F. (1959)

J. Mol. Biol. 1, 301.

Lampen, J.O. (1966)

Symp. Soc. gen. Microbiol., vol. 16, p. 111. Ed. by Newton, B.A. & Reynolds, P.E. Cambridge : Cambridge University Press.

Lark, K.G. (1966)

Bact. Rev. 30, 3.

Leggate, J. (1967)

Ph.D. Thesis, Glasgow University.

Leive, L. (1965)

Biochem. biophys. Res. Commun. 21, 290.

Lerman, L.S. (1961)

J. Mol. Biol. 3, 18.

Lerman, L.S. (1964)

J. Mol. Biol. 10, 367.

Lester, G. (1965)

J. Bact. 90, 29.

Maaløe, O. & Hanawalt, P.C. (1961)

J. Mol. Biol. 3, 144.

Maaløe, & Kjeldgard, N.O. (1966)

In "Control of Macromolecular Synthesis : A Study of DNA, RNA, and Protein Synthesis in Bacteria" No. 4 of Microbial and Molecular Biology Series Ed. by Davis, B.D. New York : W.A. Benjamin, Inc.

Meselson, M. & Stahl, F.W. (1958)

Proc. nat. Acad. Sci., Wash. 44, 671.

Mitchell, P.D. & Moyle, J. (1956)

In "Bacterial Anatomy" Symp. Soc. gen. Microbiol., vol. 6, p. 150. Ed. by Spooner, E.T.C. & Stocker, B.A.D. Cambridge : Cambridge University Press.

Moore, C. & Pressman, B.C. (1964)

Biochem. biophys. Res. Commun. 15, 562.

McIlwain, H. (1941)

Biochem. J. 35, 1311.

Nathans, D. (1964)

Fed. Proc. 23, 984.

Nathans, D. & Lipmann, F. (1961)

Proc. nat. Acad. Sci., Wash. 47, 497.

Nathans, D., Notami, G., Schwartz, J.H. & Zinder, N.D. (1962)

Proc. nat. Acad. Sci., Wash. 48, 1424.

Neidhard, F.C. & Gros, F. (1957)

Biochim. biophys. Acta 25, 513.

Neuhaus, F.C. & Lynch, J.L. (1962)

Biochem. biophys. Res. Commun. 8, 377.

Newton, B.A. (1953)

J. gen. Microbiol. 9, 54.

Newton, B.A. (1958)

Symp. Soc. gen. Microbiol. 8, 62.

Newton, B.A. (1965)

Ann. Rev. Microbiol. 19, 209.

Nirenberg, M.W. & Matthaei, J.H. (1961)

Proc. nat. Acad. Sci., Wash. 47, 1588.

Nonoyama, M. & Ikeda, Y. (1964)

Biochem. biophys. Res. Commun. 15, 87.

Ochoa, S., Burma, D.P., Krüger, H. & Weil, J. (1961)

Proc. nat. Acad. Sci., Wash. 47, 670.

Parks, J.T. (1966)

In "Biochemical Studies of Antimicrobial Drugs",

Symp. Soc. gen. Microbiol., vol. 16, p. 70. Ed. by Newton,

B.A. & Reynolds, P.E. Cambridge : Cambridge University Press.

Pollock, M.R. (1960)

Brit. med. Bull. 16, 16.

Powell, E.O. & Errington, F.P. (1963)

J.R. micro. Soc. 82, 39.

Prevost, C. & Moses, V. (1966)

J. Bact. 91, 1446.

Reich, E. (1964)

Science 143, 684.

Reich, E., Franklin, R.M., Shatkin, A.J. & Tatum, E.L. (1961a)

Science 134, 556.

Reich, E., Shatkin, A.J. & Tatum, E.L. (1961b)

Biochim. biophys. Acta 53, 132.

Rendi, R. & Ochoa, A. (1962)

J. Biol. Chem. 237, 3711.

Reynolds, P.E. (1966)

In "Biochemical Studies of Antimicrobial Drugs", Symp. Soc. gen. Microbiol., vol. 16, p. 47. Ed. by Newton, B.A. & Reynolds, P.E. Cambridge : Cambridge University Press.

