BACTERICIDAL EFFECTS OF

CHLOROQUINE ON ESCHERICHIA COLI

The effects of chloroquine on the viability of growing and non-growing Escherichia co1i in a MOPS (3-(N-Morpholino)-propanesulphonic acid) buffered medium, and its effect on thymidine utilisation.

A Dissertation submitted by

Philip Folaranmi Olurinola, B.Sc.(Pharm.), Ph.C., M.P.S.N.

to the University of Bradford

for the degree of Doctor of Philosophy

Postgraduate School of Studies in Pharmacy,

University of Bradford

May, 1979

BACTERICIDAL EFFECTS OF CHLOROQUINE ON ESCHERICHIA COLI

P. F. OLURINOLA

ABSTRACT

The bactericidal effects of chloroquine on growing and non-growing cells of Escherichia coli NCTC 1093 were investigated under controlled experimental conditions in a MOPS buffered minimal salts medium. Changes in cell mass and viable cell numbers were determined by measurements of absorbance at 600nm, and by viable cell counts respectively.

At pH 7.4, the approximate minimum bactericidal concentration of chloroquine for the organism was 1.0×10^{-3}M. This concentration caused about 3 log cycles of kill within 2 hours, with an initial short lag before kill commenced. Variation in the drug concentration up to a ten-fold increase had no significant effect en the rate of kill, although the time of maintenance of that rate was increased. In non-growing (glucose-starved) cells treated with the same drug concentrations, however, there was virtually no loss in viability for at least 3 hours, except with the highest drug concentration used. Removal of glucose from cultures treated with chloroquine did not appear to affect bactericidal activity.

The effect of pH on the action of chloroquine was also examined. A four-fold increase in activity was observed with increase in pH from 6.7 to 7.7, and calculations indicated that both the monoprotonated and diprotonated forms of chloroquine were biologically active, but that the diprotonated form was only l/25th as effective as the monoprotonated.

Cellular uptake studies of chloroquine by a direct method, using C-labelled chloroquine suggested a biphasic uptake process, a rapid initial passive uptake followed by a slower active one. The latter apparently did not occur in the absence of glucose.

The effect of the drug on the utilisation of thymidine by E. coli cells was investigated. Chloroquine rapidly and strongly inhibited both the uptake and incorporation of ^{14}C-thymidine into the cells, and caused a breakdown of preformed DNA.

This work establishes the bactericidal action of chloroquine against growing cells of E. coli, and indicates that its antibacterial activity has some similarity to the phenomenon of thymineless death.

ACKNOWLEDGMENTS

I wish to express my most profound gratitude to Dr. D. Wiseman for his guidance, encouragement and supervision throughout this project.

I am grateful to Professor J. M. Rowson, Professor G. Osuide and the academic staff of this Pharmaceutics department, through whom the plan for this work originated.

My thanks go to other members of the academic and technical staff for their support and friendliness, to Dr. D. B. Cowell and Miss J.M. Bagnall for their paper translation assistance, and to my colleagues and friends for their useful discussions.

I am grateful to the Ahmadu Bello University, Nigeria, for the Study Fellowship award, and to the Federal Government of Nigeria for part of my sponsorship.

I must thank Mrs. L. Carberry for her expertise in typing this thesis, and Mr. S. Wood for preparing the diagrams.

Finally, I thank my wife, Omowumi, and other members of my family for their patience, support and co-operation, without which this work could not have been completed.

P. F. Olurinola

May, 1979.

TO JESUS CHRIST

CONTENTS

	Page No.
ABSTRACT	(i)
ACKNOWLEDGEMENTS	(iii)
CHAPTER 1. INTRODUCTION.	
Historical Background of Chloroquine	1
Description	2
Malaria	3
Chloroquine and Other Antimalarials	9
Other Clinical Uses of Chloroquine	13
Absorption, Distribution and Metabolic Disposition	15
Toxicity	19
Structure-activity Relationship	20
Mode of Action	21
Prelude to present study	29
CHAPTER 2. MATERIALS AND GENERAL METHODS.	31
Materials	32
General Methods	36
CHAPTER 3. THE EFFECT OF CHLOROQUINE ON GROWTH OF ESCHERICHIA COLI IN MOPS MEDIUM	38
Experimental	39
Results	41
Figures	43
Discussion	45

CONTENTS (Continued)

		Page No.
CHAPTER 4.	THE EFFECT OF CHLOROQUINE ON THE VIABILITY OF GROWING CULTURES OF ESCHERICHIA COLI AT pH 7.4.	47
	Experimental	49
	Results	50
	Figures	53
	Discussion	64
CHAPTER 5.	EFFECT OF GLUCOSE ON THE BACTERICIDAL ACTION OF CHLOROQUINE	67
	Experimental	70
	Results	73
	Figures	79
	Discussion	94
CHAPTER 6.	EFFECT OF pH ON THE ANTIBACTERIAL ACTIVITY OF CHLOROQUINE	98
	Experimental	100
	Results	102
	Figures	105
	Discussion	114
CHAPTER 7.	UPTAKE OF CHLOROQUINE BY ESCHERICHIA COLI	120
	Experimental	122
	Results	124
	Figures	127
	Discussion	134

CONTENTS (Continued)

	Page No.
CHAPTER 8. EFFECT OF CHLOROQUINE ON THE UTILISATION OF THYMIDINE	138
Experimental	141
Results	146
Figures	152
Discussion	160
GENERAL DISCUSSION AND EPILOGUE	163
General Discussion	165
Epilogue	171
APPENDIX	176
REFERENCES	186

CHAPTER ONE

INTRODUCTION

	Page No.
Historical Background of Chloroquine	1
Description	2
Malaria	3
Chloroquine and other Antimalarials	9
Other Clinical Uses of Chloroquine	13
Absorption, Distribution and Metabolic Disposition	15
Toxicity	19
Structure-activity Relationship	20
Mode of Action	21
Prelude to Present Study	29

HISTORICAL BACKGROUND

Chloroquine, an outstanding antimalarial drug, was one of the first synthetic compounds employed in chemotherapy, and is still a very useful drug today. Its history goes back to the years following the first World War. Before that war, the only specific antimalarial drugs available were the Cinchona alkaloids, the most commonly used of which was Quinine (Covell et al., 1955). During the war, however, the Germans were cut off from all sources of Quinine, and this was the stimulus which led them to search for synthetic substitutes. The first fruit of their search was the synthesis of Plasmochin (Pamaquine) in 1925, followed by the synthesis of the acridine compound, Quinacrine (Mepacrine) in 1930. Chloroquine (Resochin), their third success, was first synthesised in 1934 by Andersag, and became the prototype of the 7-halogenated 4-aminoquinoline antimalarials which were patented by Andersag et al. (1939). It was at first thought to be too toxic for general use, and was discarded for Sontoquine, its relative, having an extra methyl group (Coatney, G.E., 1963; Hahn, F.E., 1975).

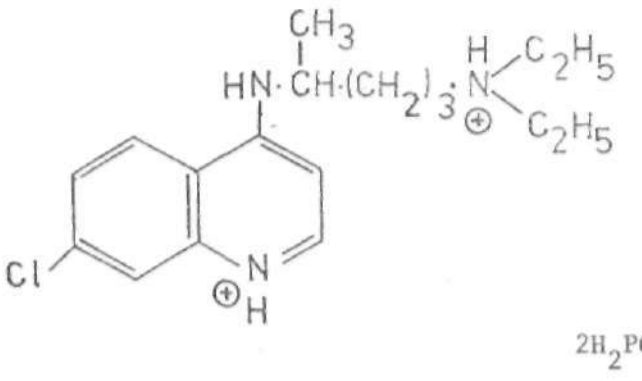
During the Second World War, the Allies (France, Britain, Russia, U.S.A.) in their turn were cut off from the main sources of Quinine, which was still the most commonly used antimalarial drug, and intensive research was undertaken by these nations. This led to improved dosage schedules for mepacrine, and to its general acceptance, a better knowledge of Sontoquine, and a rediscovery of chloroquine in the United States, the latter being introduced into practical Medicine in 1947 (Covell et al., 1955; Coatney, 1963; and Hahn, 1975).

Chloroquine was found to be the most active antimalarial drug among about 16,000 compounds tested in the United States by 1946, and remained the most important drug for the radical cure of malaria for over a decade, until 1961 when chloroquine-resistant falciparum malaria was first reported in Latin America (Moore and Lanier, 1961; Maberti, 1960), and later in South east Asia (Harinasuta et al., 1962; Powell and Trigertt, 1968). An example of the wide use and large scale distribution of chloroquine was given by Coatney (1963), who reported that the United States International Cooperation Administration (now USAID) purchased 88,240,000 tablets of chloroquine diphosphate and 184,500 pounds of its powder for use in medicated common salt in 1960, followed in 1961 and 1962 by the purchase of 75,500,000 and 137,250,000 tablets respectively. Since the introduction of chloroquine into medicine, other drugs such as Proguanil, Pentaquine, Primaquine, Pyrimethamine, Dapsone, Amopyroquine and Trimethoprim have been used as antimalarials. Despite these developments, and the appearances of chloroquine resistant plasmodia, chloroquine is probably still the most generally useful of all currently available antimalarial drugs.

DESCRIPTION

Chloroquine is currently classified in Chemical Abstracts under the heading "Pentanediamines" and designated as 1,4-Pentanediamine, N^4-(7-chloro-4-quinolynyl)-N-diethyl-. However, it will be described in this thesis by its former classification as a quinoline derivative, since most references still describe it as such, and also because the basis of its therapeutic value is "quinolinic" rather than "pentanediaminic" (Coatney et al., 1953; Thompson and Werbel, 1972). Chloroquine is commercially available as the phosphate, sulphate and hydro-

chloride salts (Hong, 1976). The chemical structure of chloroquine diphosphate is shown in Figure 1.1.



$C_{18}H_{26}ClN_3.2H_3PO_4$ Molecular Weight 515.87

Figure 1.1. Structure of Chloroquine Diphosphate.

MALARIA

Aetiology

Malaria is a febrile protozoan infection, caused by members of the genus Plasmodium, which undergo a cyclical development both in the mammalian host, and in the vector, the female Anopheles mosquito (Pinder, 1973). Although there are over 100 varieties of malaria parasites, only four of them are known to infect man, namely *Plasmodium falciparum*, *P. malariae*, *P. ovale*, and *P. vivax*. There are other species which affect birds, mice and monkeys. Interested readers are directed to Garnham (1966) for fuller details. The complicated life cycle of the malarial plasmodia can be divided into two main stages: schizogony, which takes place in the primate host, and sporogony, which matures in the mosquito. Figure 1.2 illustrates some details of the schematic life cycle of plasmodia in lower primates and man, and the drugs which are effective against its various stages.

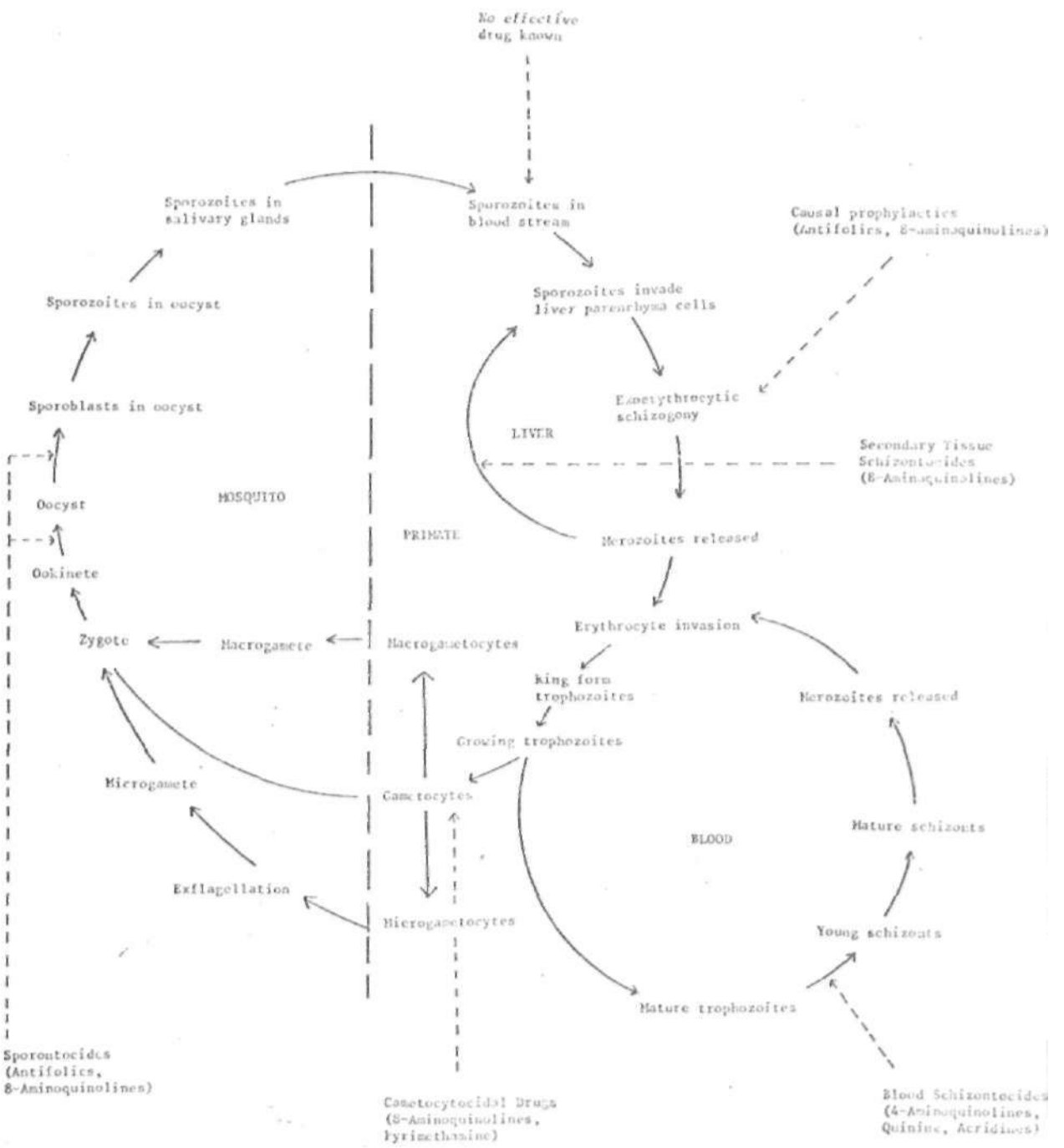


Figure 1.2. Schematic life cycle of Plasmodia in lower primates and man, showing points of action of various antimalarial drugs.

P. falciparum (malignant tertian) malaria is found in warm moist climates, most of tropical Africa, India, Pakistan, South-east Asia, and South and Central America. P. vivax (benign tertian) malaria is widespread in the tropics, subtropics and some temperate regions. P. malariae (quartan) malaria occurs widely in the tropics, especially East and West Africa, India, Sri Lanka, South and Central America. P. ovale (ovale tertian) malaria is uncommon and is found irregularly in East, Central and West Africa, and South America.

Clinical Patterns

The clinical manifestations of malaria result from the invasion of the erythrocytes by plasmodia. P. falciparum infection causes malignant malaria, an acute progressive disease associated with complications, and which is often fatal. The other forms of malaria infection caused by P. vivax, P. malariae, and P. ovale are more benign, have less complications, and rarely lead to death. Their acute attacks are self-limited, and relapses are common.

The incubation period, that is, duration from time of bite by infected mosquitoes to time of appearance of parasites in the human blood stream, is roughly 10 to 15 days for all forms, although it may be several weeks for P. vivax and P. malariae in some temperate regions. The febrile paroxisms in fevers may occur every 48 or 72 hours, that is, every third or fourth day in tertian or quartan malarias respectively, as these are the periods taken for the blood schizonts to mature.

Signs and Symptoms.

1. Infection with P. vivax and P. ovale (vivax or benign tertian malaria and ovale tertian malaria).

A primary attack is often preceded by symptoms of headache, backache, limb pains, anorexia, nausea and sometimes vomiting. Onset of attack is associated with a rise in temperature to about 38.3°C (101°F) or higher; this marks the appearance of parasites in the blood. For the first few days or first week of the primary attack, fevers occur intermittently with peaks of 39.4 - 40.6°C (103 to 105°F), but without clear periodicity. In a majority of cases periodic intermittent fevers follow, and may last up to three months if untreated. In relapses, fevers are periodic from onset.

Febrile paroxisms have three typical stages: the cold, the hot, and the sweating. In the cold stage, the patient feels increasingly cold and shivery, and finally develops rigors. His temperature rises rapidly, skin remains pale and may feel cold. The pulse is fast and thready; blood pressure rises, nausea and vomiting may develop. External warmth such as blankets and hot water bottles may be helpful up to this point. The hot stage then suddenly comes in; the patient feels hot and feverish, rigors stop, the pale dry skin flushes, the pulse is full and bounding, and the blood pressure falls. Nausea and vomiting increase. Temperature may be as high as in the cold stage or higher. The patient becomes restless, possibly incoherent and delirious. Blankets and hot water bottles are no longer needed. This lasts longer than the cold stage, and is succeeded by the sweating stage. Sweating starts at the temples, and spreads rapidly over the whole body, soaking bed clothes. Fever symptoms then disappear, and the patient feels greatly relieved and falls into an exhausted sleep from which he wakes refreshed.

The temperature remains normal for some time, except for a few low peak fevers, and he feels well until onset of the next paroxysm. Parasites are present in the blood throughout an attack, the stage of the predominant erythrocytic cycle depending on which stage of the paroxysm the patient has reached.

Anaemia may be present, especially in long attacks, with relapses. The spleen increases in size, and may be palpable 10-14 days after the onset of the attack. Untreated cases may last six weeks or longer.

2. *Infection with P. malariae.*

This is similar to that described above, but has some differences. The incubation period is often longer (3-4 weeks); paroxysms occur every four days usually, but may include daily or alternate days attack, depending on time of maturation of blood schizonts. Paroxysms last longer, and the sweating stage may be succeeded by prostration. Parasites are fewer. Normocytic anaemia may develop. Untreated attacks may last two or more months.

3. Infection with falciparum malaria may be complicated or uncomplicated.

(a) Uncomplicated falciparum malaria.

Duration of incubation varies from 8-15 days. Onset of attack may be clearcut or insidious, at times with symptoms not suggesting malaria. The patient may develop moderate fever with headache, bone and joint pains, and backache; or he may appear moderately ill with influenza symptoms, a dangerous type that may easily lead to death, due to inappropriate treatment.

In severe cases there may be anxiety, excitement or maniacal outbursts. Light or deep coma may develop, but if it is the latter, the case is better treated as a complicated one. In some cases periodic fevers may never develop; in others there may be quotidian, tertian or subtertian paroxysms (i.e. daily, every other day or slightly less). These resemble those of vivax malaria, but are often shorter. Skin may be moist throughout, and the 'well-being feeling' may be absent.

The parasites are often present in large and increasing numbers in the blood, first peripherally, and later in deep tissue vessels. Anaemia is often severe. The spleen is rapidly enlarged, and the liver, enlarged and tender. Urinary problems may set in. The blood pressure is low, and the pulse full, bounding and fast.

Uncomplicated cases respond well to specific treatment, but untreated attacks often end fatally. If the patient survives a partial immunity is developed against the causal strain.

(b) Complicated falciparum malaria.

Complications are more likely to develop in anaemic patients, or in those where more than 5% of their erythrocytes are infected. Such complications may result in cerebral malaria - with signs and symptoms related to disorders of the CNS; dysenteric malaria - in which the patient passes frequent stools with blood and mucus; choleraic malaria - with acute watery diarrhoea, vomiting and dehydration; bilious remittent fever - with increasing jaundice; or algid malaria - associated with circulatory failure or shock. For details of these complications and for a full description of malaria the reader is directed to Woodruff (1978).

In certain cases of P. falciparum infection, Blackwater Fever may develop; this is a syndrome in which waves of acute haemolysis with both haemoglobinaemia and haemoglobinuria occur.

Malaria in Partial Immunes

Individuals who have acquired immunity or resistance to local parasite strains as a result of past infections or drug prophylaxis can be described as "partial immunes". In them clinical features of malaria differ from those of non-immune adults described above. Generally they consist of malaise, mild fever, headache, backache, anorexia, sweating, feeling unwell and ineffective.

Malaria during pregnancy can cause abortion or premature labour. It may also occasionally be transmitted across the placenta to the foetus. Antimalarials reduce the risk, and prevent post-partum fever due to the disease.

Chloroquine and other Antimalarials.

The activity of an antimalarial drug is usually restricted to one or two stages of the life cycle of the plasmodium (Thompson and Werbel, 1972). Chloroquine acts mainly against the asexual erythrocytic forms, and is, therefore, described as a blood schizontocide. It is also effective against the gametocytes of P. vivax and P.malariae, but is ineffective against those of P. falciparum. It is employed both in the treatment and suppression of malaria.

Antimalarial drugs in general can be classified according to the stage of the life cycle on which they act.

1.

1. Primary Tissue Schizontocides - the antifolics: Proguanil, Chlorproguanil, Cycloguanil, Pyrimethamine, and Trimethoprim; and, to some extent, the 8-aminoquinolines. These drugs act on the pre-erythrocytic forms of the parasite, and are used as causal prophylactics.

2. Blood Schizontocides - Amodiaquine, Chloroquine and other 4-aminoquinolines, Mepacrine, and Quinine. These act on the asexual erythrocytic forms; they are effective suppressants of all forms of human malaria, and effect radical cure in most cases of falciparum infection.

3. Secondary Tissue Schizontocides. The 8-aminoquinolines - Pamaquine, Primaquine, Pentaquine, Plasmocid, and Quinocide - act on late exoerythrocytic forms. They are used as anti-relapse drugs.

4. Gametocytocides - Pamaquine and other 8-aminoquinolines have a powerful destructive action on all stages of the gametes of P. falciparum and P. vivax. Quinine, Mepacrine and Chloroquine are also of some value against some gametocytes.

5. Sporontocides - Proguanil and other antifolics, and the 8-aminoquinolines are effective against the sporogonic forms in the mosquito and are used as clinical prophylactics.

The superiority of Chloroquine over Quinine and Mepacrine is shown by the fact that it is more active both as a suppressive and as a therapeutic agent; it does not have to be as frequently administered for effective results; it causes less tissue reaction following intramuscular administration; and it does not stain the skin or conjunctivae

(Covell et al., 1955). It has a rapid action, which gives it an advantage over the 8-aminoquinolines, Pyrimethamine, and Proguanil (De Carlo and Russo, 1970). Chloroquine is also more active than other 4-aminoquinolines except Amodiaquine.

Chloroquine has been marketed under several names such as Resochin (Bayer), Aralen (Winthrop), and Avloclor (I.C.I. - Pharmaceutical) - all of which are the phosphate salts; and also Nivaquine B (May and Baker), which is the sulphate salt. However, the dose is usually expressed as the base equivalent to remove confusion of dosage.

For the treatment of acute malaria, the loading dose principle first put forward by Shannon et al. (1944) is followed, as recommended by Covell et al. (1955). 600 mg. base is given initially, followed by 300 mg. after 6 hours, and 300 mg. daily for the next two days. It is usually given orally in the form of tablets or syrup, unless these forms are not acceptable by the patient, in which case parenteral administration may be used either wholly, or as the initial form of therapy. This treatment regimen has remained virtually the same over the years, the only slight alteration being an increase in the period of treatment to 4 days (Martindale, 1977; Woodruff, 1978).

In grave infections, intramuscular or intravenous injections may be given in single doses of 200-300 mg.,up to a total of 800 mg.spaced over 24 hours, oral therapy being resumed as soon as practicable. In cerebral malaria, only the parenteral administration is applicable for the first 24 hours.

For semi-immune dispensary patients, a single adult dose of 600 mg. base orally is recommended. The drug is relatively well tolerated in children, and 3 to 6 year olds can take 300 mg. as a single dose, or alternatively 5 mg./kg. body weight.

As a suppressive in endemic areas, chloroquine can be given in weekly doses of 300 mg. base orally.

Chloroquine-Resistant Malaria.

The use of chloroquine in the chemotherapy of malaria has been hampered in recent years by the increasing prominence of chloroquine-resistant strains of P. falciparum, first reported in South America and then in South-East Asia. The area affected in South America extended from Columbia and Guayana in the North, to Bolivia and Uruguay (Calvao et al., 1961; da Silva, 1966; Giglioli, Rutten and Ramjattan, 1967; Comer et al., 1968; Rieckmann and Lopez-Antunano, 1971). The area affected in South-East Asia stretched from Thailand and Vietnam to Sumatra (Pinder, 1973; Peters, 1970; Harinasuta et al., 1962; Bourke et al., 1966; McKelvey et al., 1968; Ng et al., 1969; Colwell et al., 1972). However, chloroquine-resistant falciparum malaria has not yet been confirmed in any part of the malarious areas of Africa (Woodruff, 1978).

Chloroquine-resistant malaria of type (RI) in South America has been treated by increasing the dose of chloroquine up to a total of 2.4 g, instead of the usual 1.5 g(Garcia-Martin, 1965). Resistant strains of the more serious type (R III) found in Malaysia have been treated with intravenous Quinine (Laing, 1968), whilst others have been found to be sensitive to Amodiaquine and Amopyroquine (Rieckmann, 1971).

Multiple resistance to Chloroquine, Pyrimethamine and Dapsone has been reported in Cambodia by Verdrager, Riche, Chheang, 1967, 1969). Generally the more drugs are involved in the resistance, the more difficult the infection is to treat. The current practice seems to be the use of drug mixtures to which the infecting strains are still sensitive. For example, strains resistant to 4-aminoquinolines respond to a full dose (1.3 g daily for 10 days) of Quinine, or a combination of Sulphormethoxine 1.0 g and Pyrimethamine 50 mg. given at once (Woodruff, 1978). Similarly for suppressive regimens, one tablet of Maloprim (12.5 mg. Pyrimethamine plus 100 mg. Dapsone) weekly is effective in areas where chloroquine-resistant strains are prevalent.

Other approaches have been made to overcome drug-resistant malaria. Through these came the development and clinical uses of the new antimalarial sulphonamides such as Sulphamonomethoxine, Sulphalene, and Trimethoprim, which have been found of value in treating chloroquine-resistant falciparum malaria (Harinasuta et al., 1967; Laing, 1970; Pirame et al., 1968; Baruffa, 1966; Michel, 1968; Yoshinaga et al., 1970). But the ease with which plasmodia and microbes develop resistance against Pyrimethamine and Sulphonamides suggests that these "new generation" sulphonamides may not be of use for long, and may only serve as temporary remedies. There is a need for the continuation of the search for a permanent solution.

Other Clinical Uses of Chloroquine.

In addition to its antimalarial uses, chloroquine has been employed in the treatment of several other diseases.

Hepatic Amoebiasis - Alone, or in combination with emetine hydrochloride, dehydroemetine, or diiodohydroxyquinoline, it has been found effective (Wilmot et al., 1959; Martindale, 1977). The reviews on the therapeutic applications of chloroquine by De Carlo and Russo (1970) have also shown that the drug has been used successfully in the treatment of various forms of hepatitis. The recommended dose for the treatment of hepatic amoebiasis, when used alone, is the equivalent of 300 mg. of chloroquine base orally twice daily for two or more days, followed by a daily 300 mg. dose for two or three weeks. Children may be given 6 mg/kg body weight. Alternatively, when parenteral administration is advisable, intramuscular injections of 160-200 mg. daily are given for 10 to 12 days. Children can again be given 6 mg/kg body weight (Martindale, 1977). Chloroquine is claimed to be as effective as metronidazole in treating this disease, although it takes a longer time, but it is ineffective against intestinal amoebiasis (Cohen and Reynolds, 1975).

Discoid or Subacute Lupus Erythematosus Chloroquine has also been found of value in the treatment of this disease, the recommended dose being 300-450 mg. of chloroquine base daily, in divided doses, initially, later reduced to 150 mg. once daily.

Rheumatoid Arthritis - Chloroquine has been used extensively in the treatment of this disease, and remission of symptoms has been effected (Freedman and Sternberg, 1960; Popert et al. , 1961; Lansbury and Menduke, 1963). The B.P.C. 1973 dose recommendation is 120-900 mg. daily. However, it is not very effective and is coming to be regarded as obsolete because of the high incidence of toxic side effects observed with high doses and prolonged use (B.N.F. 1976-78).

Other uses of chloroquine include the control of cardiac arrhythmias, with a dose of 1.0 g. daily in divided doses (Martindale, 1977), in giardiasis, and in some skin diseases such as rosaceae, localised scleroderma, solar dermatitis, lymphocytosis facci, acne, and sarcoidosis (Goldman and Preston, 1957; Knox and Owens, 1966).

Many other apparently unrelated actions of chloroquine have been reported by research workers, which have not been applied to clinical uses yet. Ayitey-Smith and Boye (1974) reported its protective ability against histamine induced bronchial asthma in rabbits; Norman and Gombe (1975), its stimulatory effect on the respiration and motility of bovine spermatozoa; Tarayne and Lauressergues (1978), its value in the treatment of Bordetella pertussis infections in rats. Chandra et al. (1978) have reported its use for fertility control in female rhesus monkeys, and Shoemaker (1978) achieved some success in using it with 5-fluoro-uracil in the treatment of mammary carcinoma in mice. Unrelated as these reports may appear, many of them were observations made in the course of investigating the mode of action of chloroquine, an event that is still in progress.

ABSORPTION, DISTRIBUTION AND METABOLIC DISPOSITION.

Absorption

Absorption from the human gastrointestinal tract is rapid and essentially complete within a short time (2 to 4 hours) after oral administration of the soluble salt forms of chloroquine (Berliner et al., 1948; De Carlo and Russo, 1970; Thompson and Werbel, 1972). In monkeys, Wiselogle (1946) reported an absorption of up to 90% of a soluble salt within two hours of administration. Absorption is more rapid in the intestines than in the stomach because of pH differences -

1.2 in the latter, and 6.1 - 7.3 in the former (Sorsby, 1970).

Distribution in Blood.

With acceptable regimens of malaria treatment, using the loading dose principle orally, effective chloroquine concentrations in the plasma are reached within two to three hours; when given intramuscularly, within 15 minutes (Covell et al. , 1955). Effective plasma concentrations for malaria therapy for P. falciparum and P. vivax vary from 0.02 x 10^{-6}M to 0.39 x 10^{-6}M (Berliner et al., 1948; Most et al., 1946). Other plasma levels have been reported by different workers: McChesney, Fasco and Banks (1967) reported 0.39 x 10^{-6}M after 14 days of medication at 310 mg. per day, whereas Zvaifler and Rubin (1962) reported plasma levels of 11.6 to 15.5 x 10^{-6}M after 4 to 14 days medication with doses varying from 125 mg. to 750 mg. daily. These differences were most likely due to differences in the methods of extraction used. A substantial portion (about 50%) of administered drug has been shown to be bound to plasma proteins (Berliner et al., 1948; Martindale, 1977), in which form it is transported to different parts of the body, and slowly released.

In normal erythrocytes, the chloroquine concentration is about twice that in the plasma (McChesney et al., 1966, Berliner et al., 1948), but in parasitised erythrocytes the drug has been shown to be concentrated hundreds of times at low doses. For example, Polet and Barr (1968) reported 300 to 600 times the concentrations found in normal erythrocytes in monkeys infected with P. knowlesi. Macomber, O'Brien and Hahn (1966) found a hundred fold increase over the plasma concentrations in erythrocytes infected with P. berghei, and Fitch (1969) a 600-fold increase over an external concentration of 1.0 x 10^{-8}M, but only a 15-fold increase in the presence of 1.5 x 10^{-3}M. However, red

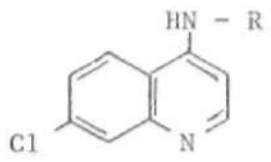
blood cells parasitised by chloroquine-resistant plasmodia accumulate only a half to a third of the drug accumulated by cells infected by chloroquine-sensitive parasites (Macomber, O'Brien and Hahn, 1966), an effect suggested to be due to differences in capabilities of the plasmodia to permit passage or accumulation of critical concentrations of the drug.

Tissue Distribution.

Apart from its binding to plasma proteins, chloroquine or its metabolites is stored in several different tissues of the body, following single or prolonged administration of the drug. In man the distribution follows the general order of liver>spleen>heart>kidney >brain (Prouty and Kuroda, 1958). In animals there is a difference in distribution between albino and pigmented species. In the albino, the highest concentrations are found in nucleated tissues such as liver, heart, and kidney (Berliner et al., 1948; McChesney et al., 1965),whereas in pigmented animals, the eyes have the highest concentrations. In rhesus monkeys, McChesney et al., (1966) found the tissue distribution to be in the order: retina-choroid>adrenal>liver = pituitary>lung>kidney = spleen = bone marrow >heart>skin = sclera>muscle. But in terms of total amounts, the liver and muscle were the most important storage organs of the drug. Recent findings by Fischer and Fitch (1975), from their autoradiographic studies with rat and chicken tissues, have shown that the drug has considerable affinity for bone. This tissue may be a potentially significant storage organ for chloroquine and its metabolites, and may account for the maintenance of plasma levels long after stopping administration of the drug.

Metabolism and Excretion.

Metabolic products of chloroquine in human tissues and urine have been studied by Kuroda (1962). He found that the drug undergoes slow metabolic conversions along a major pathway involving oxidation on the side chain, which finally leads to the formation of 4-amino-7-chloroquinoline. However, McChesney et al. (1966),



1. R = $-CH.(CH_2)_3.N(C_2H_5)_2$ (CH$_3$)
2. R = $-CH.(CH_2)_3.NH.C_2H_5$ (CH$_3$)
3. R = $-CH.(CH_2)_3.NH_2$ (CH$_3$)
4. R = $-CH.CH_2.CH_2.CH_2OH$ (CH$_3$)
5. R = H

Metabolic products of chloroquine in man (Kuroda, 1962).

while confirming a degradation product to be the 4-amino-7-chloroquinoline, pointed out that it was a relatively minor metabolite in man. The proportions of chloroquine and its metabolites in urine, following daily administration of the drug to man, were found to be chloroquine 60%, secondary amines 37%, primary amines 3%. Their later work (McChesney, Fasco and Bank, 1967) showed that about 23% of the excreted drug in the urine was monodesethylchloroquine.

Of the total amount of drug administered to human patients at 310 mg. per day, over a 14-day period, the same workers found 10% excreted in the faeces, and 56% in the urine; 34% was not recovered, and its fate in the body could not be ascertained. It is likely that

a substantial part of the drug or its metabolites was stored in a remote tissue, such as bone, as later shown to be possible by Fischer and Fitch (1975); or that there were metabolites present which were undetectable by their methods. Essien (1978), for example, has recently reported the detection of two new metabolites - chloroquine-N-oxide, and chloroquine-N-dioxide in human urine following the administration of the drug. It is possible if not probable that there are other metabolites still undetected.

Toxicity.

Chloroquine is generally well tolerated at the doses required for the treatment and suppression of malaria, but reported side effects include pruritis, headache, blurring of vision, occasional nausea and vomiting, diarrhoea, abdominal cramps and urticaria. More severe side effects may include rare psychotic episodes, convulsions, hypotension and cardiovascular collapse, EEG changes, double vision and difficulty in focussing the eyes.

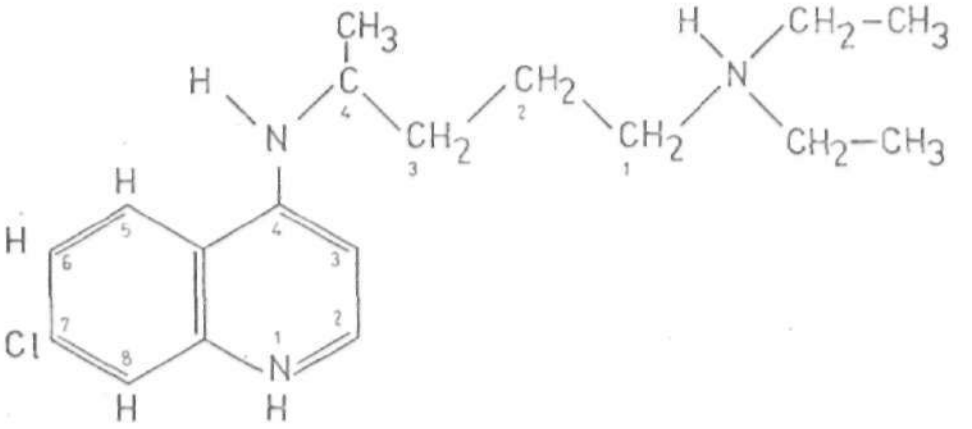
Overdosage, especially in children, can lead to headache, drowsiness, arrhythmias, shock, visual disturbances, severe gastrointestinal disturbances possibly leading to convulsions,respiratory and cardiovascular depression, and death.

Prolonged administration and high doses as used in rheumatoid arthritis may lead to irreversible serious retinal changes. Other uncommon toxic effects from prolonged use include bleaching of the hair pigment, bluish-black pigmentation of mucous membranes and skin, photosensitivity, nerve deafness, neuromyopathy and myopathy (Martindale, 1977).

This long list of the possible side effects of chloroquine appears frightening, and could deter people from taking the drug, but the author can state from personal experience and observation that, at the doses used for malaria therapy and suppression, side effects are rare and almost always much milder than the tortures of the infection (Olurinola, 1967-72).

Structure-Activity Relationship.

Studies of the structure-activity relationship of chloroquine and related compounds show that variations in nuclear substituents seem more consistently to affect the biological activity than changes in the side chain (Thompson and Werbel, 1972). The halogen



substituent in position 7 of the nucleus is important for activity; a halogen in any other position, or dihalo-substitution reduces antimalarial activity (Coatney et al., 1953). Variation in the basic side chain generally has less influence on biological activity, as do variations in the terminal amines. With some exceptions aryl branching of the side chain reduces activity, while alkyl substitution other than methyl lowers the therapeutic index. Activity seems to be retained so long as the carbon skeleton basic side chain has two to five carbon atoms, and decreases with greater chain lengths; the

latter also increases toxicity. Large alkyl substituents (C_6H_{13} or greater) on the terminal nitrogen also tends to decrease activity and increase toxicity (Wiselogle, 1946).

Mode of Action.

Although much is known about the structure, therapeutic uses and toxicity of chloroquine, there are still uncertainties about its mode of antiplasmodial and anti-inflammatory actions. Several research workers have been involved in tackling the problem,with the resultant emergence of several views and postulates.

One of the first approaches to uncovering the mode of action of chloroquine was observation of the morphological changes which occur in plasmodia exposed to chloroquine. Thurston (1953) and other early observers, using the light microscope, reported aggregation of hemozoine - the malaria pigment, which has been identified as the residues of haemoglobin chains incompletely degraded by plasmodial cells. The formation of such aggregations was reported to be the first and most conspicuous change in plasmodial morphology following exposure to the drug. Similar observations had been made much earlier with quinine and with quinacrine treated plasmodia by Marchiafava and Bignami (1894), Bock,(1939), and Mackerras and Ercole (1949). More recent work by Macomber et al. (1967) showed that hemozoine aggregation can be observed as early as 30 minutes after administering the drug to mice infected with P. berghei, and that it is completed after four hours. It was reported that these drugs caused first the clumping of the phagosomes in which the haemoglobin metabolism took place, and later their expulsion from the parasite. Aggregation in the phagosomes has been

shown to reach a peak at the same time as maximum chloroquine concentrations are achieved in the plasmodial infected erythrocytes (Warhurst and Hockley, 1967), and it is possible that this clumping is related to the accumulation of chloroquine in infected erythrocytes, and to the mode of antiplasmodial action of chloroquine.

Another major approach in studying the mode of action of chloroquine was to determine its effect on macromolecular biosynthesis. Chloroquine, in dilute aqueous solution, is known to exist mainly as a doubly protonated cation at physiological pH (Irvin and Irvin, 1947). It is this ionic species that has been shown to form molecular complexes with nucleic acids (Parker and Irvin, 1952), and to inhibit in vitro reactions catalysed by DNA and RNA polymerases (Cohen and Yielding, 1965a). These actions of chloroquine have been confirmed by the use of equilibrium dialysis (Stollar and Levine, 1963), viscosimetry (Kurnick and Radcliffe, 1962), and spectrophotometry (Cohen and Yielding, 1965b). The latter reported from their work that chloroquine binding to DNA involves both electrostatic attraction between the protonated ring system of chloroquine and the anionic phosphate groups of DNA and a more specific interaction apparently involving the aromatic ring portion of chloroquine and the nucleotide bases.

Search for a test organism for use in investigating the mechanism of action of chloroquine led Ciak and Hahn (1966) to a strain of B. megaterium, against which the drug was found to be bactericidal at a concentration of 1.6×10^{-3}M in growth medium. In the presence of this concentration of chloroquine, the biosyntheses of DNA and RNA was stopped, and ribosomal RNA and ribosomes themselves were degraded,

thus preventing any further synthesis of proteins. From the results of this work, they proposed the mechanism of antimicrobial action of chloroquine to be inhibition of DNA replication.

In the same year, O'Brien et al.(1966) (including Hahn) proposed a hypothetical structure of the DNA-chloroquine complex, and postulated that the quinoline ring of chloroquine is intercalated between base pairs of double stranded DNA involving electrostatic interactions, while the 1,4-diaminopentane side chain falls outside the contour of the base pairs and bridges the minor groove of the double helix by binding electrostatically to DNA phosphates. In this way the electropositive 2-amino group of the guanine is brought into close contact with the 7-chloro atom of the quinoline ring of chloroquine; this interaction being thought to be critical for complexation with DNA.

Polet and Barr (1968), using intraerythrocytic P. knowlesi, showed that chloroquine at 10^{-7}M in the medium inhibited DNA and RNA syntheses strongly and protein synthesis much less. But Warhurst and Williamson (1970), with a concentration of 18 mg. of chloroquine base per kg. of monkey body weight observed dissimilation of ribosomal RNA in the parasites.

Chloroquine was also found to inhibit nucleic acid biosynthesis in erythrocytic P. berghei by Van Dyke et al. (1969), an effect shown to be due to inhibition of incorporation of adenosine into the nucleic acid of the organisms. A similar confirmation was later reported by Theakston et al., (1972) who reported the effect of chloroquine on the incorporation of tritiated adenosine, uridine and methionine into P. berghei. Their work showed strong inhibition of incorporatic of ^{3}H-adenosine by chloroquine.

Other findings on the effect of chloroquine on the biosynthesis of macromolecules have been reported. It inhibited the uptake of ^{32}P into both DNA and RNA of chick red blood cells infected by P. gallinaceum (Schellenberg and Coatney, 1961), a process probably more related to substrate utilisation than to nucleic acid synthesis. It inhibited (like Quinine, Primaquine and Quinacrine) uptake of amino acids by protozoa (Conklin and Chou, 1970). The same workers also reported inhibition of the uptake of thymidine, uridine and amino acids by Tetrahymena pyriformis two years later.

The effects of chloroquine on the metabolism of carbohydrates in plasmodia have also been looked into. Glucose is an essential source of energy for intracellular growth of plasmodia, particularly for the de novo synthesis of folates which depends on the active glycolytic mechanism. It is also involved in the de novo synthesis by plasmodia of aspartate, glutamate, and alanine. Polet and Conrad (1969) and Polet et al., (1969), in their uptake studies with P. knowlesi, found that while some amino acids such as methionine and isoleucine are taken up by these plasmodia, aspartate and glutamate are not taken up at all. The production of these two amino acids and alanine by avian plasmodia has been demonstrated by Sherman and Mudd (1966), and Sherman and Ting (1969) by using ^{14}C labelled bicarbonate as a precursor. P. knowlesi was reported by Polet et al. (1969) to demonstrate similar ability to synthesise their own aspartic and glutamic acids and alanine from ^{14}C labelled glucose. These findings together with those of Siu (1967), Nagarajan (1968) and Sherman and Ting (1968) apparently show that P. lophurae, P. berghei, and P. knowlesi synthesise their own aspartic and glutamic acids by the fixation of carbon dioxide. These processes

are suggested to involve the activities of phosphoenolpyruvic carboxylase and also phosphoenolpyruvic carboxikinase. Chloroquine and Quinine, but not spermine, inhibit those enzymes (Siu, 1967). Chloroquine has also been reported to inhibit both the uptake and glycolysis of glucose by plasmodia (Ali et al., 1969).

In recent years, the presence of protozoan mitochondria in avian plasmodia, and of equivalent organelles in mammalian plasmodia (evidenced by the presence of cytochrome oxidase in the trophozoites of mammalian parasites) have been demonstrated (Theakston et al., 1969; Howells, Peters and Fullard, 1970; Schiebel and Miller, 1969a, b). Chloroquine has been shown to inhibit in vitro the mitochondrial oxidation of reduced nicotinamide adenine dinucleotide (NADH) and succinate by coenzyme Q (Skelton et al., 1968, 1970). This inhibition may be by interfering with the biosynthesis of CoQ_8, or by competitive inhibition at its site of action (Turnbull et al.,1971). This effect may contribute to the mode of antimalarial action of chloroquine.

A critical appraisal of these reports and findings on the mode of antimalarial action of chloroquine indicates that it is capable of attacking the malarial plasmodia at many points. The review of Pinder (1973) on the mode of action of the antimalarials is helpful in this respect. In his review, Pinder described four of the major metabolic reactions occurring in plasmodia and ways, from several quoted works, that various antimalarials attack or block stages in this metabolism.

1. Plasmodial Synthesis of Purines and Pyrimidines.

Plasmodial syntheses of these passes through the synthesis of folic acid, starting from PABA. Only the antifolic antimalarials -

Proguanil, Pyrimethamine, Trimethoprim, and the sulphones act at points in these syntheses.

2. Plasmodial Utilisation of Purines and Pyrimidines.

Chloroquine, together with Dapsone and Quinacrine, inhibits the uptake of adenosine at the level of the red-cell membrane. It inhibits the incorporation of adenosine monophosphate into parasite DNA and RNA (Van Dyke et al., 1969; Lantz and Van Dyke, 1971).

3. Plasmodial Synthesis of Proteins.

Chloroquine interrupts the synthesis of messenger RNA in the parasite nucleus (Morris et al., 1970; Roskoski and Jaskunas, 1972), thus causing lesions in cytoplasmic ribosomes. It forms molecular complexes with nucleic acids, thus inhibiting DNA replication primarily, and RNA transcription secondarily.

Chloroquine causes amino-acid starvation by interfering with haemoglobin degradation by plasmodia during clumping. It also blocks incorporation of isoleucine into parasite proteins (Gutteridge et al., 1972). It, together with Primaquine, Quinacrine and Quinine, inhibits uptake of amino acids by protozoa.

4. Carbohydrate Metabolism in Plasmodia.

As mentioned above, glucose is an essential energy source for intracellular growth of plasmodia and for de novo syntheses of folates and some amino-acids. Chloroquine inhibits both uptake and glycolysis of glucose. It also inhibits in vitro mitochondrial oxidation of NADH and succinate by Coenzyme Q.

The evidence for intercalation of chloroquine with parasite DNA is strong, and this may be an important component of its mode of action.

However, from the volume of other findings, intercalation may not be the only, or even the primary mode of antiplasmodial action of chloroquine. Quinacrine binds much more strongly by intercalation to parasite DNA than chloroquine, and inhibits uptake of precursors about ten times more effectively than chloroquine, yet it is not as effective antimalarially as chloroquine. It is only more toxic. The results of some workers, showing the effects of pH on the antimicrobial action of chloroquine (Wiseman, 1972a; Middleton, 1976), revealed that the drug was less effective when the 'intercalator' - the double protonated cations of the drug were the main form in solution. Activity of the drug increased with increase in the proportion of monoprotonated ionic species of the drug (Middleton, 1976). Also, see Chapter 6 of this thesis. The clumping of the parasite phagosomes, leading to amino-acid starvation in the parasite, is an effect distinct from complexation with nucleic acids. The emergence of new effective non-intercalating antimalarials like Mefloquine (Davidson et al., 1977) indicates that intercalation with DNA is not a prerequisite for antimalarial activity as a blood schizontocide.

One may argue that a major attack on an important metabolic reaction in the plasmodia will eventually affect all other processes. However, it is becoming clear that chloroquine is doing more than one thing at a time in the malarial plasmodia.

Mechanism of Action of Chloroquine-resistance.

On the mechanism of resistance to chloroquine by plasmodia, several views have been put forward. Macomber et al. (1966) reported marked differences in the uptake of ^{14}C chloroquine by erythrocytes parasitised by P. berghei c.s. (Chloroquine sensitive) and P. berghei

c.r. (Chloroquine-resistant), and suggested that the differences were due to differences in cell capabilities to permit passage or accumulation of critical concentrations of the drug. Later works by Fitch (1969, 1975 and 1978), using different techniques to investigate accumulation of chloroquine into erythrocytes parasitised by P. berghei c.s. and P. berghei c.r., seem to indicate that the mechanism of resistance to chloroquine lies in the ability of the resistant parasite to alter the surface of the erythrocyte membrane to limit the process of chloroquine accumulation.

Mode of Anti-inflammatory Action.

The modes of action of chloroquine in the treatment of rheumatoid arthritis and lupus erythematosus are still not clear. Stollar and Levine (1963) proposed that its action against lupus erythematosus may be due to its interaction with DNA by preventing the latter from binding to antiserum. Its action against rheumatoid arthritis may be similar. Whitehouse (1962), and Whitehouse and Bostrum (1962, 1965) in a series of studies on antiinflammatory drugs postulated that compounds need to be lipophilic to be effective in the treatment of rheumatoid arthritis.

Forsdyke (1975) found a relationship between chloroquine and complement from his studies on lymphocyte mitogens. He reported that chloroquine increased the inhibition of cultured lymphocytes by high concentrations of phytohemaglutinin (PHA), or Concanavalin A (Con A), an inhibition also increased by complement. He concluded from his own results and other published data, including those of Handemaker and co-workers, that chloroquine exerts its therapeutic effects in auto-immune diseases (lupus erythematosus and rheumatoid arthritis)

and in malaria by enhancing or simulating the action of complement upon autologous cells (erythrocytes or lymphocytes). More work may be needed before a final conclusion can be drawn.

A lot more, in several volumes, can be written about chloroquine and its relationship with malaria and other forms of therapy. The little that has been mentioned above is to enlighten or remind the reader of some of what has been known about chloroquine, and how researchers are still marching on to unravel the 'mysteries' behind its multi-purpose actions, but with emphasis on malaria therapy.

Many of the studies already done are difficult to compare because of differences in techniques, organisms and tissues used, media used in growing or maintaining these, concentrations of the drug used, and other environmental conditions such as temperature and pH. It would have been ideal to study the effects of the drug on various aspects of the human malarial plasmodia under conditions similar to those that obtain during the disease. This has not, however, been possible because of the complicated life cycle of plasmodia, and the great difficulty of growing the organisms in vitro. The alternative that researchers have taken is to study the effects of the drug on isolated aspects of the metabolic reactions of plasmodia, often under dissimilar experimental conditions. Under such varying conditions, results which demonstrate recurrence of a particular drug effect on a parasite system serve as a confirmation of that particular drug action, but disagreeing results are difficult to correlate.

Another reasonable alternative is to work on a single micro-organism, and to study the effects of the drug on various aspects of

its metabolism, growth and viability under controlled experimental conditions. Middleton (1976) has done precisely this, working on a strain of E. coli. He studied mainly the effect of chloroquine on the growth and respiration of E. coli.

The present work has some similarities to the work of Middleton, but the emphasis this time is on the effect of the drug on the viability of E. coli. Although the work is on a bacterial species instead of a plasmodium, the insight gained from the results of this work may be applicable to what obtains in plasmodia, and thereby shed further light on the mechanism of antimalarial action of chloroquine.

CHAPTER TWO

MATERIALS AND GENERAL METHODS

	Page No.
Materials	32
Organism	32
Growth Medium	32
Diluting and Plating Media	35
Chemicals	35
General Methods	36
Cultivation of Organism	36
Viable Cell Counts	36
Absorbance Measurements	37
Chloroquine Solution	37
pH Measurements	37

1. MATERIALS

(a) Organism

The organism used was Escherichia coli NCTC 1093, obtained from the National Collection of Type Cultures, London, and adapted for growth in a MOPS buffered minimum salts medium (see below). Purity of the bacterial cells was maintained by continual subculturing, regular microscopic examinations, or plating out. New freeze-dried cultures were germinated whenever doubts about purity arose.

(b) Growth Medium

The growth medium used was a chemically defined one, buffered with MOPS 3-(N-Morpholino)-propanesulphonic acid .

MOPS is one of the organic zwitterionic buffers which possesses many of the properties of the 'ideal' biological buffer, as prescribed by Good, N.E., et al. (1966). Its pK_a varies only from 7.2 at 20°C to 7.01 at 37°C, a change in pK_a of 0.011 per °C. Its useful buffering range is pH 6.5 to 7.9 (Good, N.E. and Izawa, S. 1972, Hopkins and Williams, 1978).