Rosenkranz, H.S. & Carr, H.S. (1966)

J. Bact. 92, 178.

Rosenkranz, H.S., Carr, H.S. & Rose, H.M. (1965)

J. Bact. 89, 1354.

Rosenkranz, H.S., Garro, A.J., Levy, J.A. & Carr, H.S. (1966)

Biochim. biophys. Acta 114, 501.

Rosenkranz, H.S. & Levy, J.A. (1965)

Biochim. biophys. Acta 96, 181.

Shiba, S., Terawaki, A., Taguchi, T. & Kawamata, J. (1958)

Biken's J. 1, 179.

Shockman, G.D. & Lampen, J.O. (1962)

J. Bact. 84, 508.

Silver, S. & Wendt, L. (1967)

J. Bact. 93, 560.

Slepecky, R.A. (1963)

Biochem. biophys. Res. Commun., 12, 369.

Smillie, E.J. & Eason, R.

unpublished data.

Smith-Kielland, I. (1964)

Biochim. biophys. Acta 91, 360.

Smith, M.R. & Woods, W.B. (1956a)

J. Exp. Med. 103, 499.

Smith, M.R. & Woods, W.B. (1956b)

J. Exp. Med. 103, 509.

Stevens, A. (1960)

Biochem. biophys. Res. Commun. 3, 92.

Strominger, J.L. (1957)

J. Biol. Chem. 224, 509.

Strominger, J.L., Ito, E. & Threnn, R.H. (1960)

J. Am. chem. Soc. 82, 998.

Strominger, J.L., Threnn, R.H. & Scott, S.S. (1959)

J. Am. Chem. Soc. 81, 3803.

Suzuki, H. & Kilgore, W.W. (1967)

J. Bact. 93, 675.

Suzuki, H. & Pangborn, J. & Kilgore, W.W. (1967)

J. Bact. 93, 683.

Szybalski, W. & Iyer, V.N. (1964)

Fed. Proc. 23, 946.

Thimann, K.V. (1963)

In "The Life of Bacteria", 2nd ed., p. 758.

New York : The Macmillan Co.

Tomchick, R. & Mandel, H.G. (1964)

J. gen. Microbiol. 36, 225.

Traut, R.R. & Monro, R.E. (1964)

J. Mol. Biol. 10, 63.

Treick, R.W. & Konetzka, W.A. (1964)

J. Bact. 88, 1580.

Umbreit, W.W., Burris, R.H. & Stauffer, J.F. (1957)

"Manometric Techniques" 3rd ed. Minneapolis :

Burgess Publishing Co.

Vazquez, D. (1964)

Biochem. biophys. Res. Commun. 15, 464.

Vazquez, D. (1966a)

In "Biochemical Studies of Antimicrobial Drugs", Symp. Soc.

gen. Microbiol., vol. 16, p. 169. Ed. by Newton, B.A. &

Reynolds, P.E. Cambridge : Cambridge University Press.

Vazquez, D. (1966b)

Biochim. biophys. Acta 114, 289.

Vazquez, D. & Monro, R.E. (1967)

Biochim. biophys. Acta 142, 155.

Ward, D.C., Reich, E. & Goldberg, I.H. (1965)

Science 149, 1259.

Waring, M.J. (1964)

Biochim. biophys. Acta 87, 358.

Weidel, W. & Pelzer, H. (1964)

Advanc. Enzymol. 26, 193.

Weiss, S.B. & Gladstone, L. (1959)

J. Am. chem. Soc. 81, 4118.

White, J.R. & Flaks, J.G. (1962)

Fed. Proc. 21, 412.

Wise, E.M. Jr. & Park, J.T. (1965)

Proc. nat. Acad. Sci., Wash. 54, 75.

Woodruff, H.B. & Miller, I.M. (1963)

In "Metabolic Inhibitors", vol. 2, p. 23. Ed. by Hochster, R.M. & Quastel, J.H. New York : Academic Press Inc.

Woods, D.D. (1940)

Brit. J. Exp. Path. 21, 74.

Yarmolinski, M.B. & de la Haba, G.L. (1959)

Proc. nat. Acad. Sci., Wash. 45, 1721.

Young, C.W. & Hodas, S. (1964)

Science 146, 1172.

Young, C.W., Schochetman, G. & Karnofsky, D.A. (1967)

Cancer Res. 27, 527.