Compared with the more commonly used Tris, tris (hydroxymethyl)-aminomethane , it has a more useful buffering range, it is less toxic, and the change of pH with change in temperature is smaller. According to Middleton, K.R. (1976), the change in pH per degree centigrade was 0.031/°C for Tris, whereas with MOPS, it was found to be -0.0083/°C. (See Figure 2.1.)

Since the antibacterial action of chloroquine is very sensitive to variations in pH, MOPS was considered a more suitable buffering agent than the Tris used by previous workers in these laboratories (Rye, R.M., 1969; Wiseman, D., 1969; Salt, W.G., 1970; and Middleton,

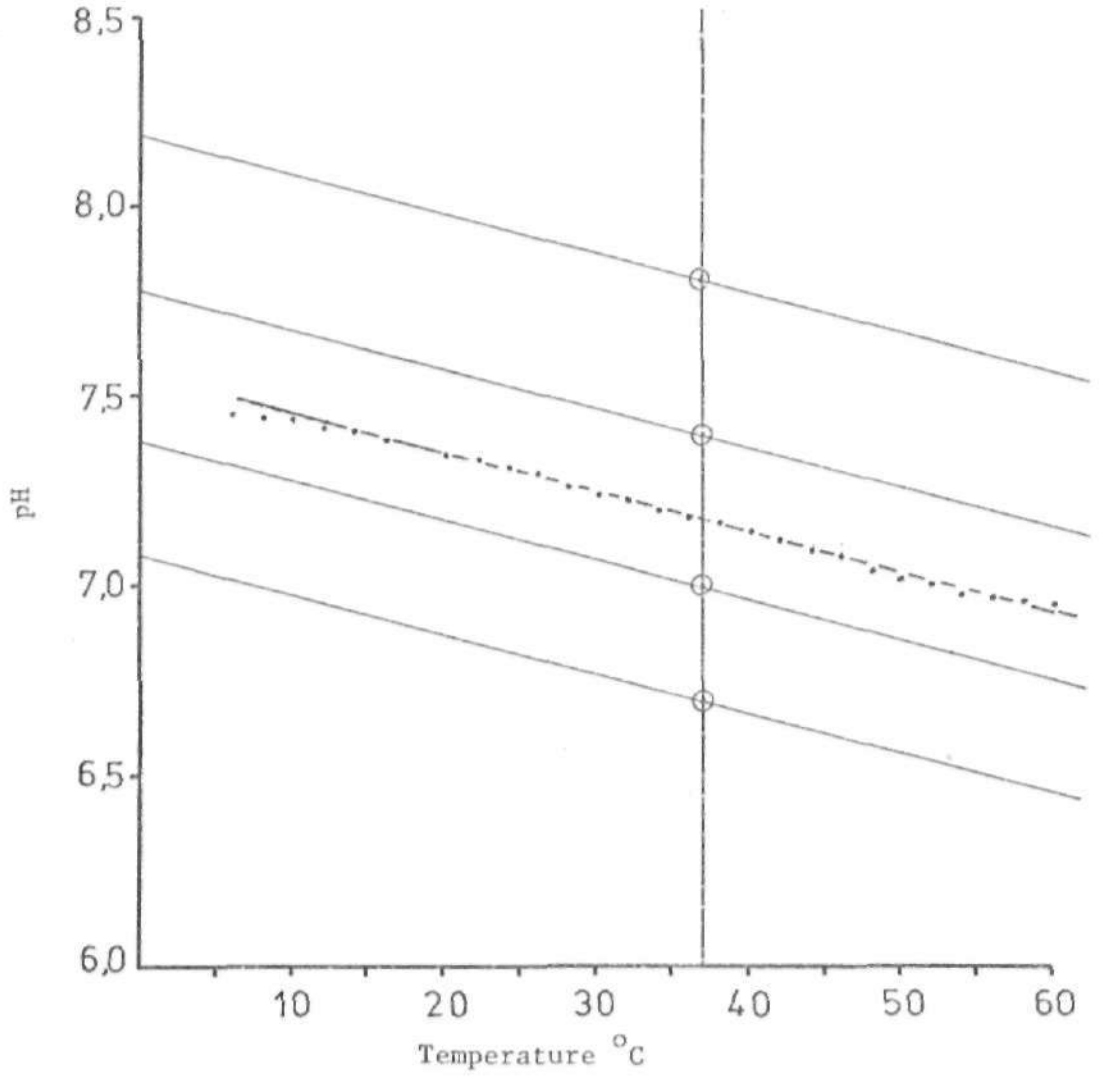


Figure 2.1. The effect of change of temperature on pH of glucose-free MOPS medium.

———— Experimental result

—⊙— pH Isotherms

K.R., 1976).

The structure, properties and preparation of MOPS were described by Good, N.E. and Izawa, S. (1972). An example of its use was demonstrated by Neidhart, F. (1974), who used the potassium salt of MOPS in conjunction with Tricine, ammonium chloride, and a mixture of metallic salts to prepare a medium for growing E. coli and S. typhimurium. He recommended this medium for universal acceptance for such biological work. Another example of its use was reported by Dougherty, et al.(1976), who used it in a chemically defined medium for susceptibility testing of antimicrobial agents.

The composition of the standard MOPS medium as used in the work described in this thesis was as follows:

MOPS	1.0×10^{-1}M
Ammonium chloride	3.7×10^{-2}M
Sodium sulphate (anhydrous)	7.0×10^{-4}M
Magnesium chloride (hexahydrate)	5.9×10^{-4}M
Potassium dihydrogen orthophosphate	8.8×10^{-4}M
Potassium chloride	2.0×10^{-3}M
Glucose	2mg/ml

The medium was normally prepared in batches of 4 to 5 litres by dissolving the ingredients in freshly distilled water. The magnesium chloride was dissolved in a separate portion of water before adding it to the other chemicals in solution, to prevent the precipitation of phosphates (Middleton, K.R., 1976). The pH was adjusted with concentrated solution of sodium hydroxide to give pH 7.4 at 37°C. The medium was distributed in volumes of 100mls or 200mls into 250ml

conical flasks, sterilised by autoclaving, and stored at room temperature until required. The glucose was prepared separately as 10 mls of 20% solution, and also sterilised by autoclaving, being normally added to the main medium immediately before use.

(c) Diluting and Plating Media

For viable cell count experiments, Tryptone-soya broth was used for serial dilutions, and Nutrient agar as the plating medium.

(d) Chemicals

Chloroquine diphosphate, unlabelled Thymidine, and Deoxyadenosine were obtained from the Sigma Chemical Company, St. Louis, Missouri, U.S.A.

MOPS was obtained from Hopkins and Williams, Biological Buffers, Romford, England.

Glucose and Trichloroacetic acid were obtained from British Drug Houses, Ltd., Poole, Dorset.

2-C^{14} Thymidine was obtained from the Radiochemical Centre Ltd., Amersham.

Chloroquine Ring 3-C^{14} was obtained from NEN Chemicals GmbH, Dreieich, West Germany.

Ampicillin sodium (Penbritin) was from Beecham Research Laboratories, Brentford, England.

All other chemicals used were of 'Analar' grade.

2. GENERAL METHODS

(a) Cultivation of Organism

Escherichia coli cells from overnight cultures were subcultured into fresh MOPS medium in 50ml or 250ml conical flasks, incubated at 37°C in a Gallenkamp shaking water bath, and aerated by shaking at 120 throws per minute until the cells were well established in the exponential growth phase. The cells were then prepared for experimental use by one of two methods:

(i) diluting a small volume of the culture with appropriate volume of fresh medium, previously adjusted to the required pH at 37°C, or

(ii) filtering the culture through a pre-wetted 0.45µm mean pore size membrane filter, supported on sintered glass pads, washing the retained cells with, and resuspending them in fresh medium as above.

For experiments involving non-growing cells, the second method was used, the suspending medium in these cases being glucose-free.

(b) Viable cell count

The number of viable cells in the control and chloroquine-treated cultures was estimated by the pour-plate method.

Initially, serial dilutions were made in quarter strength Ringer's solution, but as it gave unsatisfactory results, even with the control cultures, it was replaced with Tryptone-soya broth. Care was taken to ensure that dilution and plating were completed within 20 minutes of sampling. An automatic fin tip pipette was used in taking

sample cultures, and for transferring diluted cells into dilution tubes and petri dishes. A Fisons Whirlmixer (Loughborough, England) mixer was used. The diluted samples for counting were plated out in Nutrient Agar, colonies being counted after 36 hours of incubation at 37°C.

(c) Absorbance Measurements

Absorbance measurements were made at 600nm with a 1cm path length, using a SP500 series 2 ultraviolet and visible spectrophotometer (Pye Unicam Ltd., Cambridge, England).

(d) Chloroquine Solution

The diphosphate salt of chloroquine was dissolved in glucose-free MOPS medium to give required molar concentrations. The solution was sterilised by autoclaving, and stored in the refrigerator until required.

(e) pH Measurements

A Corning-EEL Model 109 pH meter with digital display was used for most pH measurements. A PYE Dynacap pH meter No. 11087 was used occasionally whenever the former instrument broke down.

CHAPTER THREE

THE EFFECT OF CHLOROQUINE ON GROWTH OF *ESCHERICHIA COLI* IN MOPS MEDIUM

CHAPTER THREE

THE EFFECT OF CHLOROQUINE ON THE GROWTH OF ESCHERICHIA COLI IN MOPS MEDIUM

	Page No.
Experimental	40
Growth Medium	40
Inhibition	40
Results	41
Figures	43
Discussion	45

Previous workers (Wiseman, 1972a, and Middleton and Wiseman, 1974) have reported on the growth inhibitory effects of chloroquine against E. coli. A phosphate buffered minimal salts medium was used in the first of these studies, and a Tris buffered one in the second. From the results obtained by the above workers, differences in the inhibitory effects were apparently observed between these two media. It could not be assumed, therefore, that concentrations of chloroquine having particular effects in either of those media would have the same effects in the MOPS buffered medium chosen for the work described in this thesis.

Thus, as a preliminary to studying bactericidal actions of chloroquine on E. coli, experiments were carried out to determine the concentrations of chloroquine required to cause inhibition of growth of this organism in MOPS medium at pH 7.4.

EXPERIMENTAL

Growth Medium.

The medium used was MOPS medium, adjusted to give pH 7.4 at 37°C.

Inhibition of Growth.

An overnight culture of E. coli grown from a low inoculum was subcultured into fresh medium and incubated with shaking until well established in the exponential phase of growth. The culture was then filtered through a 0.45μm mean pore size membrane filter, and the retained cells washed with and resuspended at 37°C in fresh medium containing twice the normal concentration of glucose. The absorbance of the resulting suspension was adjusted to 0.05 with more medium, and

incubation with shaking resumed. After one hour, when the absorbance had reached about 0.100, 10ml portions of this culture were added to 50ml conical flasks containing 10ml volumes of glucose-free medium, previously warmed to 37°C, containing twice the finally required concentrations of chloroquine. The flasks were then incubated with aeration by shaking, and growth followed by taking absorbance measurements at intervals for up to 3 hours.

RESULTS

The results shown in Figure 3.1 are typical of the changes in absorbance with time that followed the addition of chloroquine in the concentration range 2.0 to 13.0 x 10^{-4}M to exponentially growing cultures of E.coli at pH 7.4 at 37°C, together with an untreated control culture.

The addition of 2.0 x 10^{-4}M chloroquine had only a slight effect on the rate of increase in absorbance, which appeared to be exponential throughout the period of the experiment, with a growth rate constant equivalent to about 94% of that of the control culture.

The addition of 4.0, 8.0 and 10.0 x 10^{-4}M chloroquine to cultures caused rapid but small primary decreases in the rate of increase of absorbance followed after between 50 and 70 minutes by further decreases to give secondary inhibited exponential growth rates, which continued until the cell concentrations became limiting for balanced growth.

It was generally observed that at low concentrations (below 4.0 x 10^{-4}M) there was no obvious division into primary and secondary inhibited growth rates, but such divisions became apparent at higher

concentrations.

In the presence of 12.0×10^{-4}M chloroquine, the absorbance increased for about 60 minutes after addition of the drug, by which time it had increased by about 52%, and then remained fairly constant for the duration of the experiment.

In the presence of 13.0×10^{-4}M chloroquine, there was a small increase in absorbance during the first 30 minutes, followed by a slow decrease during the next few hours.

Figure 3.2 illustrates the relationship between the concentration of chloroquine and the degree of inhibition, calculated as the decrease in growth rate after 120 minutes, relative to the control. This figure shows that the degree of inhibition increased with increase in concentration of chloroquine, the rate of increase in degree of inhibition being more rapid than the rate of increase in concentration.

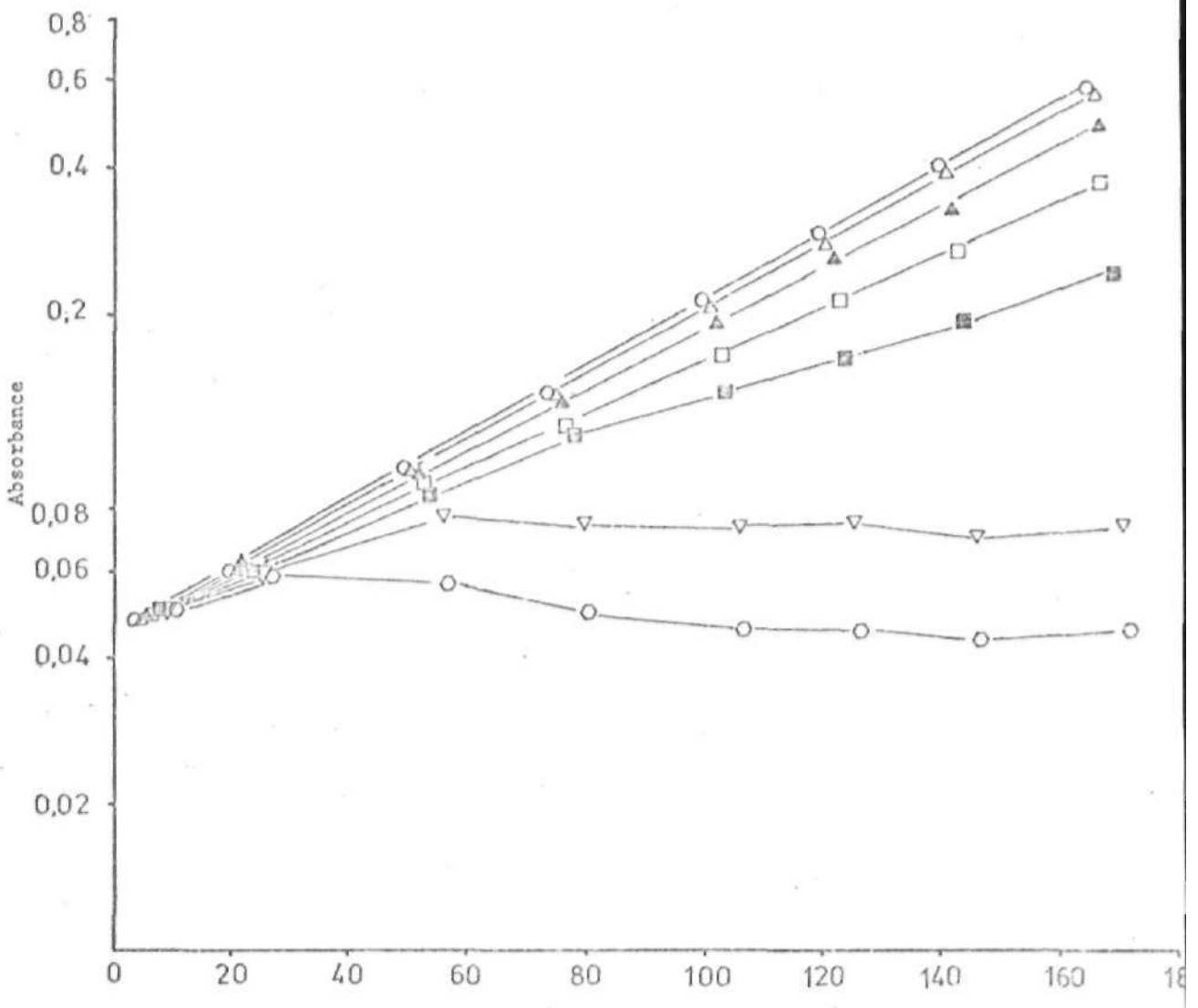


Figure 3.1. Effect of Chloroquine on changes with time of absorbance of E. coli cultures treated with 0.0, (O——), 2.0 (Δ——), 4,0 (Δ——), 8.0 (□——), 10.0 (▣ —), 12.0 (▽——) and 13.0 x 10^{-3} M (O ——).

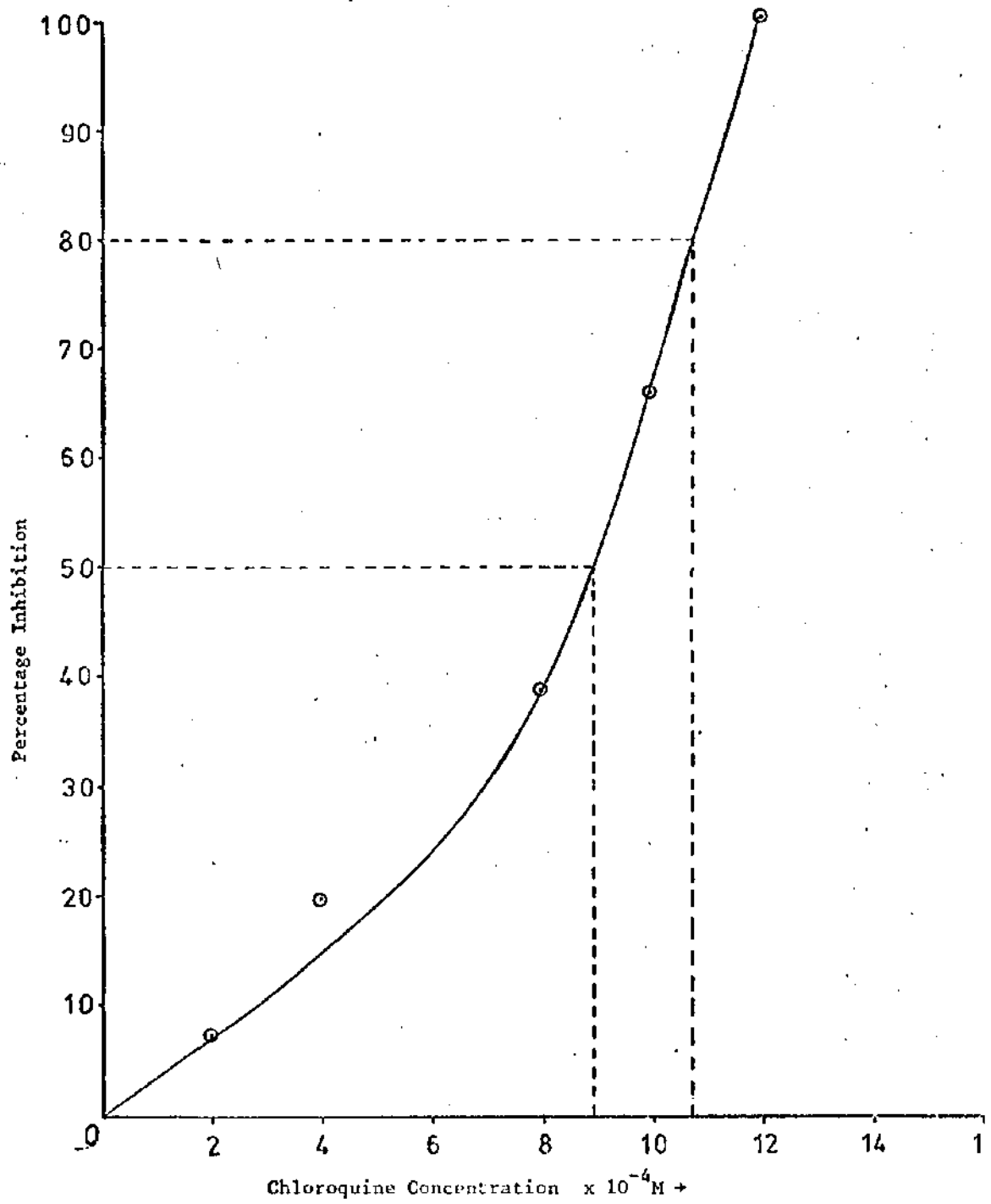


Figure 3.2. The relationship between chloroquine concentration and the degree of growth inhibition of E. coli cultures in MOPS medium at pH 7.4.

DISCUSSION

Growth of bacterial cultures can be followed by measuring changes with time in cell numbers or cell mass in the cultures, and many methods have been used for studying growth inhibition, including the use of total and/or viable cell counts, the determination of the dry weight or carbon content of cells in cultures, and the use of absorbance measurements.

Of these methods, the measurement of absorbance is the most convenient, and has been shown to be reliable for following changes in total cell mass in cultures of E. coli partially inhibited by treatment with chloroquine (Middleton, K.R., 1976). This worker showed that the rate of incorporation of ^{14}C labelled glucose into growing cells of E. coli was directly proportional to the rate of increase in absorbance in both control cultures, and in those treated with chloroquine. Absorbance measurements were used in the work described in this thesis to study growth inhibition.

The ability of chloroquine to cause the inhibition of growth of E. coli has been reported previously (Wiseman, D. 1972[a], and Middleton, K.R., 1976). In both studies the inhibition of growth was reported to follow a biphasic pattern, the addition of chloroquine causing an immediate slowing to a primary exponential inhibited growth rate, followed after about 60 minutes by a further slowing to a secondary exponential inhibited growth rate. The results reported here gave essentially the same biphasic pattern of growth inhibition for individual concentrations of chloroquine, but the concentrations of drug

required to give particular effects were higher in the MOPS buffered medium, as illustrated in Table 3.1.

Also, the shape of the dose response curve of concentration of chloroquine with percentage inhibition was distinctively different from that reported by Middleton (1976) at pH 7.4. This point will be discussed further in Chapter 6.

Medium	Chloroquine Conc. causing 50% growth inhibition	Chloroquine Conc. causing 80% growth inhibition
Phosphate buffered	2.5×10^{-4}M	3.0×10^{-4}M
Tris buffered	4.5×10^{-4}M	5.5×10^{-4}M
MOPS buffered	8.4×10^{-4}M	11.0×10^{-4}M

Table 3.1. Effect of Medium on effective growth inhibition concentrations of chloroquine on E. coli.

CHAPTER FOUR

THE EFFECT OF CHLOROQUINE ON THE VIABILITY OF GROWING CULTURES OF E. COLI AT pH 7.4

CHAPTER FOUR

THE EFFECT OF CHLOROQUINE ON THE VIABILITY OF GROWING CULTURES OF ESCHERICHIA COLI AT pH 7.4

	Page No.
Experimental	49
Viable cell counting	50
Results	50
Effect on viability	50
Relationship between concentration of chloroquine and its effect on cell viability	51
Effect of slow growth on bactericidal action	52
Figures	53
Discussion	64
Effect on viability	64
Effect of drug concentration on viability	65
Choice of a working chloroquine concentration	65

Ciak and Hahn reported in 1966 that chloroquine, in high concentrations, can be bactericidal for vegetative cells of B. megaterium whilst more recently, Middleton (1976) has demonstrated that concentrations high enough to cause complete inhibition of the growth of E. coli also cause some loss in viability. Despite this, there has been no reported comprehensive study of the bactericidal activity of chloroquine. This thesis as a whole describes an attempt at such a study, whilst this Chapter in particular reports studies on the relationship between the concentration of chloroquine and its bactericidal activity against E. coli at pH 7.4 and 37°C. A concentration range of 1.5 to 16.0 x 10^{-3}M was chosen for this study, 1.5 x 10^{-3}M being the lowest concentration that consistently caused complete inhibition of growth, and a ratio of 10 to 1 between the maximum and minimum concentrations studied being thought sufficient to demonstrate any effect of change in concentration on bactericidal activity.

EXPERIMENTAL

Overnight cultures of E. coli were inoculated into fresh medium and the cells allowed to grow with aeration by shaking at 37°C until well established in the exponential phase of growth. The cultures were then filtered through a 0.45μm mean pore size membrane filter and the retained cells washed with and resuspended in fresh medium containing twice the normal concentration of glucose and adjusted to give pH 7.4 at 37°C. The absorbance of the cultures was adjusted to either 0.100 or 0.200 at 600 nm by addition of more medium, and incubation with shaking was continued. After about an hour when the absorbance had reached either 0.200 or 0.400, 20 ml portions of the

cultures were added to 20 ml volumes of glucose free medium (pH 7.4), containing twice the finally required concentrations of chloroquine, in 250 ml conical flasks, and the suspensions incubated with shaking at 37°C. Absorbance measurements were made and samples taken for viable cell counting at intervals for up to 5 hours.

Viable Cell Counting.

Viable counts were determined by the pour plate method using tryptone soya broth as the diluent and nutrient agar as the plating medium; the chloroquine was inactivated by dilution, and colonies were counted after 36 hours incubation at 37°C (Middleton, 1976).

RESULTS

Effect on Viability.

Figure 4.1 shows a typical set of results for the changes with time of absorbance and viable cell count per ml of an E. coli culture following the addition of chloroquine at a concentration of 1.5×10^{-3}M.

The absorbance of the chloroquine treated culture increased slightly for the first 50 minutes, after which it gradually decreased, becoming lower than the original value after about 100 minutes. The viable cell count began to decrease soon after the addition of the chloroquine. A short initial lag phase was followed by a period of exponential loss in viability which lasted for at least two hours, after which there was some tailing off in the death curve.

A number of repeat experiments were carried out using a concentration of 1.5×10^{-3}M chloroquine. Average results are illustrated in Figure 4.2 which shows the changes in percentage viability with time for both untreated controls and chloroquine treated cultures. The shape of the curve is essentially the same as in Figure 4.1, and shows that the results are fairly reproducible.

Relationship between Concentration of Chloroquine and its Effect on Cell Viability.

Figures 4.3 to 4.6 illustrate the effect of variation in concentration of chloroquine on its bactericidal action against E. coli. A quick glance indicates that concentrations of 2.0, 4.0, 8.0, and 16.0×10^{-3}M chloroquine were all bactericidal, and produced similar patterns of death curves. A closer examination revealed however that although the curves all showed some evidence of an initial lag phase, this became less pronounced with increase in chloroquine concentration. In addition, the duration of the exponential death phase became longer with increase in drug concentration, although the rate of kill during this exponential phase was similar for all concentrations. These similarities and differences are better illustrated in Figures 4.7 and 4.8.

Generally the rate of kill is similar for all the concentrations for about the first 120 minutes, which roughly marks the beginning of the 'tailing off' of the curve for the 1.5×10^{-3}M chloroquine concentration. By this time, all the drug concentrations considered had killed at least 99% of the cell population.

Effect of Slow Growth on Bactericidal Action.

An important observation made in some experiments was the inhibiting effect of slow growth on the bactericidal action of chloroquine (Figure 4.9). It was found that both the rate and the extent of kill were greatly reduced in chloroquine treated cells when their parallel untreated controls grew more slowly than normal. Control cultures usually grew with a mean generation time of about 60 minutes, but occasionally for unknown reasons cultures grew more slowly with an MGT of about 80 min. Figure 4.9 and Table 4.1 show that 1.5×10^{-3}M chloroquine effected a kill of 99.97% by the end of 4 hours with normally growing cells, whereas the same drug concentration effected a kill of only 80% after the same period with slow growing cells. Similarly, with 4.0×10^{-3}M chloroquine 99.997% and 96.60% of cell populations were killed after 4 hours with normal and slow growing cells respectively.

PERCENTAGE CELL VIABILITY PER ML

Time in Minutes	Cells treated with 1.5×10^{-3}M chloroquine		Cells treated with 4.0×10^{-3}M chloroquine	
	Normal (MGT~60)	Slow (MGT>80)	Normal (MGT~60)	Slow (MGT>80)
0	100.00	100.00	100.00	100.00
60	15.00	60.00	5.00	70.00
120	8.00	36.00	0.34	27.00
180	0.12	26.50	0.035	9.70
240	0.027	20.00	0.0028	3.40

Table 4.1.

Effect of slow growth of E. coli cultures on bactericidal action of chloroquine.

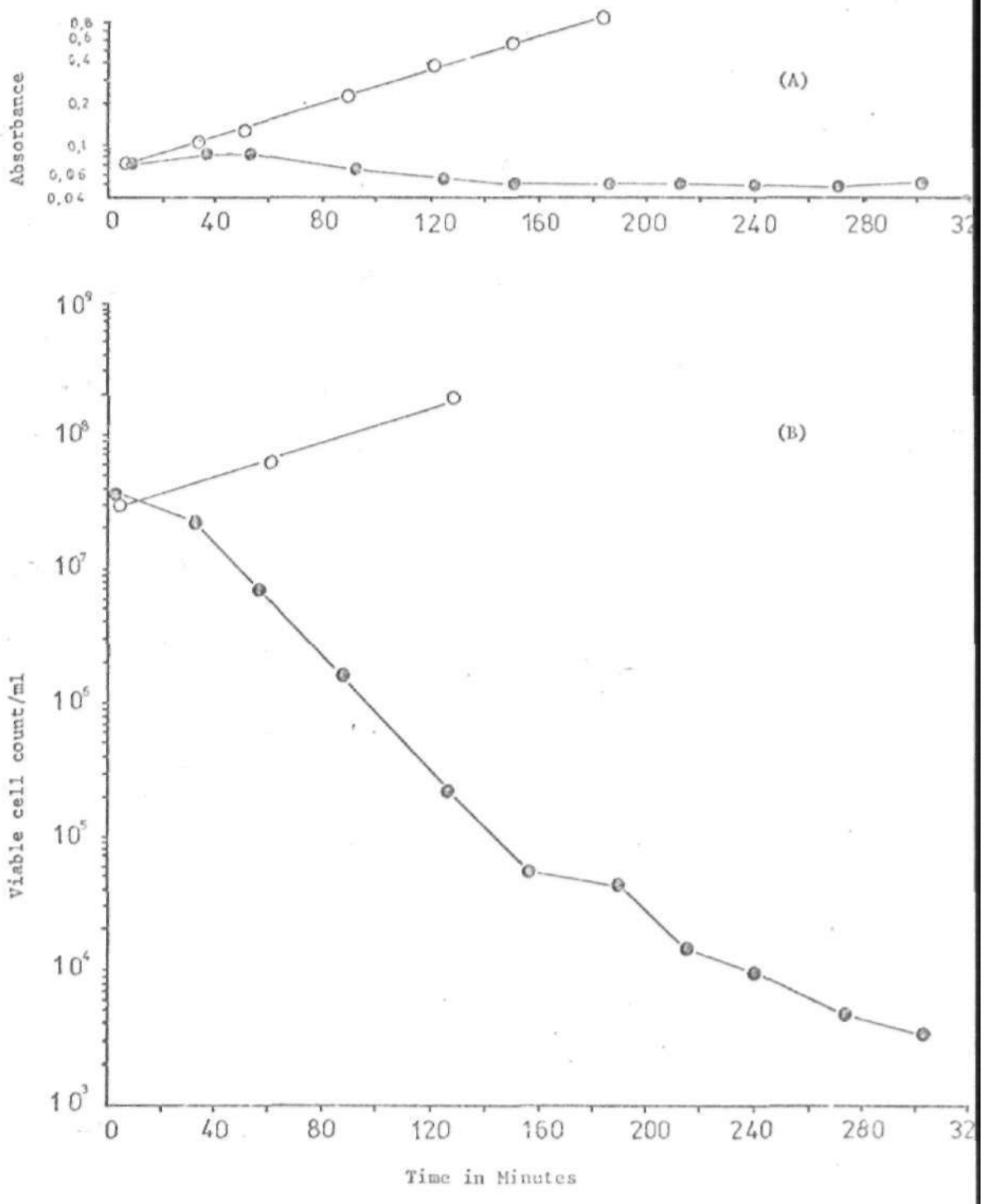


Figure 4.1. The effect of chloroquine on the changes with time of the absorbance (A) and viable cell counts/ml (B) of E. coli cultures treated with 0.0 (O——), and 1.5×10^{-3}M (O——) chloroquine.

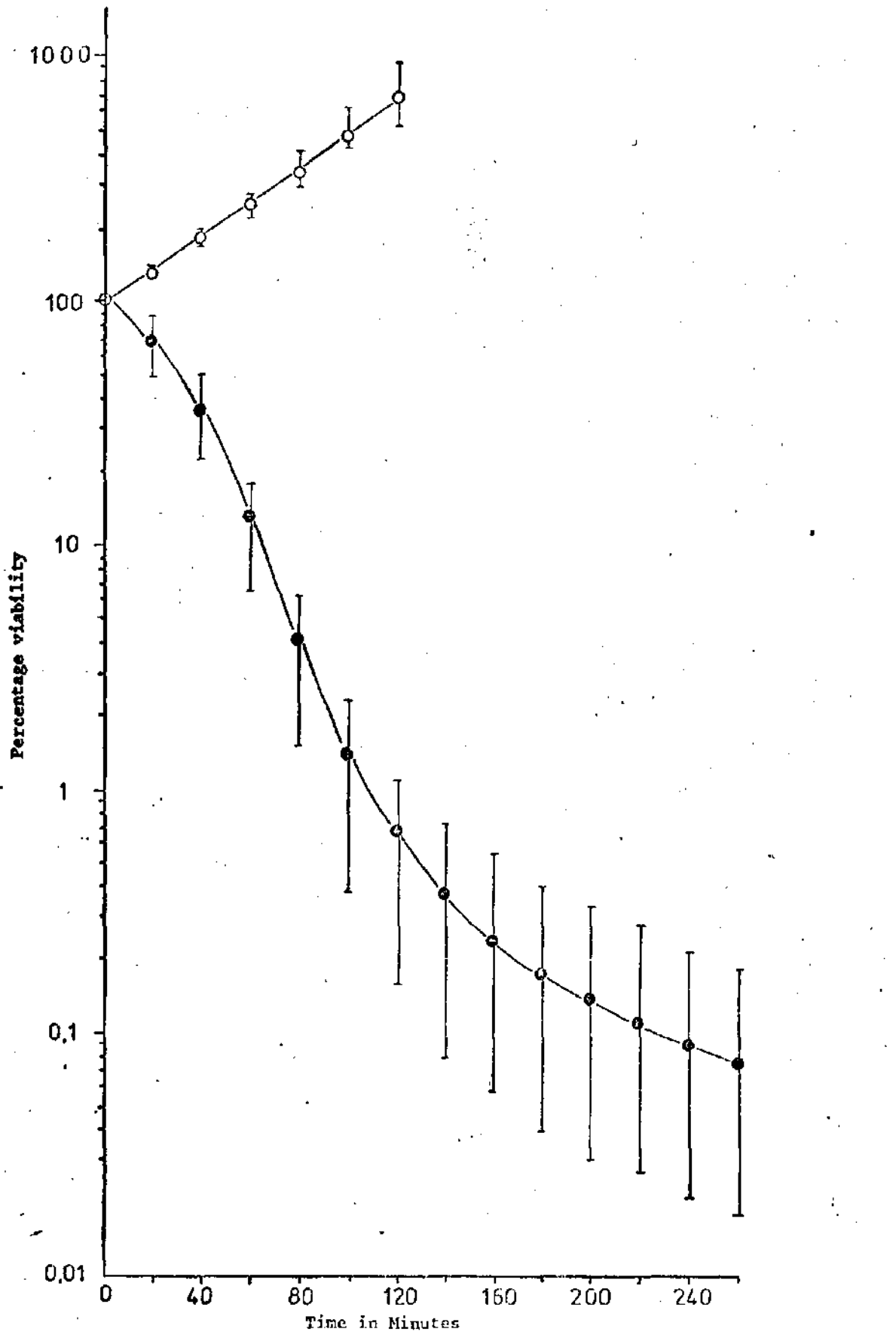


Figure 4.2. The effect of chloroquine on the changes with time of percentage viability of E.coli cultures treated with 0.0 (O——), and 1.5×10^{-3}M (O ——) chloroquine.

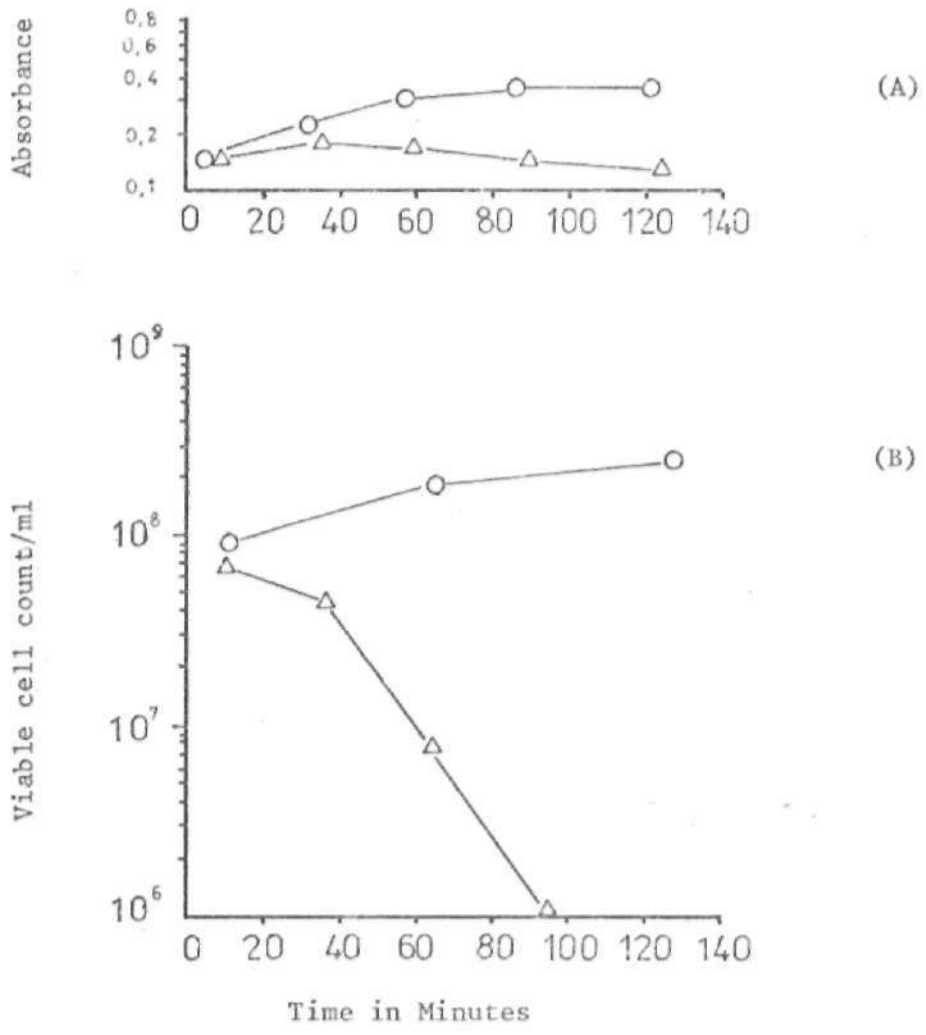


Figure 4.3. The effect of chloroquine on the changes with time of the absorbance (A) and viable cell counts/ml (B) of E. coli cultures treated with 0.0 (O——), and 2.0 x 10^{-3}M (Δ——) chloroquine.

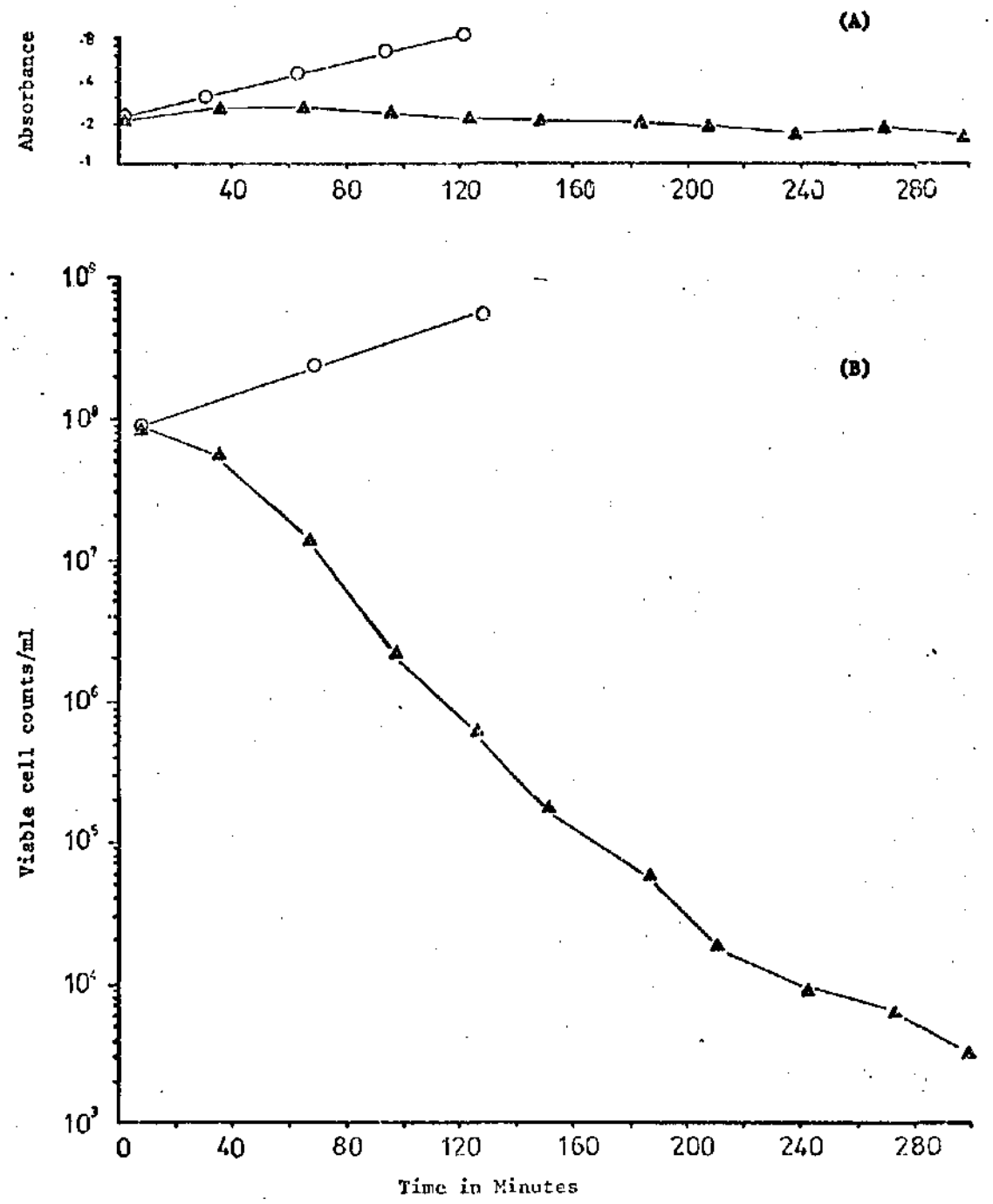


Figure 4.4. The effects of chloroquine on the charges with time of the absorbance (A) and viable cell counts/ml (B) of E. coli cultures treated with 0.0 (O—), and 4.0 x 10^{-3}M (▲—) chloroquine.

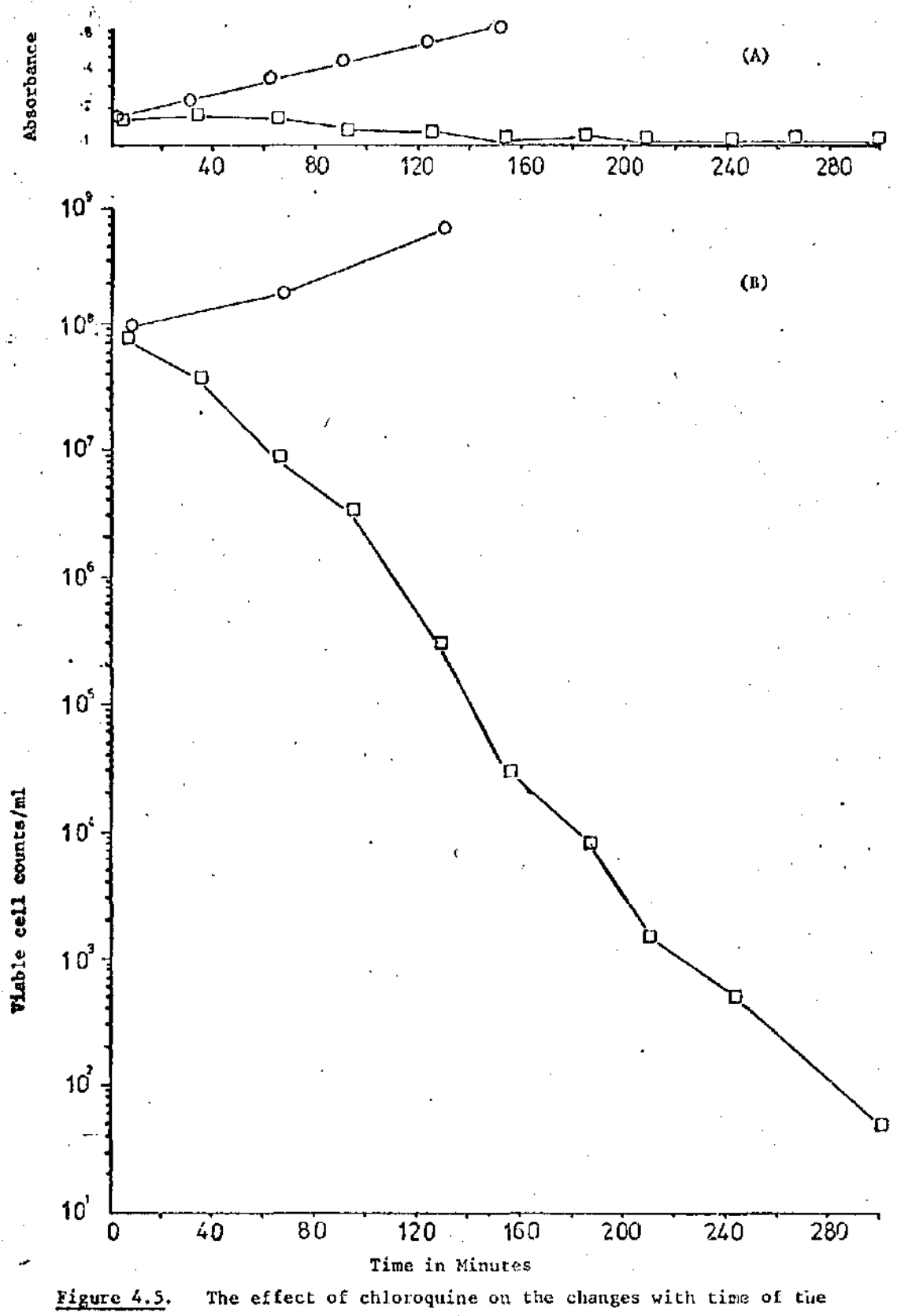


Figure 4.5. The effect of chloroquine on the changes with time of the absorbance (A) and viable cell counts/ml of E. coli cultures treated with 0.0 (O—), and 8.0 x 10^{-3}M (□——) chloroquine.

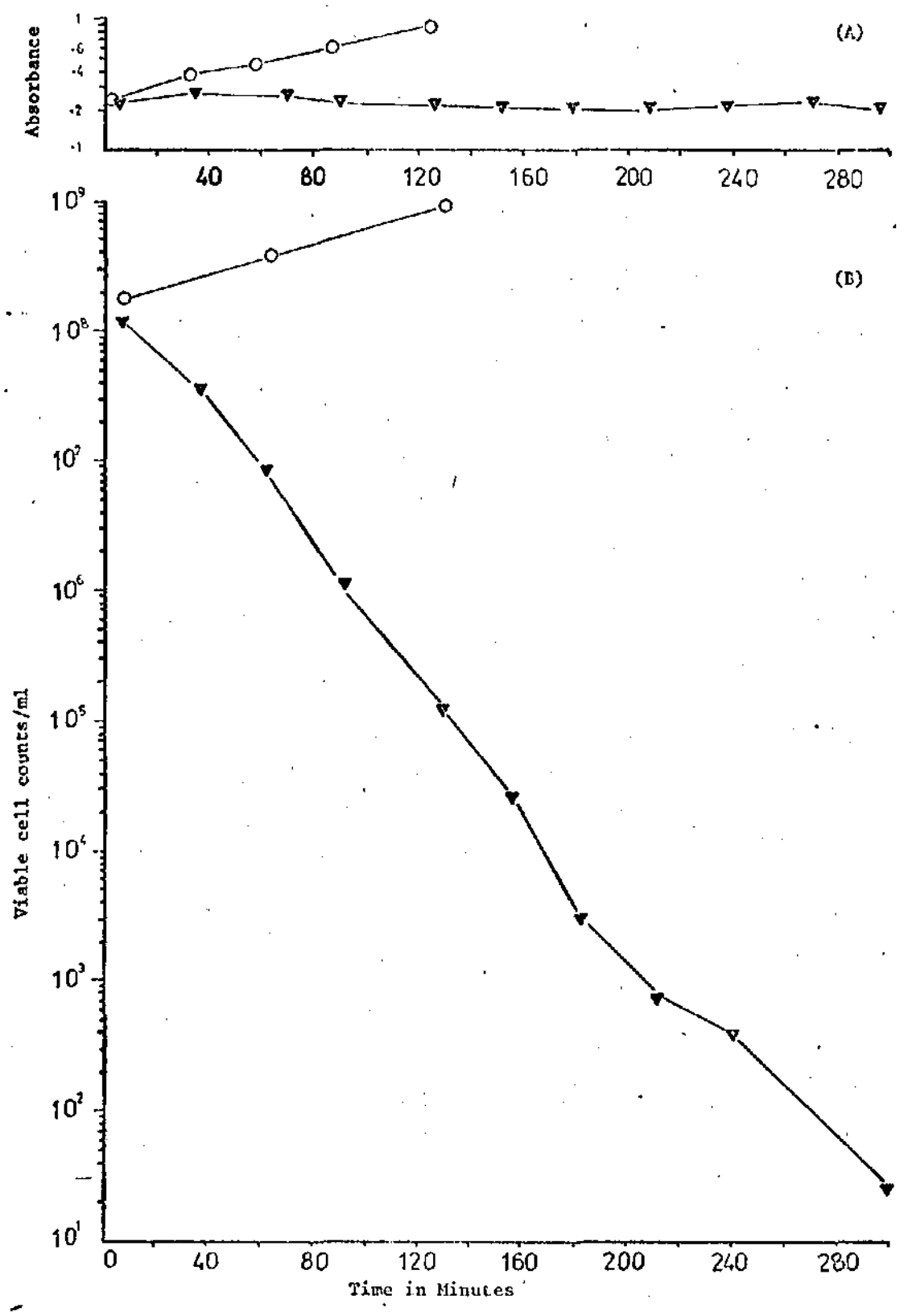


Figure 4.6. The effect of chloroquine on changes with time of the absorbance (A), and viable cell counts/ml (B) of E. coli cultures treated with 0.0 (O——), and 16.0 x 10^{-3}M (▼——) chloroquine.

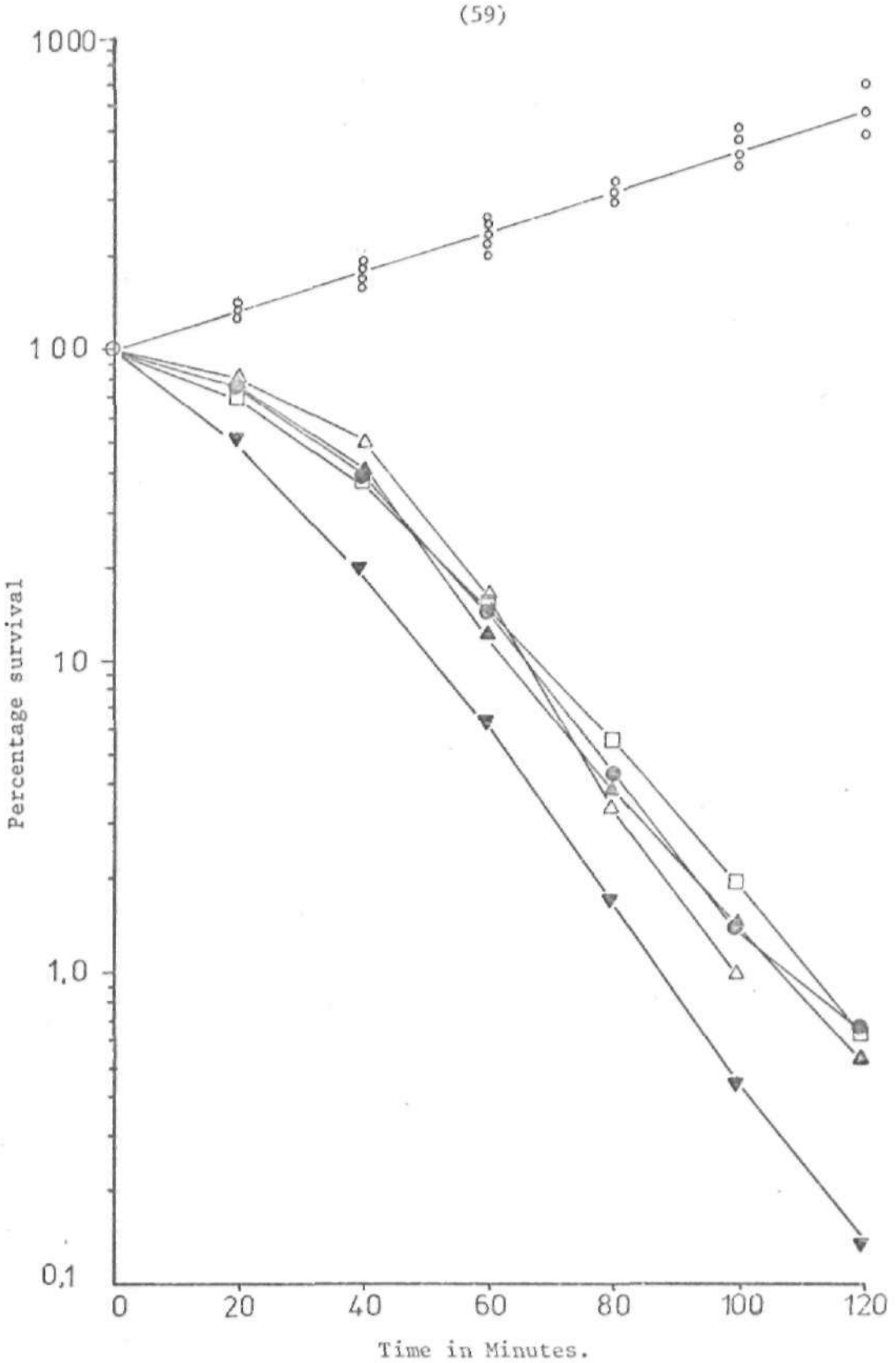


Figure 4.7. The effect of changes in chloroquine concentrations on the rate of kill of E. coli cultures. 0.0 (o——), 1.5 (●—), 2.0 (△——), 4.0 (▲——), 8.0 (□——), and 16.0 x 10^{-3}M (▼——) chloroquine.

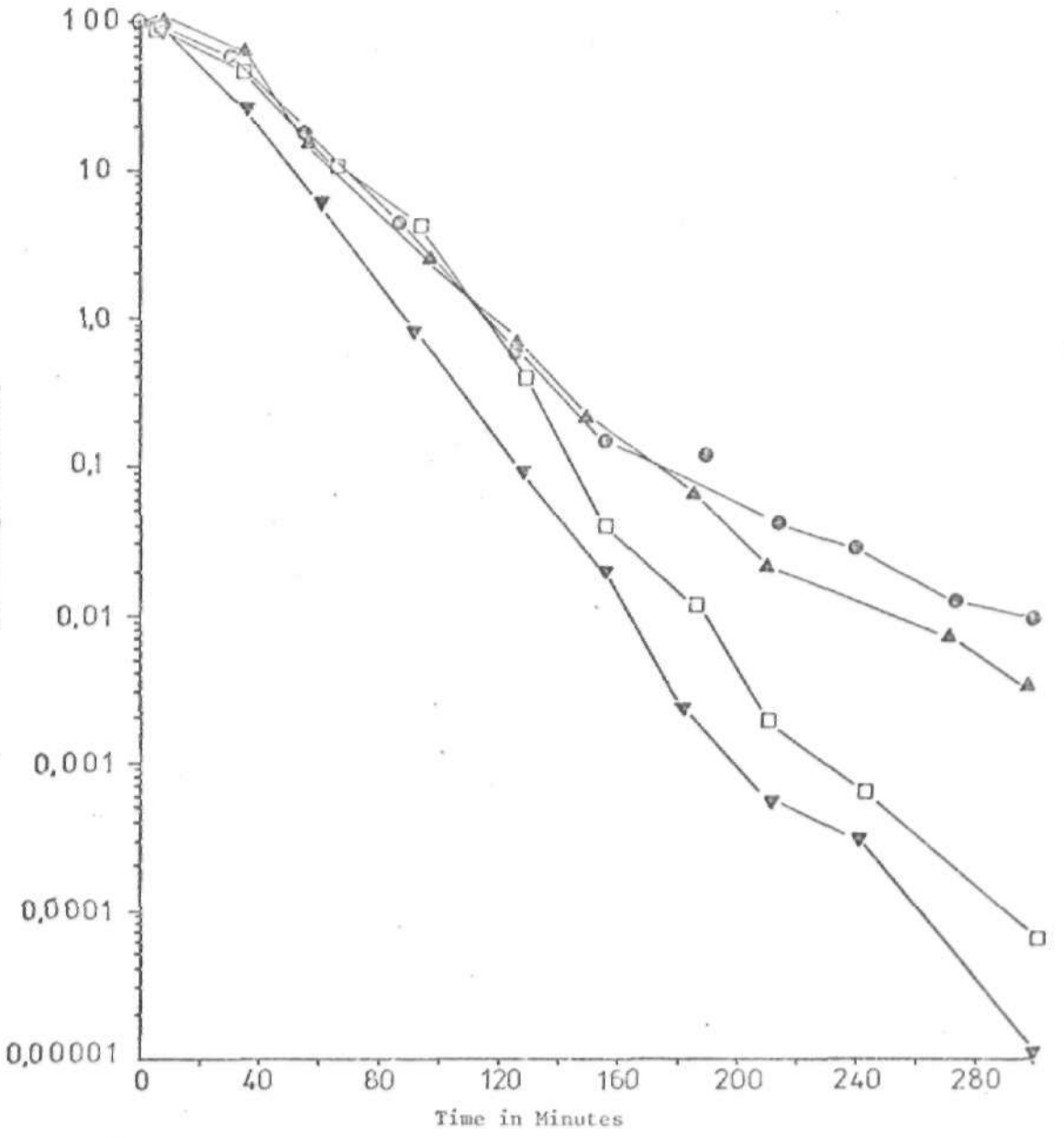


Figure 4.8. The effect of changes in chloroquine concentrations on the extent of kill of E. coli cultures, as shown by changes with time of percentage viability of E. coli cultures treated with 1.5 (●—), 4.0 (▲——), 8.0 (□——), and 16.0 x 10^{-3}M (▼——) chloroquine.

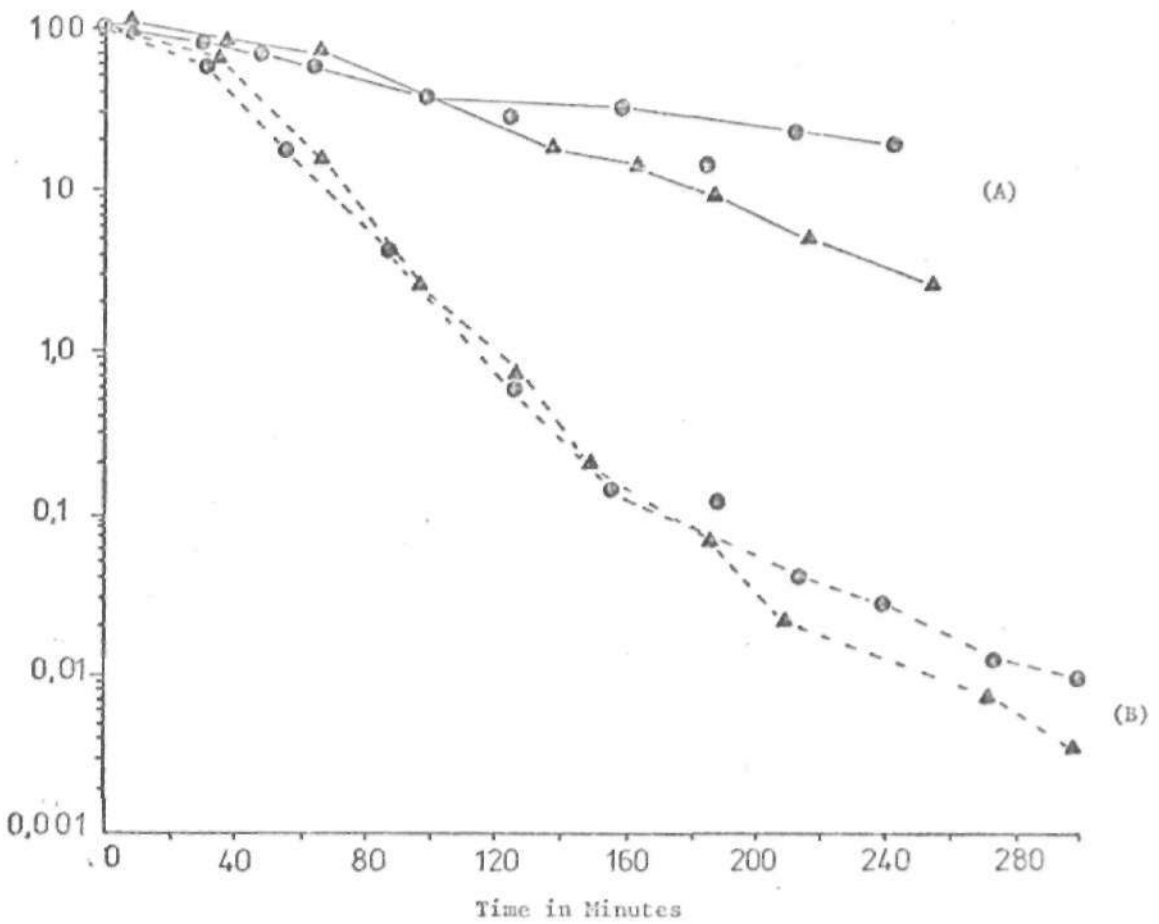


Figure 4.9. The effect of slow growth on the bactericidal action of chloroquine, as shown by the changes with time of percentage viability of slow (A) and normally (B) growing cultures of E. coli treated with 1.5 (●---), and 4.0 x 10^{-3}M (▲---) chloroquine.

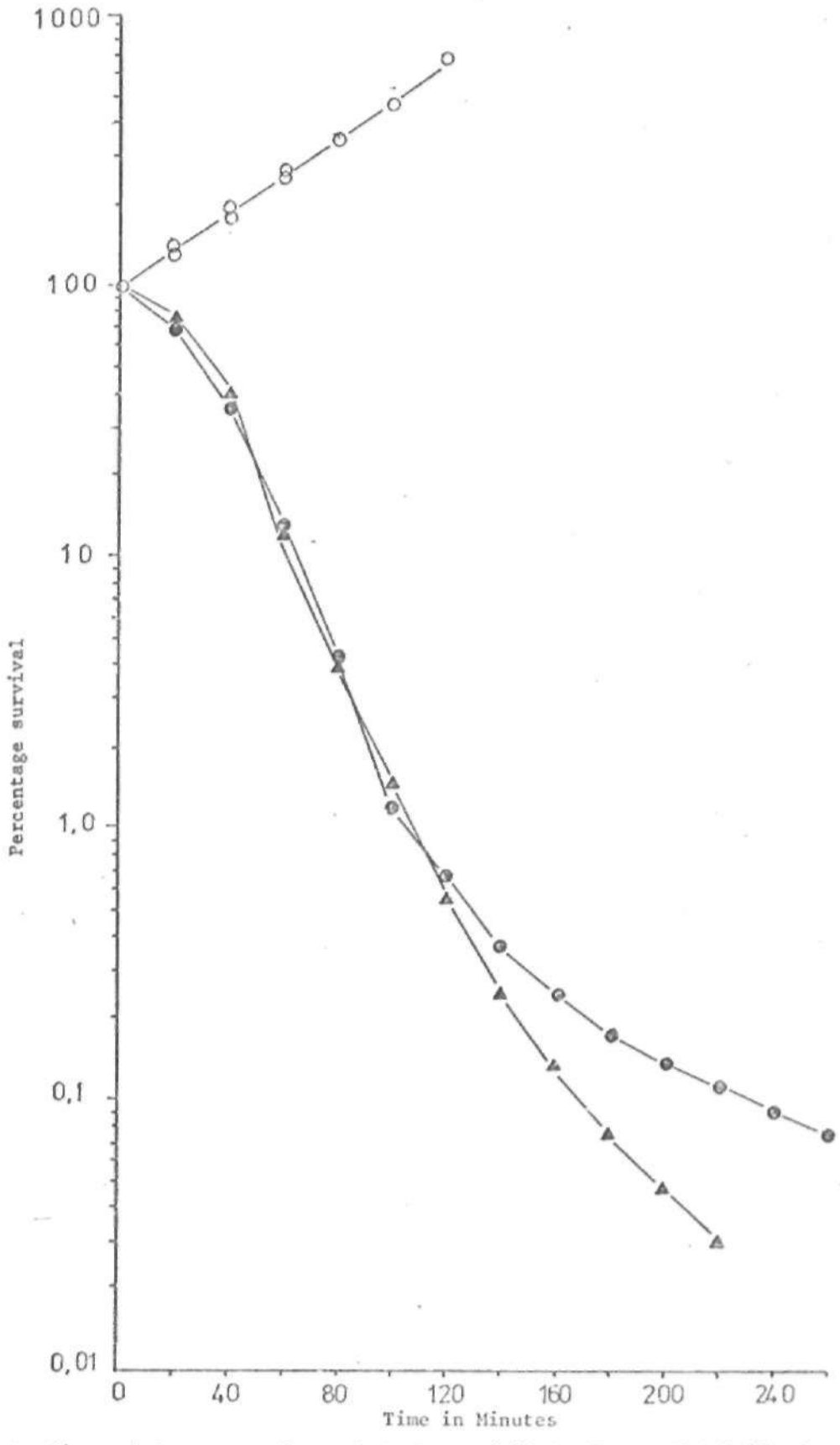


Figure 4.10. Comparison of the bactericidal effects of 1.5 (●—), and 4.0×10^{-3}M (▲——) chloroquine on E. coli cultures.

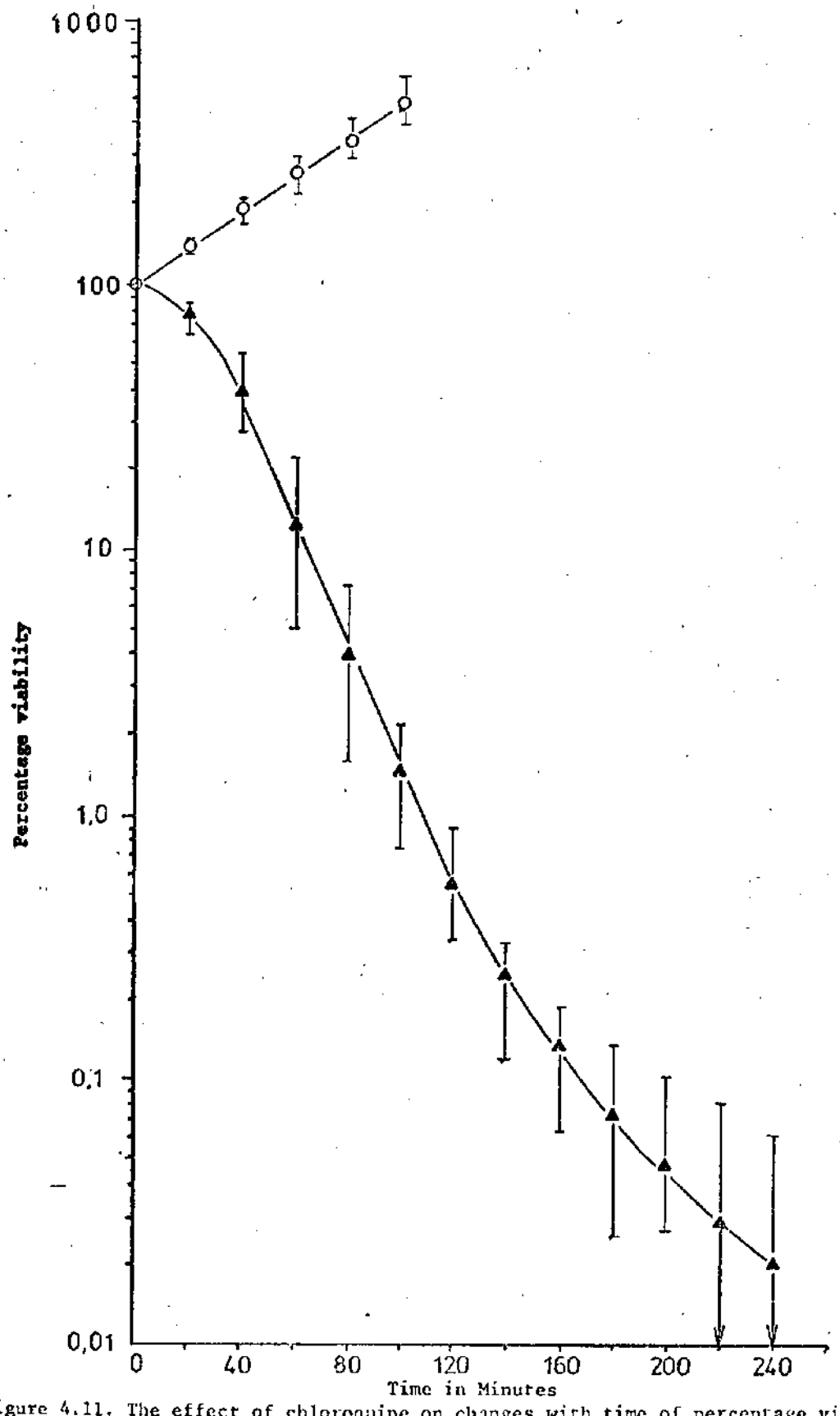


Figure 4.11. The effect of chloroquine on changes with time of percentage viability of E.coli cultures treated with 0.0 (O——), and 4.0×10^{-3}M (▲——) chloroquine.

DISCUSSION

Effect on Viability

The viable cell count and percentage viability curves illustrated in Figures 4.1 and 4.2 establish that chloroquine is bactericidal for E. coli at pH 7.4 in MOPS medium, and is another confirmation of the antibacterial action of the drug, contrary to the reports of Kradolfer and Neipp (1958), and Houba and Adam (1964). Ciak and Hahn (1966) first reported the bactericidal action of chloroquine against a strain of Bacillus megaterium, and from this effect proposed a mode of antimicrobial action of chloroquine. McDonald (1967) also reported inhibition of spore outgrowth in Bacillus subtilis by chloroquine, and pointed out the greater sensitivity of the outgrowth stage of spores in comparison with the vegetative stage of the bacteria. Middleton (1976) using the drug on E. coli (NCTC 1093) growing in a Tris-buffered medium, although mainly studying growth inhibition, reported that it was bactericidal in concentrations above 1.0×10^{-3}M. The results reported in this Chapter indicate that the same is true with E. coli cultures growing in a MOPS buffered medium.

The initial increase in absorbance of cultures treated with bactericidal concentrations of chloroquine might lead one to think that there is a delay in the drug getting to its site of action. This could not be the case, however, since the corresponding viable cell count showed a decrease in viable cell populations, whilst the absorbance was still increasing. These results are similar to those obtained by Hahn et al., (1966), who reported the dying of cells of B. megaterium within the first 20 minutes of contact with chloroquine, even

though there was increase in turbidity of the cell suspension. A possible explanation for the increase in absorbance may be continuation of protein synthesis for some time after the ability to divide has been lost.

Effect of Change in Drug Concentration on Viability.

The results illustrated in Figures 4.3 to 4.6 in which the bactericidal effects of 2.0, 4.0, 8.0 and 16.0 x 10^{-3}M chloroquine solutions are shown indicate little change in the bactericidal effects of the drug over this concentration range, the general pattern of the curve being maintained. It is a rather surprising occurrence, that such a wide range of drug concentrations produce such similar effects on cell viability. It is possible that only a small proportion of the drug in solution is taken up, and that this depends not only on the concentration in the growth medium, but also on a number of saturatable drug receptor sites in the cells, so that once the minimum concentration of drug in the medium which permits sufficient intake of drug to saturate the cell receptors has been reached, further increase in the drug concentration in the growth medium would not produce any appreciable difference in drug effects. A thorough study of the drug uptake by E. coli cells should prove valuable.

Choice of a Working Chloroquine Concentration.

Comparison of effects of the different drug concentrations used above suggests that 4.0 x 10^{-3}M should be a reliable standard bactericidal concentration of chloroquine at pH 7.4 for experimental purposes. Figure 4.10 compares the average results for a number of viable cell count experiments using normally growing cells with the 1.5 and

4.0×10^{-3}M concentrations, and shows how favourably they compare with each other. The 4.0×10^{-3}M has the advantage of better reproducibility over a wider range of experimental time, as illustrated in Figure 4.11 (cf. Figure 4.2), which illustrates the average of four separate experiments with this concentration. The fluctuations often associated with threshold bactericidal concentrations are thus removed. 4.0×10^{-3}M chloroquine was chosen as the working concentration for most of the studies described in the rest of this thesis.

CHAPTER FIVE

EFFECT OF GLUCOSE ON THE BACTERICIDAL ACTION OF CHLOROQUINE

CHAPTER FIVE

EFFECT OF GLUCOSE ON THE BACTERICIDAL ACTION OF CHLOROQUINE

	Page No.
Experimental	70
Effect of chloroquine on the viability of Escherichia coli in glucose-free medium before and after addition of glucose	70
Effect of the sequential addition and removal of glucose on the viability of cells of Escherichia coli initially treated with chloroquine in glucose free medium	70
Comparison of effect of glucose starvation on the bactericidal action of different concentrations of chloroquine	71
Effect of duration of glucose contact with growing Escherichia coli cells on the bactericidal action of chloroquine	71
Effect of filtration on cells treated with chloroquine in glucose free medium	72
Results	73
Effect of chloroquine on the viability of Escherichia coli in glucose free medium before and after the addition of glucose	73
Effect of the sequential addition and removal of glucose on the viability of cells of Escherichia coli initially treated with chloroquine in glucose free medium	73

CHAPTER FIVE (CONTINUED)

Page No.

Comparison of effect of glucose starvation on the bactericidal action of different concentrations of chloroquine 75

Effect of duration of glucose contact with growing *Escherichia coli* cells on the bactericidal action of chloroquine 77

Effect of filtration on cells treated with chloroquine in glucose free medium 78

Figures 79

Discussion 94

EXPERIMENTAL

Effect of Chloroquine on the Viability of E. coli in Glucose-free Medium Before and After the Addition of Glucose.

Cells of E. coli from cultures in the exponential phase of growth were prepared for experimental use as described in Chapter Two, using the membrane filtration method. The cells were filtered off, washed with and resuspended in glucose-free medium, and the absorbance adjusted to about 0.4 at 600nm. The resulting cell suspension was incubated at 37°C with aeration by shaking for 60 minutes to ensure that any residual glucose had been used up. 20ml volumes of this suspension were then measured into each of two 250ml flasks, one containing 20ml of glucose free medium alone, and the other 20ml of a solution of chloroquine, at twice the finally required concentration in glucose-free medium; incubation with shaking was then continued. Measurements of absorbance were made at regular intervals, and samples were taken for viable cell counting. After about two hours sufficient glucose was added to each of the two flasks to give a final concentration of 2mg/ml, and absorbance measurements and sampling for viable cell counting continued for a further two hours.

Effect of the Sequential Addition and Removal of Glucose on the Viability of Cells of E. coli Initially Treated with Chloroquine in Glucose free Medium.

The procedure was as described above, except that at a predetermined time of between 10 minutes and about 2 hours after the addition of glucose, the suspension was filtered through a membrane filter and the cells washed with and resuspended in glucose free medium containing

the same concentration of chloroquine as originally used to treat the cells. Changes in cell mass and population were again followed by making regular absorbance measurements and viable cell counts for up to a further 2 hours.

In some experiments duplicate chloroquine treated suspensions were used, one of which was maintained glucose free throughout.

Comparison of Effect of Glucose Starvation on the Bactericidal Action of Different Concentrations of Chloroquine.

The method used was the same as that described for the viability experiments in Chapter Four. However, a third flask, containing a chloroquine treated cell suspension in glucose free medium, was included. This was maintained glucose free throughout, while the other chloroquine treated suspension and the control contained the usual glucose concentration throughout, for about 4 hours.

Effect of Duration of Glucose Contact with Growing E. coli Cells on the Bactericidal Action of Chloroquine.

In these experiments chloroquine was added to growing cultures containing standard glucose concentrations. At a predetermined time, ranging from 10 to 60 minutes after the addition of chloroquine, the glucose was removed from the treated culture by filtration. The cells were washed with and resuspended in chloroquine containing glucose free medium, and measurements of absorbance and viability were continued for a further 2 hours.

Effect of Filtration on Cells Treated with Chloroquine in Glucose Free Medium.

This experiment was carried out to ensure that the filtration process had no influence on the results obtained in the experiments described above. Three cell suspensions, a control and two identical chloroquine treated ones, were prepared from cell cultures which had been filtered and resuspended in glucose free medium, as described in the first experiment. These were then incubated at 37°C with shaking. After the first hour, one of the chloroquine treated cultures was filtered, and the retained cells washed with and resuspended in glucose free medium containing the same concentration of chloroquine. Incubation with shaking was resumed and continued for a further 3 hours. The other two suspensions remained glucose-free and unfiltered for the whole period of 4 hours. Measurements of absorbance were made, and samples taken at intervals for viable cell counting.

RESULTS

Effect of Chloroquine on the Viability of E. coli in Glucose Free Medium before and after the Addition of Glucose.

Typical absorbance measurements and viable cell counts, obtained when E. coli cells were treated with 4.0×10^{-3}M chloroquine in glucose free medium for about 2 hours, are illustrated in Figure 5.1. There was little loss in viability in both the treated culture and in the untreated control during this glucose free period. Longer periods of such glucose starvation of up to 3 hours gave similar results.

After the addition of glucose to these suspensions, the untreated control cells started growing, whilst the chloroquine treated cells began dying, although at a slower rate than if there had been no initial period of glucose starvation (cf. Chapter 4).

Effect of the Sequential Addition and Removal of Glucose on the Viability of E. coli Treated with Chloroquine in Glucose Free Medium.

Figure 5.2 illustrates the effect of the addition of glucose to chloroquine treated cells followed by its removal after about 2 hours. As above, there was little loss in viability of the chloroquine treated cells prior to the addition of glucose, whilst after the addition of glucose the cells began to die. Surprisingly, after the subsequent removal of glucose the chloroquine treated cells continued to die, in fact at an increased rate.

Figures 5.3 and 5.4 illustrate the effect of variation in the duration of exposure to glucose of chloroquine treated cells. The

time of exposure to glucose in the first was 137 minutes, and in the second, 12 minutes. In both cases the viable count results are similar to those of Figure 5.2, the cells continuing to die after the removal of the glucose. However, the loss in viability was more pronounced with the longer period of exposure to glucose. This is better illustrated in Figure 5.5 and Table 5.1. The latter's point of comparison starts from the time of removal of glucose. From it, it can be observed that at 40 and 80 minutes respectively after filtration, the percentage survivals were 9.76 and 1.08 respectively with the 137 minutes exposure, whereas with the 12 minutes exposure, they were 54.19 and 21.93 respectively.

CULTURE A : 137 minutes		CULTURE B : 12 minutes	
Time after removal of glucose	Percentage Survival	Time after removal of glucose	Percentage survival
0 min	100.00	0 min	100.00
20 "	30.48	20 "	73.55
40 "	9.76	40 "	54.19
60 "	3.19	60 "	38.06
80 "	1.08	80 "	21.93

Table 5.1.

Effect of duration of exposure to glucose, after initial glucose starvation of chloroquine treated *E. coli* cells, on the bactericidal action of chloroquine.

A rather surprising result was obtained when one of the treated cultures was maintained glucose-free throughout, and the other was

supplied with glucose after 2 hours glucose starvation (Figure 5.6). The 'glucose-fed' cells continued to die after removal of glucose, but surprisingly the 'glucose-starved' cells retained up to 70% viability even after 6 hours of starvation.

Comparison of Effect of Glucose Starvation on the Bactericidal Action of Different Concentrations of Chloroquine.

The viable cell count results (Figures 5.7 to 5.10) further illustrate the effect of glucose on the bactericidal action of the drug on E. coli. Although the total cells killed in the glucose-starved cultures were in some instances appreciable, the differences in kill between them and their glucose-fed counterparts were remarkable.

The 2.0, 4.0 and 8.0 x 10^{-3}M concentrations gave fairly similar effects, but the 16.0 x 10^{-3}M concentration showed quite a difference. Table 5.2 gives a comparison. For example, by the end of 2 hours the glucose-starved cultures with the 2.0, 4.0 and 8.0 x 10^{-3}M concentrations had percentage survivals of 70.58, 82.32 and 78.86 respectively, whilst the corresponding culture with the 16.0 x 10^{-3}M concentration had only 30·55%, and by the end of 4 hours, this culture was less than one-eighth viable compared with similar glucose-starved cultures treated with the lower chloroquine concentrations.

A : Treated with 2.0×10^{-3}M CQ			B : Treated with 4.0×10^{-3}M CQ		
Time in Mins	A1 Glucose containing culture % survival	A2 Glucose free culture % survival	Time in Mins	B1 Glucose containing culture % survival	B2 Glucose free culture % survival
0	100.00	100.00	0	100.00	100.00
60	58.58	97.06	60	12.78	99.53
120	8.03	70.58	120	0.47	82.32
180	1.97	65.29	180	0.14	63.72
240	0.59	67.68	240	0.06	49.30
C : Treated with 8.0×10^{-3}M CQ			D : Treated with 16.0×10^{-3}M CQ		
Time in Mins	C1 Glucose containing culture % survival	C2 Glucose free culture % survival	Time in Mins	D1 Glucose containing culture % survival	D2 Glucose free culture % survival
0	100.00	100.00	0	100.00	100.00
60	33.33	100.00	60	16.78	70.00
120	6.11	78.86	120	2.86	30.55
180	1.61	57.14	180	0.41	12.50
240	0.34	42.28	240	0.13	5.00

Table 5.2. Comparison of effect of glucose starvation of E. coli cells on the bactericidal action of different concentrations of chloroquine.

Effect of Duration of Glucose Contact with Growing E. coli Cells on the Bactericidal Action of Chloroquine.

The results obtained after the removal of glucose have some similarity to those described above in Figures 5.3 and 5.4, as illustrated in Figures 5.11 and 5.12. The extent of kill, especially in cases where the initial exposure to chloroquine in the presence of glucose was about 60 minutes before removal of the glucose, was comparable with that normally obtained when glucose was present throughout the experiment (cf. Chapter 4). There was little difference between the 10 and 20 minutes exposures to glucose. Figure 5.14 and Table 5.3 expose the differences in the extent of kill between the 10 and 58 minutes in the presence of glucose. The Table shows that 77.44 and 98.46% of the cell population had been killed by the end of 60 and 120 minutes respectively from the culture exposed to glucose for 58 minutes, whereas only 22.22 and 43.33% were killed from the culture exposed to glucose for 10 minutes.

CULTURE A : 58 minutes in presence of glucose		CULTURE B : 10 minutes in presence of glucose	
Time in minutes after removal of glucose	Percentage kill	Time in minutes after removal of glucose	Percentage kill
0	0.00	0	0.00
30	50.00	30	11.11
60	77.44	60	22.22
90	96.61	90	42.22
120	98.46	120	43.33

Table 5.3. Effect of Duration of Glucose Contact with Growing E. coli Cells on the Bactericidal Action of Chloroquine.

Effect of Filtration on Cells treated with Chloroquine in Glucose-free Medium.

It is evident from Figure 5.15 that the filtration process itself had no adverse effect on cell viability, as there was no difference attributable to the filtration process between the two chloroquine treated suspensions.

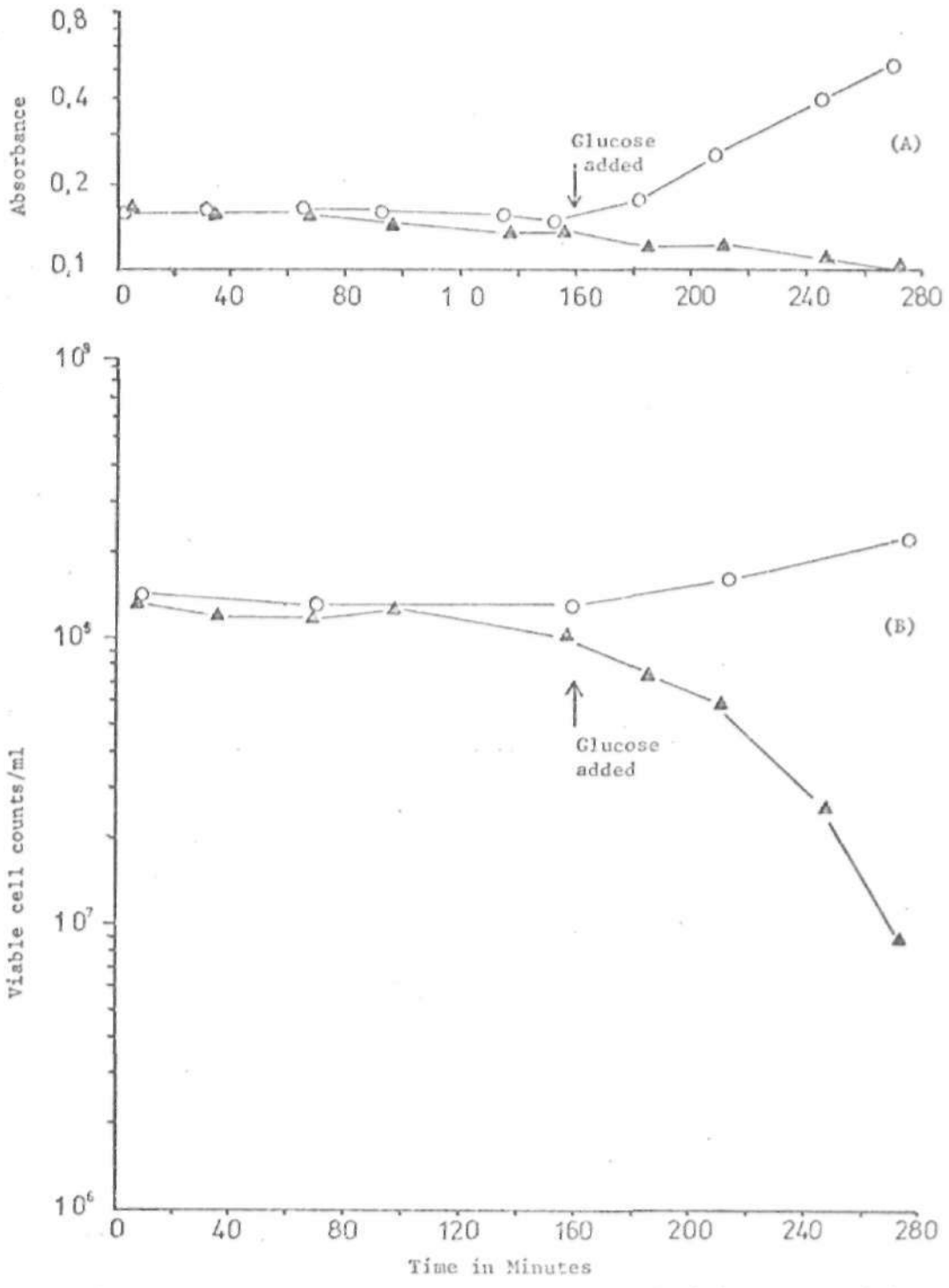


Figure 5.1. The effect of chloroquine on the viability of E.coli in glucose free medium before and after the addition of glucose, as shown by changes in absorbance (A) and viable cell counts/ml (B) of E. coli cultures treated with 0.0 (O——), and 4.0 x 10^{-3}M (▲——) chloroquine.

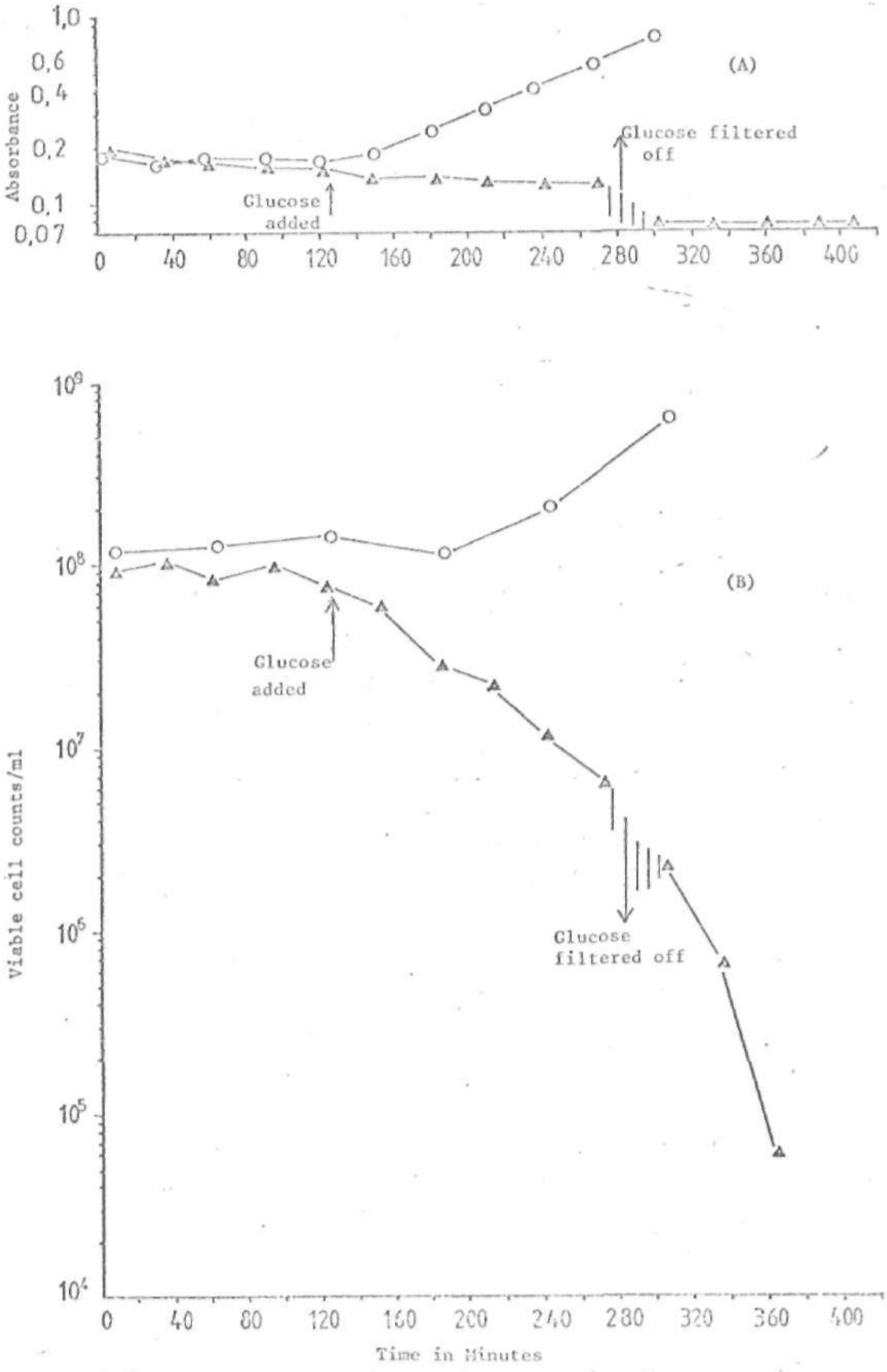


Figure 5.2. The effect of sequential addition and removal of glucose on the viability of E. coli cells treated with chloroquine in glucose free medium, as shown by changes with time of absorbance (A) and viable cell counts(ml (B) of E. coli cultures treated with 0.0 (O——), and 4.0×10^{-3}M (Δ——) chloroquine.

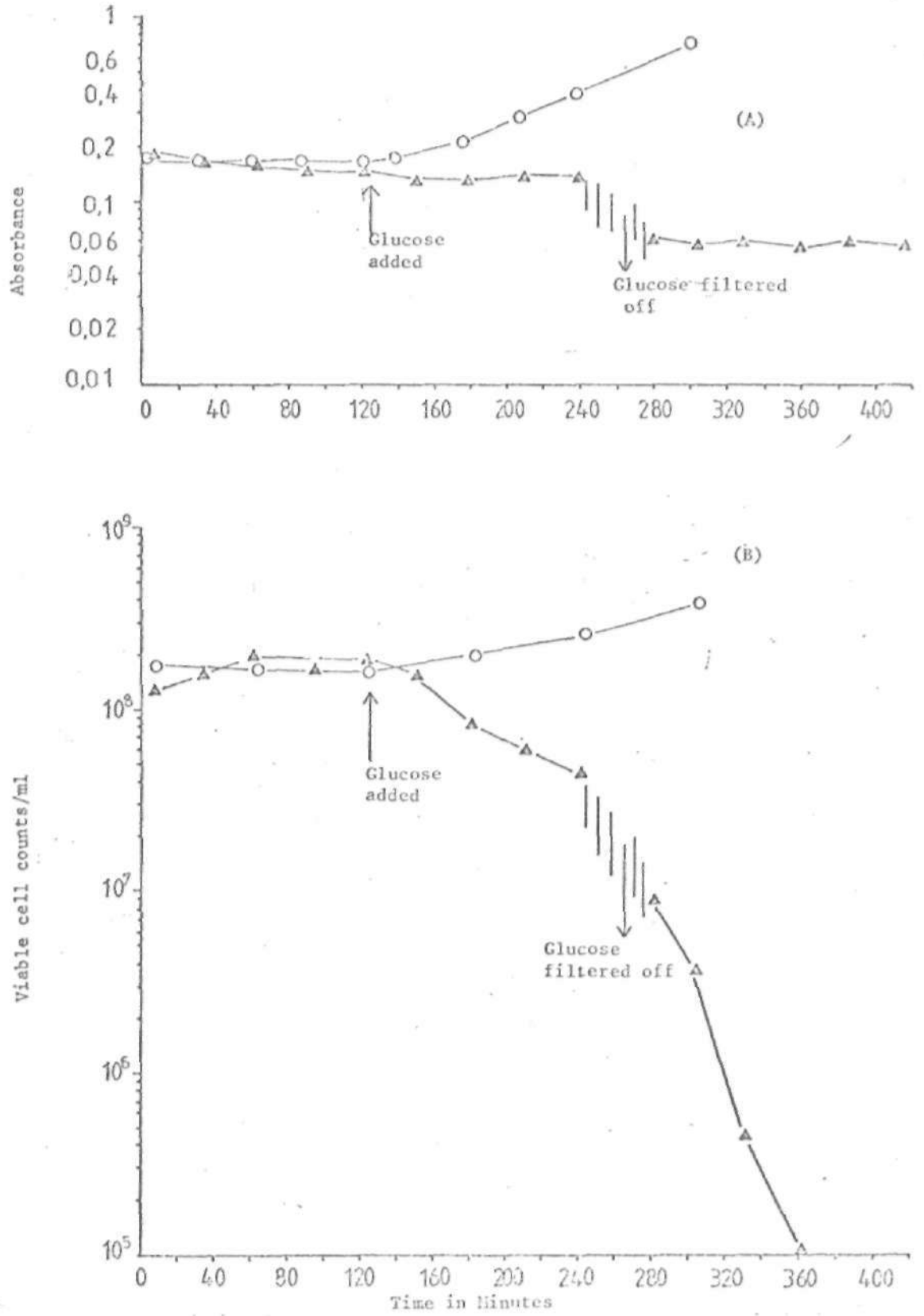


Figure 5.3. The effect of sequential addition and removal of glucose on the viability of E. coli cells treated with chloroquine in glucose free medium, as shown by changes with time of absorbance (A) and viable cell counts (B) of E. coli cultures treated with 0.0 (O——), and 4.0×10^{-3}M (▲– – –) chloroquine.

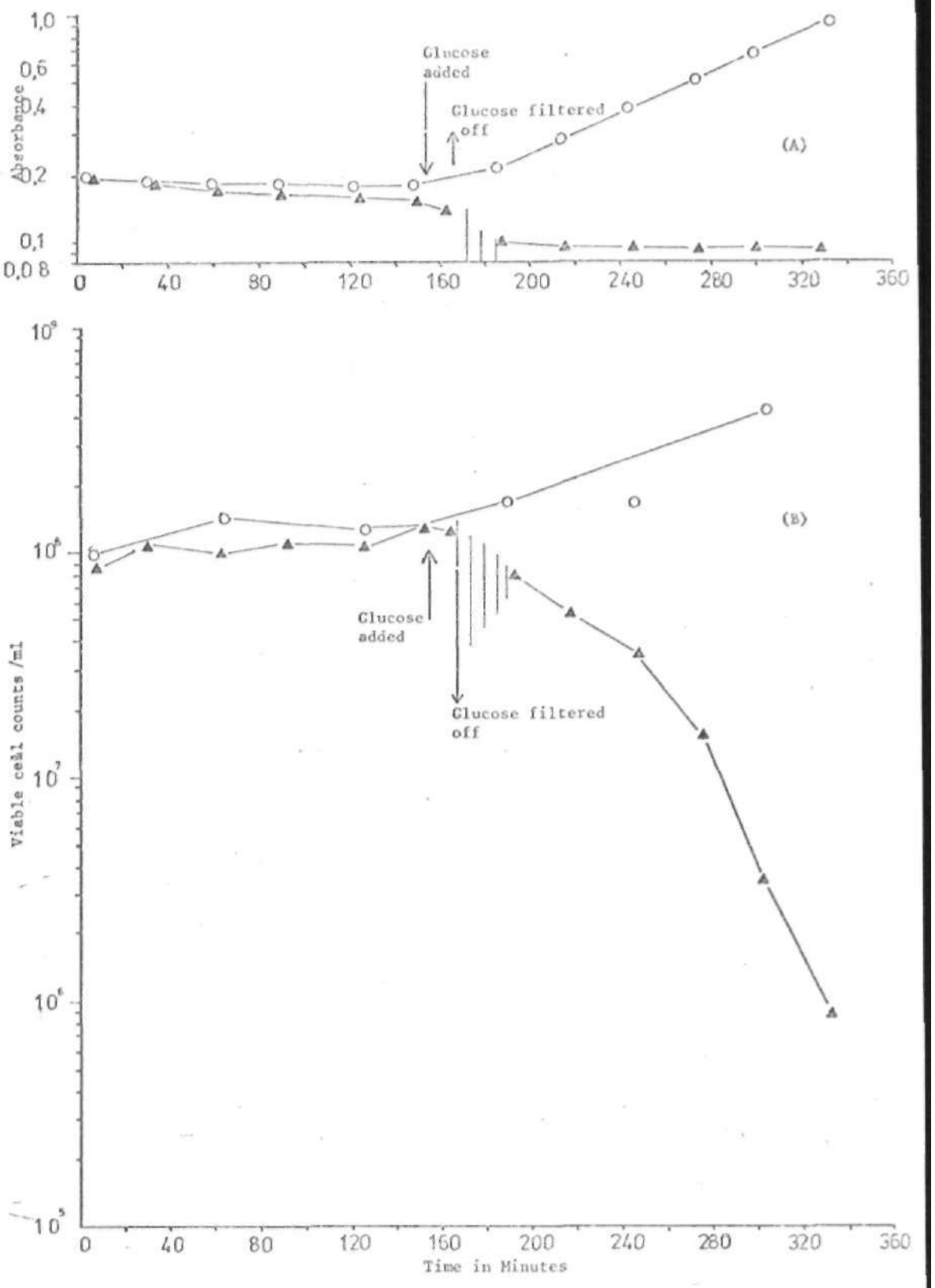


Figure 5.4. The effect of sequential addition and removal of glucose on the viability of E. coli cells treated with chloroquine in glucose free medium, as shown by changes with time of absorbance (A) and viable cell counts/ml (B) of E. coli cultures treated with 0.0 (O——), and 4.0 x 10^{-3} M (Δ——) chloroquine.

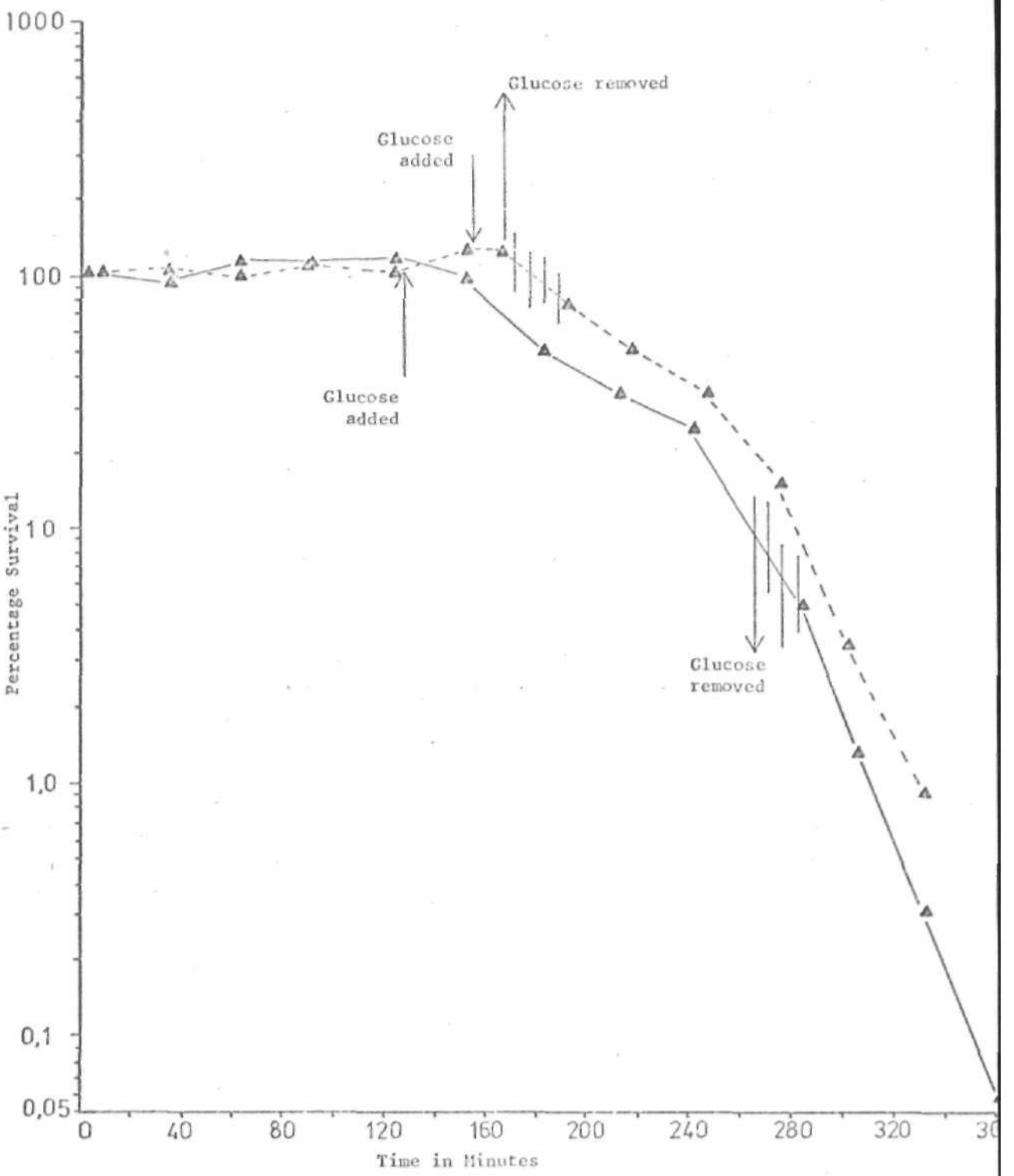


Figure 5.5. The effect of duration of exposure to glucose, after initial glucose-starvation of chloroquine treated E. coli cells, on the bactericidal action of chloroquine, as shown by changes with time of percentage survivals of E. coli cultures treated with 4.0×10^{-3}M (▲——) chloroquine.

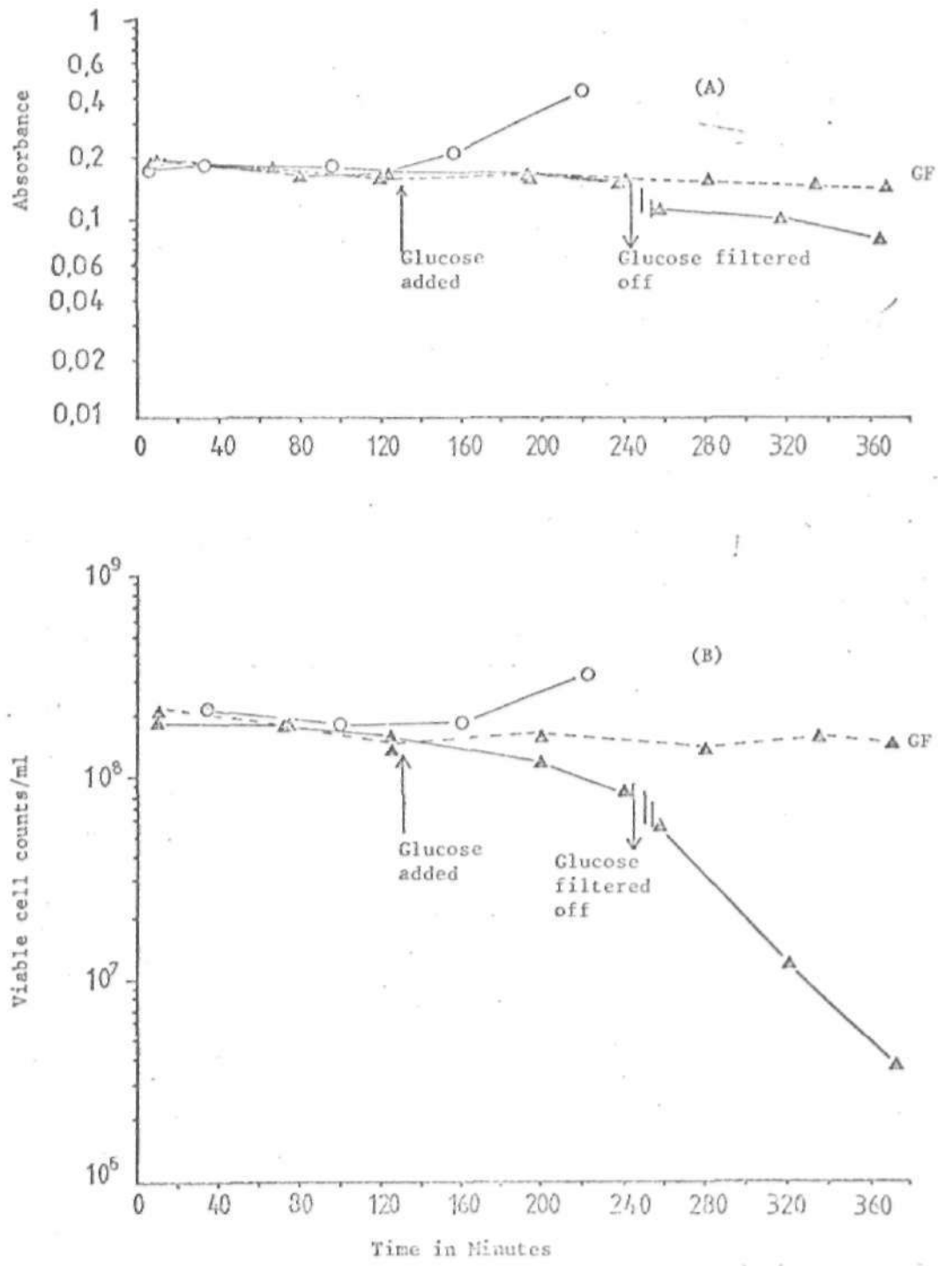


Figure 5.6. The effect of sequential addition and removal of glucose on the viability of E. coli cells treated with chloroquine in glucose free medium, as shown by changes with time of absorbance (A) and viable cell counts/ml (B) of E. coli cells treated with 0.0 (O——), and 4.0×10^{-3} M (Δ- - - -) chloroquine.
GF was maintained glucose free throughout.

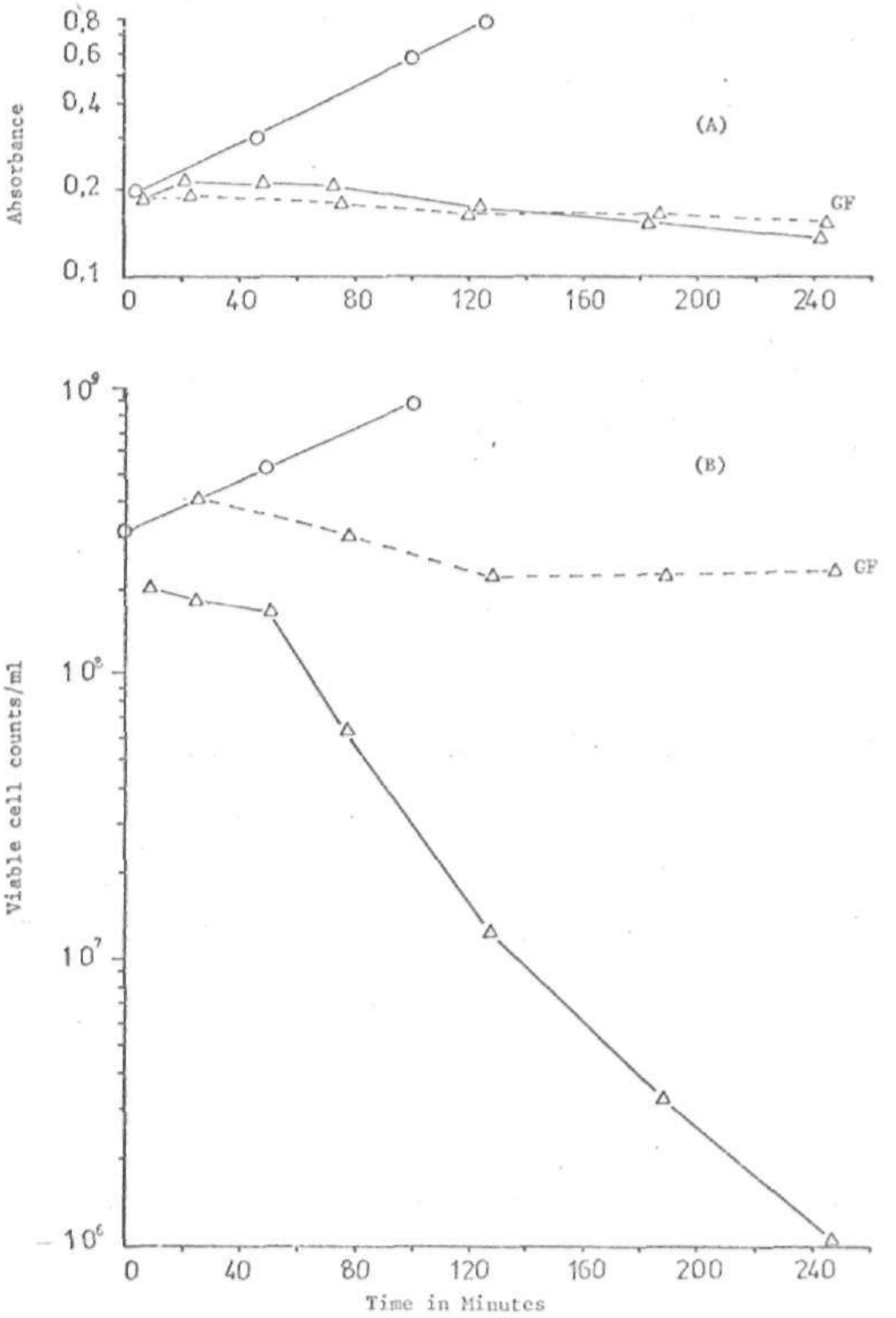


Figure 5.7. The effect of glucose-starvation of E. coli cells on the bactericidal action of 2.0×10^{-3}M chloroquine, as shown by changes with time of absorbance (A) and viable cell counts/ml (B) of E. coli cells treated with 0.0 (O——), and 2.0×10^{-3}M (Δ——— , Δ----) chloroquine. GF was maintained glucose free throughout.

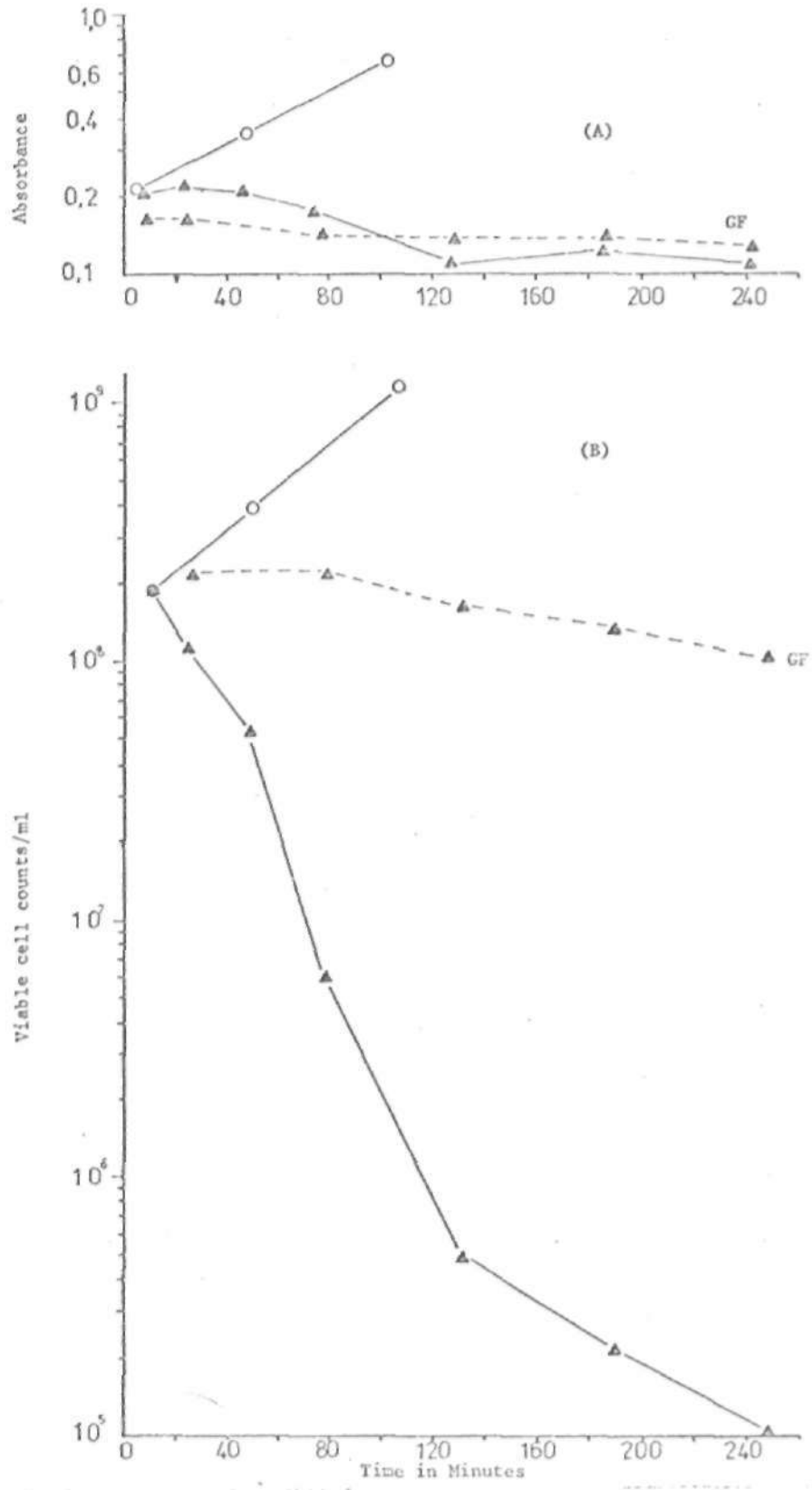


Figure 5.8. The effect of glucose starvation of E. coli cells on the bactericidal action of 4.0×10^{-3} M chloroquine, as shown by changes with time of absorbance (A) and viable cell counts/ml (B) of E. coli cells treated with 0.0 (O——), and 4.0×10^{-3} M (▲—— , ▲- - - -) chloroquine. GF was maintained glucose free throughout.

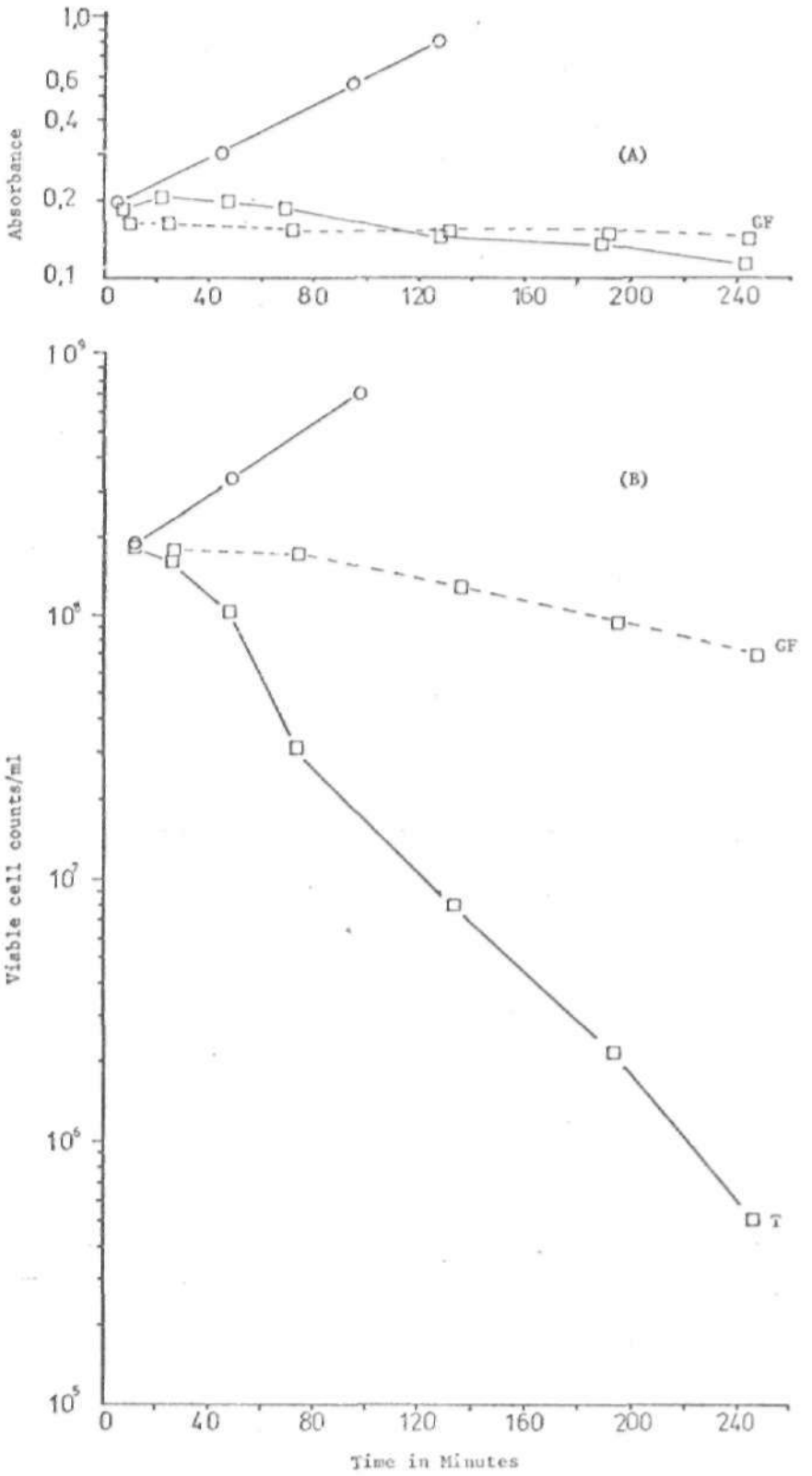


Figure 5.9. The effect of glucose starvation of E. coli cells on the bactericidal action of 8.0×10^{-3}M chloroquine, as shown by changes with time of absorbance (A) and viable cell counts/ml (B) of E. coli cells treated with 0.0 (○——) and 8.0×10^{-3}M (□——, □- - -) chloroquine. GF was maintained glucose free throughout.

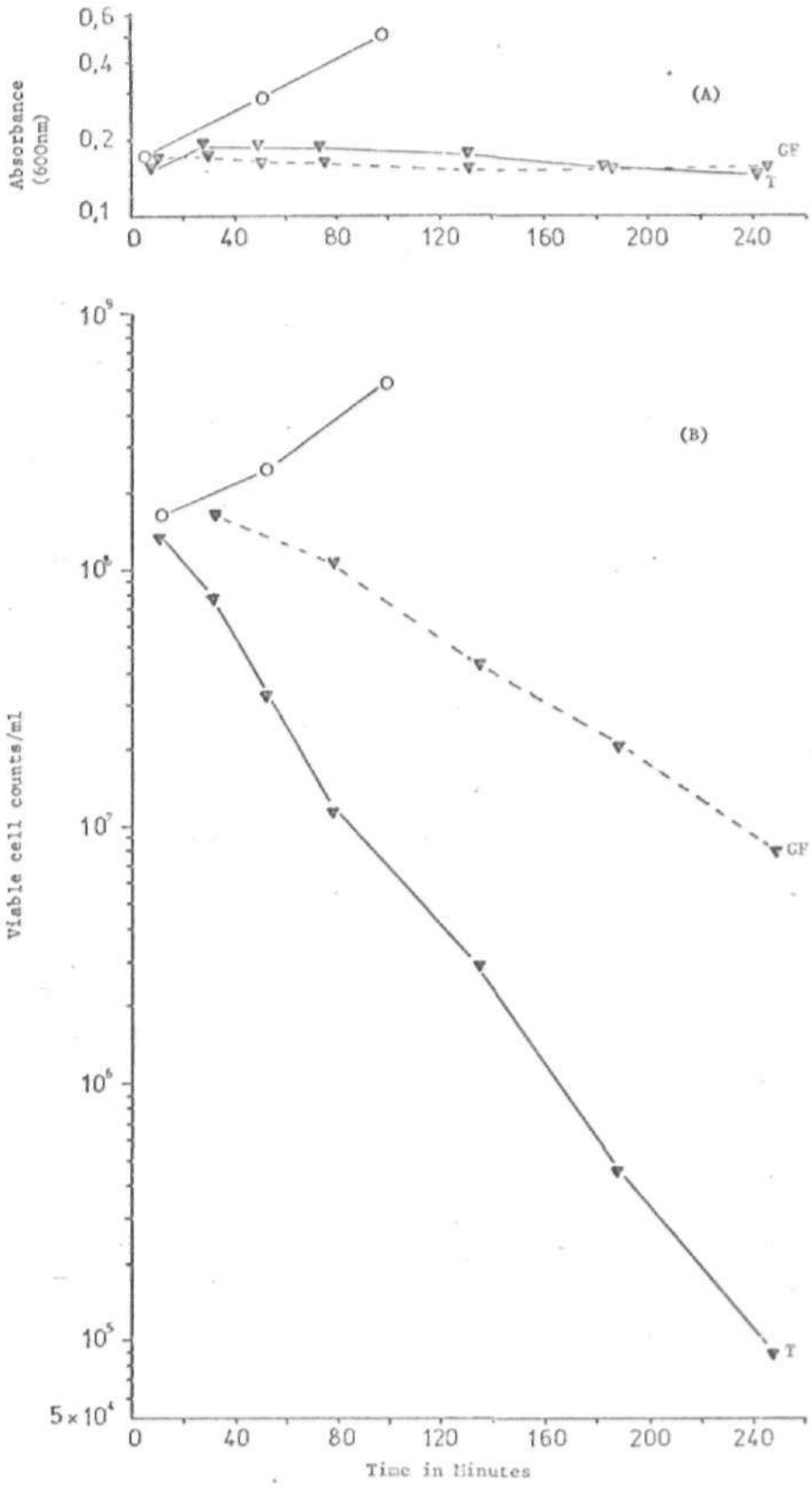


Figure 5.10. The effects of glucose starvation of E. coli cells on the bactericidal action of 16.0 x 10^{-3}M chloroquine, as shown by changes with time of absorbance (A) and viable cell counts/ml (B) of E. coli cells treated with 0.0 (O——), and 16.0 x 10^{-3}M (▼—— , ▽- - -) chloroquine. GF was maintained glucose free throughout.

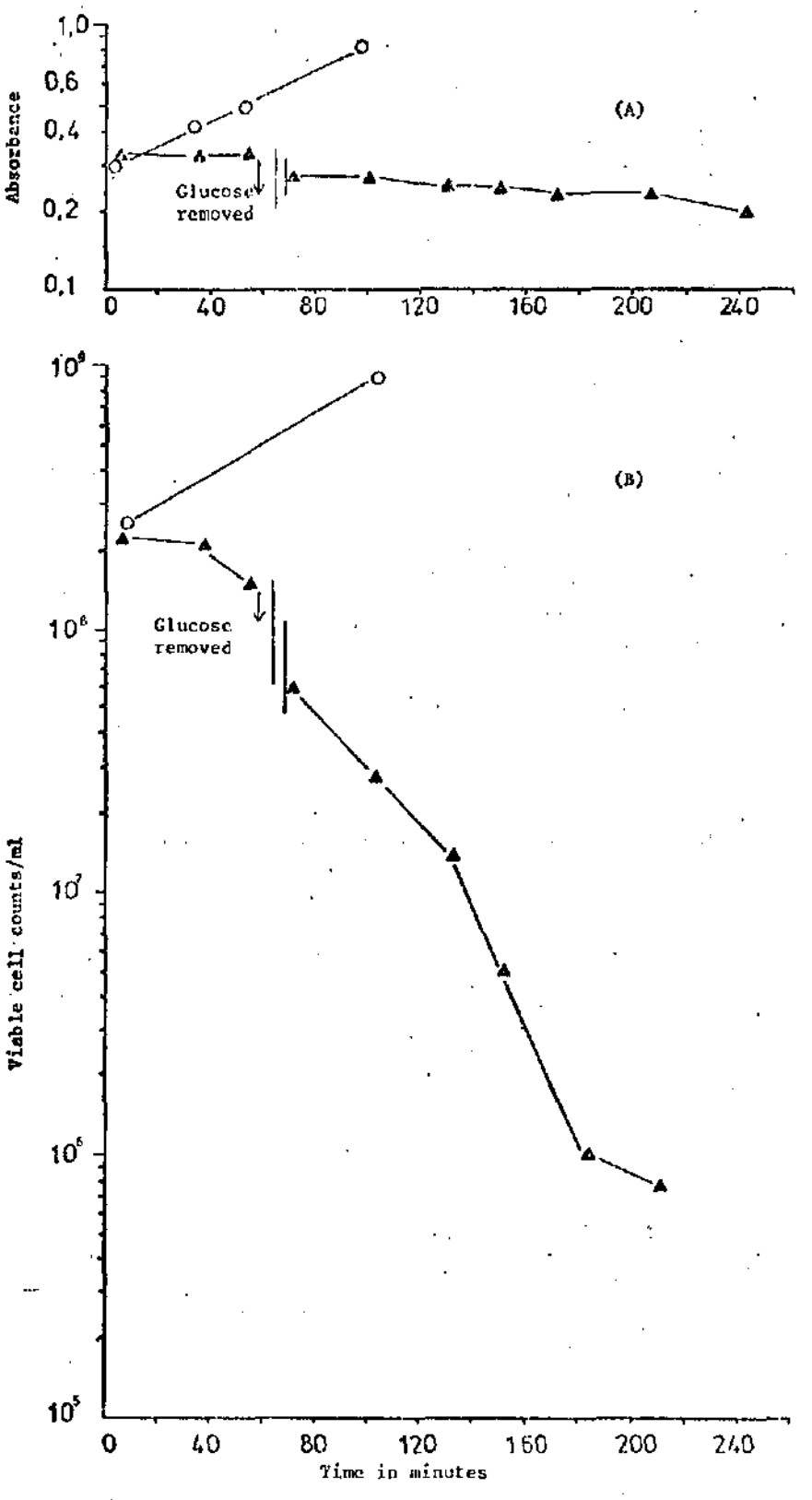


Figure 5.11. The effect of duration of glucose contact with growing cells of E. coli on the bactericidal action of chloroquine, as shown by changes with time of absorbance (A) and viable cell counts/ml (B) of E. coli cells treated with 0.0 (O——), and 4.0×10^{-3}M (▲——) chloroquine.

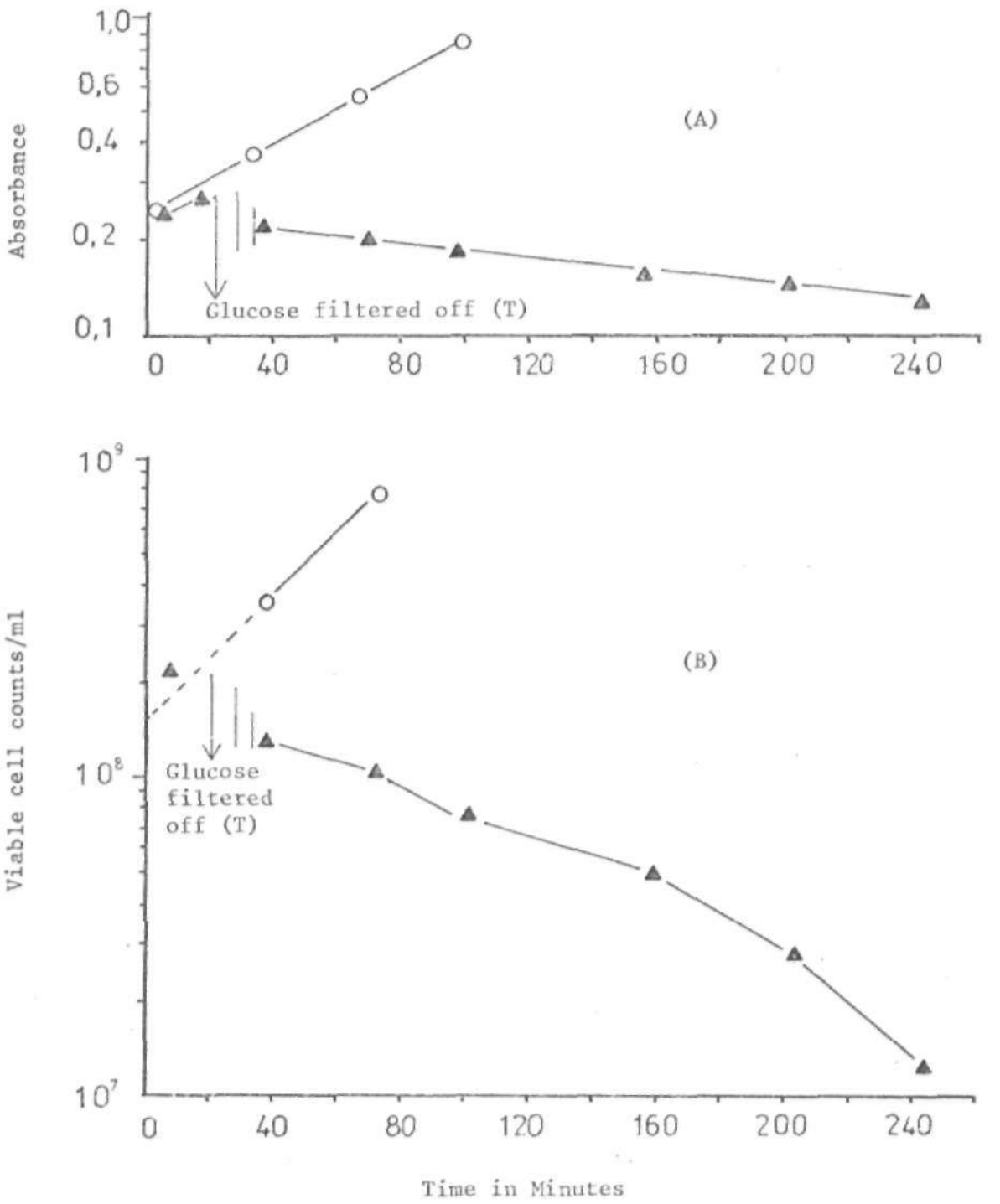


Figure 5.12. The effect of duration of glucose contact with growing cells of E. coli on the bactericidal action of chloroquine, as shown by changes with time of absorbance (A) and viable cell counts/ml (B) of E. coli cells treated with 0.0 (O——), and 4.0×10^{-3}M (▲——) chloroquine.

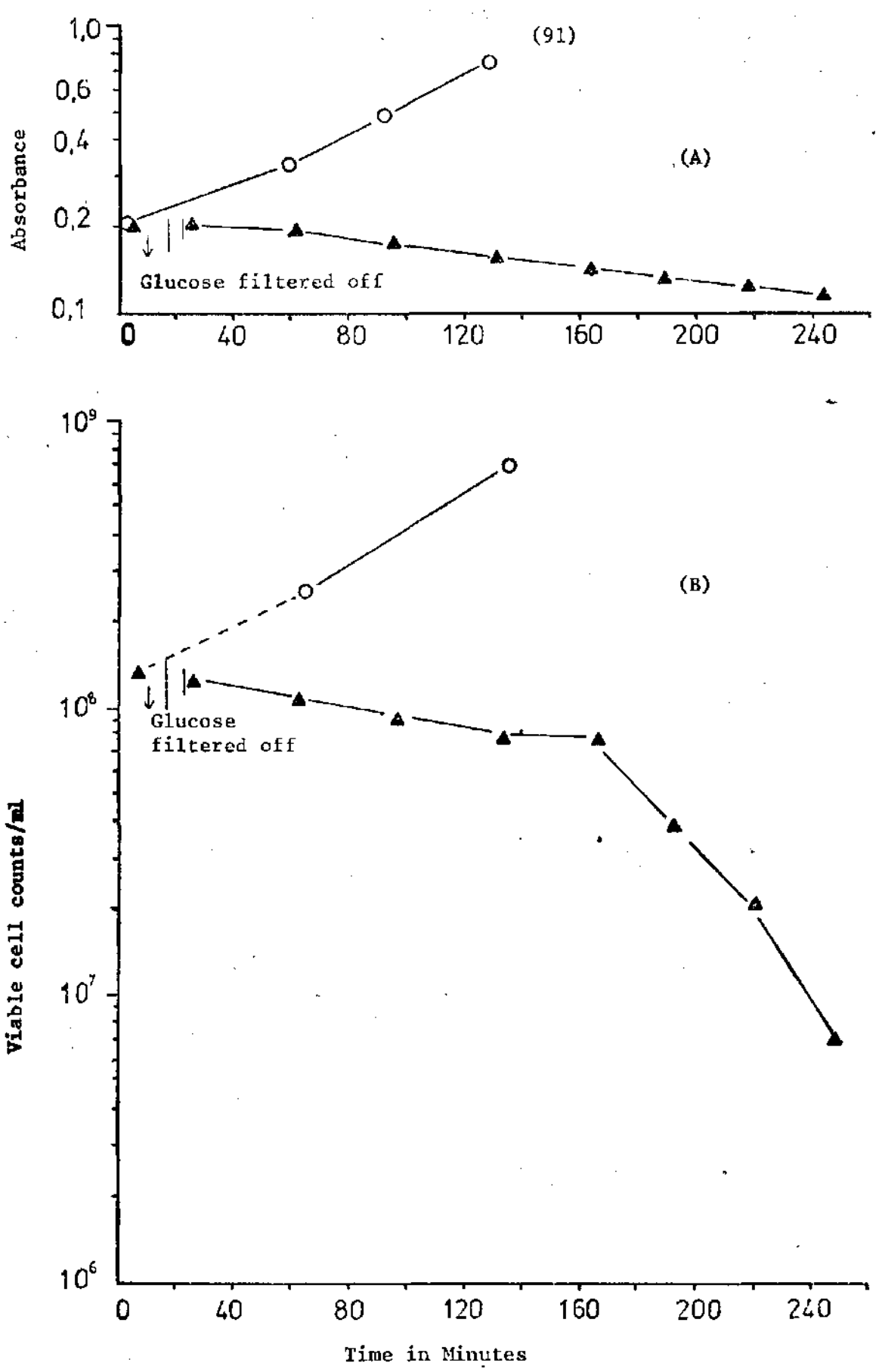


Figure 5.13. The effect of duration of glucose contact with growing cells of E. coli on the bactericidal action of chloroquine, as shown by changes with time of absorbance (A) and viable cell counts/ml (B) of E. coli cells treated with 0.0 (○——), and 4.0×10^{-3}M (▲——) chloroquine.

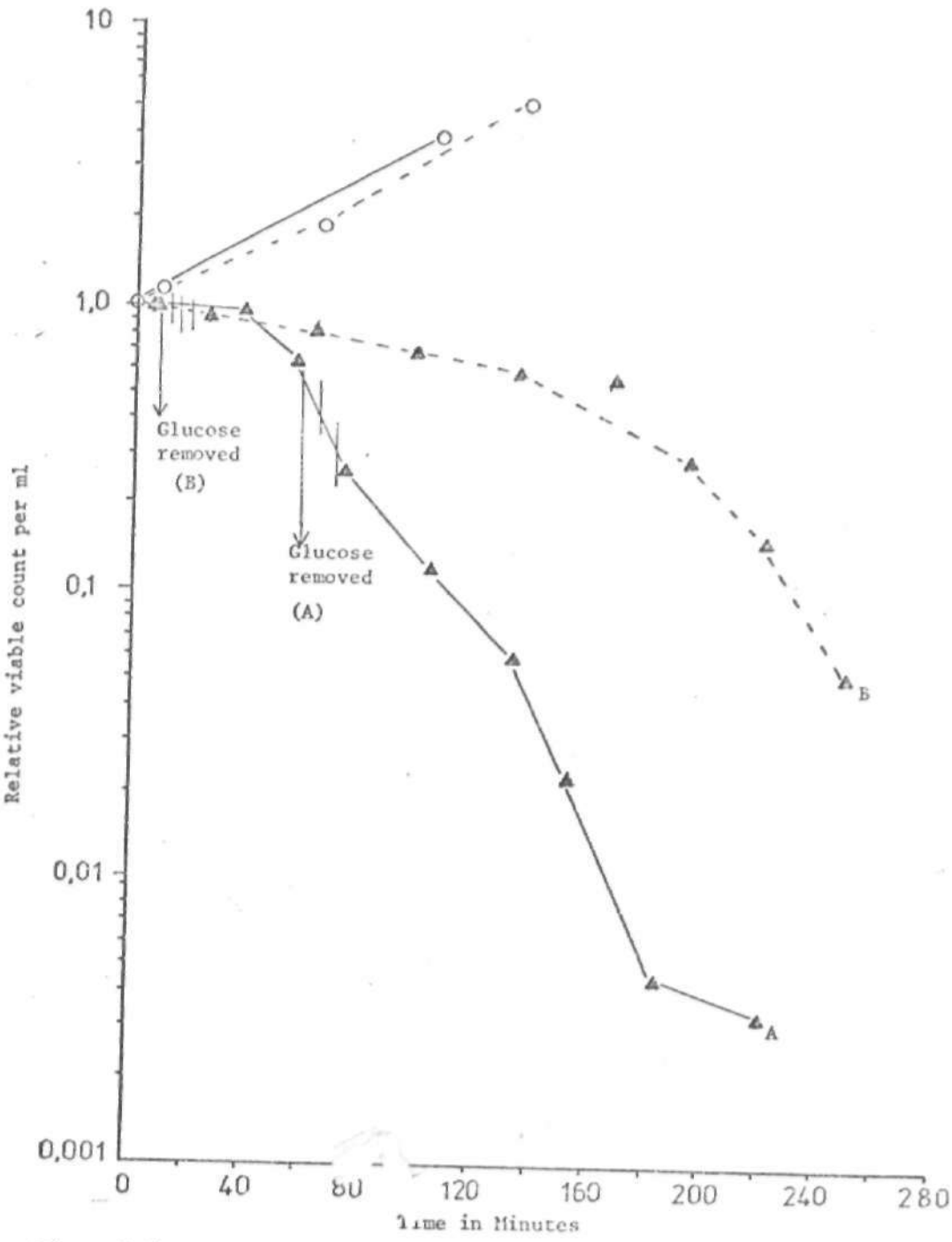


Figure 5.14. Comparison of effect of duration of glucose contact with growing cells of E. coli on the bactericidal action of chloroquine, as shown by changes with time of relative viable cell counts/ml of E. coli cells treated with 0.0 (O——) and 4.0×10^{-3}M (▲——, ▲- - -) chloroquine.

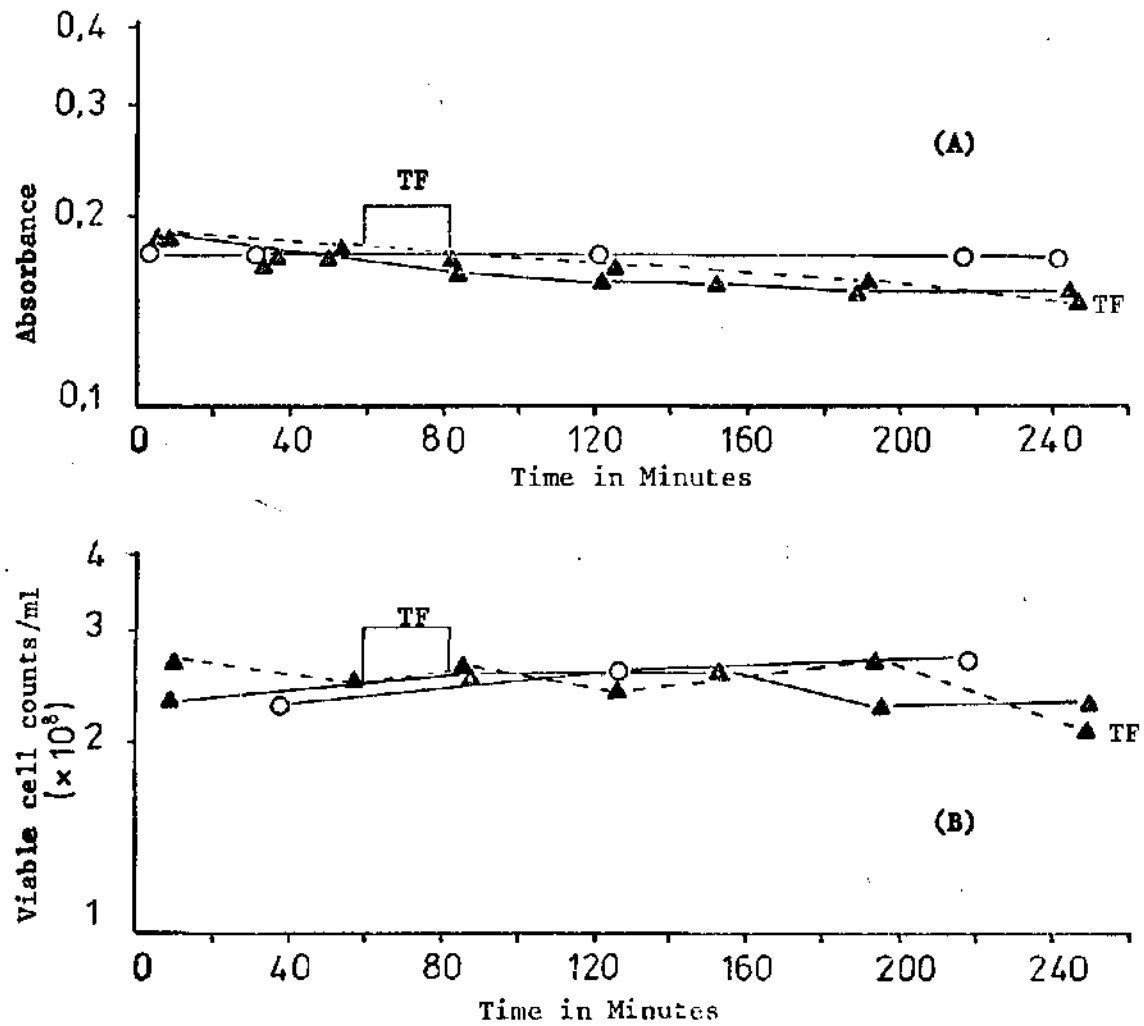


Figure 5.15. The effect of filtration on cells treated with chloroquine in glucose free medium, as shown by changes with time of absorbance (A) and viable cell counts/ml (B) of E. coli cells treated with 0.0 (O———), and 4.0×10^{-3}M (▲——— , ▲- - - -) chloroquine. TF was filtered 60 minutes after the addition of chloroquine.

DISCUSSION

Glucose and the products of its catabolism can play an important role in bacteria. For Escherichia coli growing in a minimal salts medium with glucose as the sole carbon and energy source, 35 to 40% of the glucose taken up by the bacteria is used as a building material for the formation of various cell molecules, and up to 60% is used for the synthesis of Adenosine Triphosphate (Sedlaczek et al., 1965). Glucose is also an important factor in the regulation of biosynthetic processes in the bacteria (Stasters and Winkler, 1963; Grey et al., 1966; Gershanovich, 1977). When it is added to aging cells of E.coli, death is delayed (Clifton, 1966), and when present in a bacterial culture at levels too low to support growth, it is used for energy of maintenance, that is, to fulfil the energy requirements of processes like re-synthesis of macromolecular components, repair of cell walls, membranes and other cell structures, and maintenance of cell permeability barriers (McGrew and Mallet, 1962; Rye, 1969).

Glucose being so important in the metabolism of bacterial cells, lack of it in cell cultures might be expected to at least lead to cessation of growth, and if prolonged, eventually to cell death. This was found to be the case by Ecker and Lockhart (1961), who, working with E. coli K-12 in a chemically defined medium, reported that cessation of growth was coincident with exhaustion of glucose. Rye (1969), however, working with E. coli NCTC 1093, reported a slight increase in cell number but without any increase in total cell mass in cultures deprived of glucose. The absorbance and viable cell count results reported in this Chapter for the untreated controls indicated no in-

crease in either total cell mass or cell numbers during glucose starvation, measurements of these, however, started about one hour after depletion of the glucose.

Under conditions of glucose starvation, it might be expected that E. coli cells would be more vulnerable to the bactericidal effects of chloroquine, as they are already in a weakened condition, but the results for the chloroquine treated cells, for example, Figures 5.1, 5.3, 5.4 and 5.6 prove the contrary. The glucose-starved chloroquine treated cells maintained their viability for at least two hours, unlike the situation in the presence of glucose.

One could ask why there was no bactericidal activity in the absence of glucose; whether the drug was unable to penetrate into the bacterial cells, or whether it could not act on the cells after penetration because of their physiological state. The most likely situation is that chloroquine is taken up by the bacterial cells, perhaps at a reduced rate, in the absence of glucose, as is the case with Plasmodium knowlesi (Polet and Barr, 1969). Also, results of uptake studies (see Chapter 7) indicate some uptake of the drug when E. coli cells are incubated in glucose free medium. The possible effects of glucose on uptake of chloroquine will be discussed later in Chapter 7.

It is known from the works of Hahn and associates (1966), and Cohen and Yielding (1965 a and b) that chloroquine can form molecular complexes with DNA, and thereby inhibit DNA synthesis and replication. It might therefore be expected that the drug would be more effective against cells which are actively dividing, and less effective or totally ineffective against resting cells which are not attempting to replicate their DNA.

The results depicted in Figures 5.2 - 5.5 and 5.11 - 5.14 illustrate another point of interest: the observation that cells were still dying after the removal of glucose. This was contrary to expectation, and indicated that the presence of glucose was essential only for an initial period, after which its presence was no longer a necessity for the bactericidal activity of chloroquine. Since the cells were still dying after the removal of glucose, it is possible that they were utilising endogenous sources of glucose, such as glycogen, formed during the period when the medium contained glucose. A rapid utilisation of glycogen after the removal of glucose (Dawes and Ribbons, 1962) could then explain the rapid killing observed. That being the case, it should not be surprising that the longer periods in the presence of glucose gave a greater extent of kill after glucose removal, since the longer "glucose feed" would have made greater glycogen reserves possible.

The results of the treatment of glucose-starved cells with different concentrations of chloroquine, illustrated in Figures 5.7 to 5.10, manifest a difference which had not been obvious in the presence of glucose. 2.0, 4.0 and 8.0 x 10^{-3}M chloroquine showed little bactericidal effect in the absence of glucose, whereas the 16.0 x 10^{-3}M concentration clearly did. No concrete explanation has been found for this difference. A possible explanation, however, is that the antimicrobial action of chloroquine is not based solely on its reaction with DNA, but that only one of its ionic species, namely the diprotonated, is involved with DNA interaction (Cohen and Yielding, 1965), and that one of the other species, at this high concentration, is present in sufficient quantity to manifest its mode of kill, which may be independent of growth and cell division.

The results of the work described in this Chapter strongly indicate that chloroquine, at bactericidal concentrations up to 8.0 x 10^{-3}M, is ineffective against resting non-growing cells of E. coli, and that a carbon and energy source such as glucose must be present in the growth medium concurrently for some time to initiate its bactericidal action.

CHAPTER SIX

EFFECT OF pH ON THE ANTIBACTERIAL ACTIVITY OF CHLOROQUINE

CHAPTER SIX

EFFECT OF pH ON THE ANTIBACTERIAL ACTIVITY OF CHLOROQUINE

Page No.

Experimental 100

Effect of pH on the growth inhibiting activity of chloroquine 100

Viable Cell Count 101

Results 102

Figures 105

Discussion 114

EXPERIMENTAL

It has been reported by Wiseman (1972a) that the growth inhibiting activity of chloroquine against E. coli is affected by the pH of the medium in which the organism is growing. A similar observation was made by Middleton (1976), working with the same organism but using a Tris buffered minimal salts medium, as opposed to the phosphate buffered medium used by Wiseman.

The object of the present study reported in this Chapter was to investigate the effect of pH on the antibacterial activity of chloroquine in a MOPS buffered medium, and to determine the approximate threshold bactericidal concentration of the drug at various pH values.

The choice of the range of pH values studied was based on its suitability for the growth of E. coli, and its compliance with the useful buffering range of MOPS. The pH values used were 6.4, 6.7, 7.0, 7.4 and 7.7.

Effect of pH on the Growth Inhibiting Activity of Chloroquine.

Overnight cultures of E. coli growing exponentially from a low inoculum were filtered, washed with and resuspended in fresh MOPS medium, which had previously been adjusted to the required pH. More medium was added to give an absorbance of about 0.15; the culture was then incubated with aeration in a shaking water bath at 37^{o}C for an hour, by which time the absorbance had reached about 0.300. 10ml portions of the culture were measured into each of a series of 50ml conical flasks containing 10ml volumes of either MOPS medium alone or of

solutions of chloroquine at double the finally required concentrations in MOPS medium at 37°C and the required pH. The flasks were then returned to the shaking water bath, and growth was followed turbidimetrically by regular measurements of absorbance for at least 2 hours.

The experiment was carried out at pH values of 6.4, 6.7, 7.0, 7.4 and 7.7.

Viable Cell Counts.

Viable counts were carried out on some of the treated cultures using the method described in Chapter 4.

RESULTS

Typical results of experiments carried out to determine the effects of pH on the growth inhibiting activity of chloroquine are illustrated in Figures 6.1 to 6.4. Each shows changes in absorbance with time of E. coli cultures treated with different concentrations of chloroquine at a particular pH.

Figure 6.1 for example represents the results of one experiment at pH 6.4 and shows that the absorbance of the untreated control culture increased exponentially throughout the period of the experiment with a mean generation time of about 50 minutes. The presence of 1.0×10^{-3}M chloroquine caused only a slight decrease in the rate of increase of absorbance, the mean generation time being increased to about 52 minutes. The growth curves in the presence of 2.0 and 3.0 $\times 10^{-3}$M chloroquine both showed a biphasic pattern with a primary phase where the absorbance increased exponentially at only a slightly slower rate than the control, followed after about 100 minutes by a change to a considerably slower but still exponential secondary inhibited growth rate which continued until the end of the experiment.

In the presence of 4.0×10^{-3}M chloroquine there was an initial increase in absorbance during the first 80 minutes followed by a slow decrease, whilst in the presence of 5.0×10^{-3}M there was an increase for about 40 minutes followed by a more rapid decline.

At the other pH values similar patterns of growth inhibition were obtained except that as the pH was increased, lower concentrations of chloroquine were required to cause the same degree of inhibition.

The growth rate of the untreated control cultures was unaffected by the changes in pH.

Dose response curves for the effect of chloroquine on growth rate at the different pH values were constructed by calculating the percentage decrease of the secondary inhibited growth rates relative to the controls, and are shown in Figure 6.5. This Figure represents the means of several determinations at each pH and clearly shows that the growth inhibiting activity increases markedly with increase in pH.

Some experiments were also carried out, in which single concentrations of chloroquine were studied at different pH values, and a typical result is shown in Figure 6.6. This shows that a concentration of 1.5×10^{-3}M chloroquine only partially inhibits growth at pH 6.7 and pH 7.0, but ultimately completely inhibits growth at pH values of 7.4 and 7.7.

The experiments designed to study the bactericidal effect of chloroquine showed similar patterns at all the pH values. Concentrations just sufficient to result in the absorbance remaining constant after 120 minutes had little or no effect on cell viability whilst the presence of higher concentrations which ultimately resulted in decrease in absorbance caused cell death. A typical set of results for pH 6.7 is shown in Figure 6.7.

In the presence of 2.6×10^{-3}M chloroquine, which in this experiment caused a slow decrease in absorbance after 120 minutes, there was no significant change in viability for about 60 minutes, after which there was a slow decrease in viable cell count. In the presence

of 3.0×10^{-3}M chloroquine, however, where there was a faster rate of decrease in absorbance, the viable cell count began to decline within 10 minutes of the addition of chloroquine and at a faster rate, indicating that the threshold concentration for bactericidal action at this pH was about 2.6×10^{-3}M.

Threshold bactericidal concentrations obtained for pH 7.0, 7.4 and 7.7 were approximately 1.6, 1.2 and 0.7×10^{-3}M respectively. No figure was directly determined at pH 6.4, although the absorbance curves suggest a value of just less than 4.0×10^{-3}M.

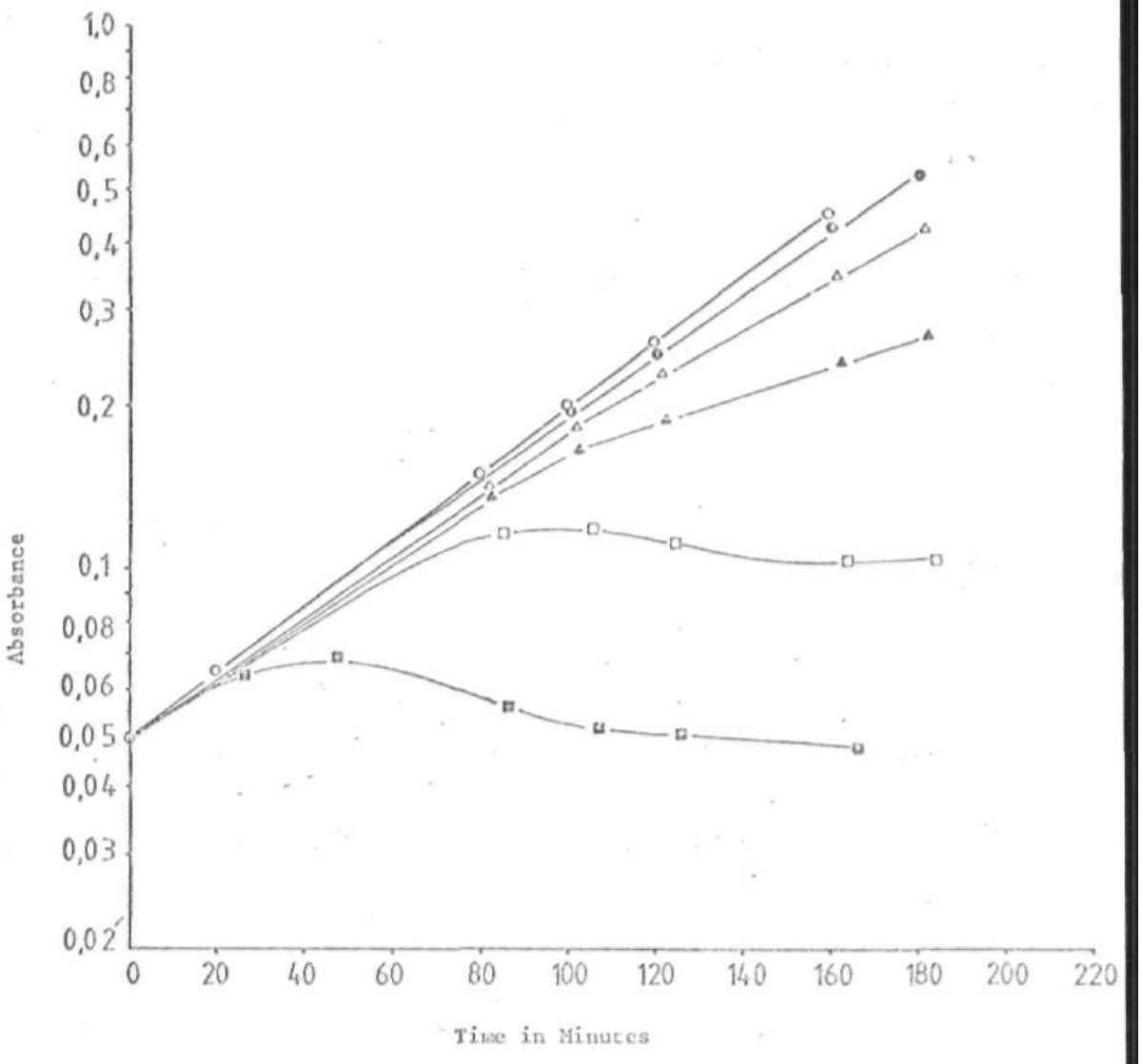


Figure 6.1. The effect of chloroquine on the changes with time of absorbance of E. coli cultures treated with 0.0 (○——), 1.0 (●——), 2.0 (△—), 3.0 (▲—), 4.0 (□——) and 5.0 x 10^{-3}M (■——) chloroquine at pH 6.4.

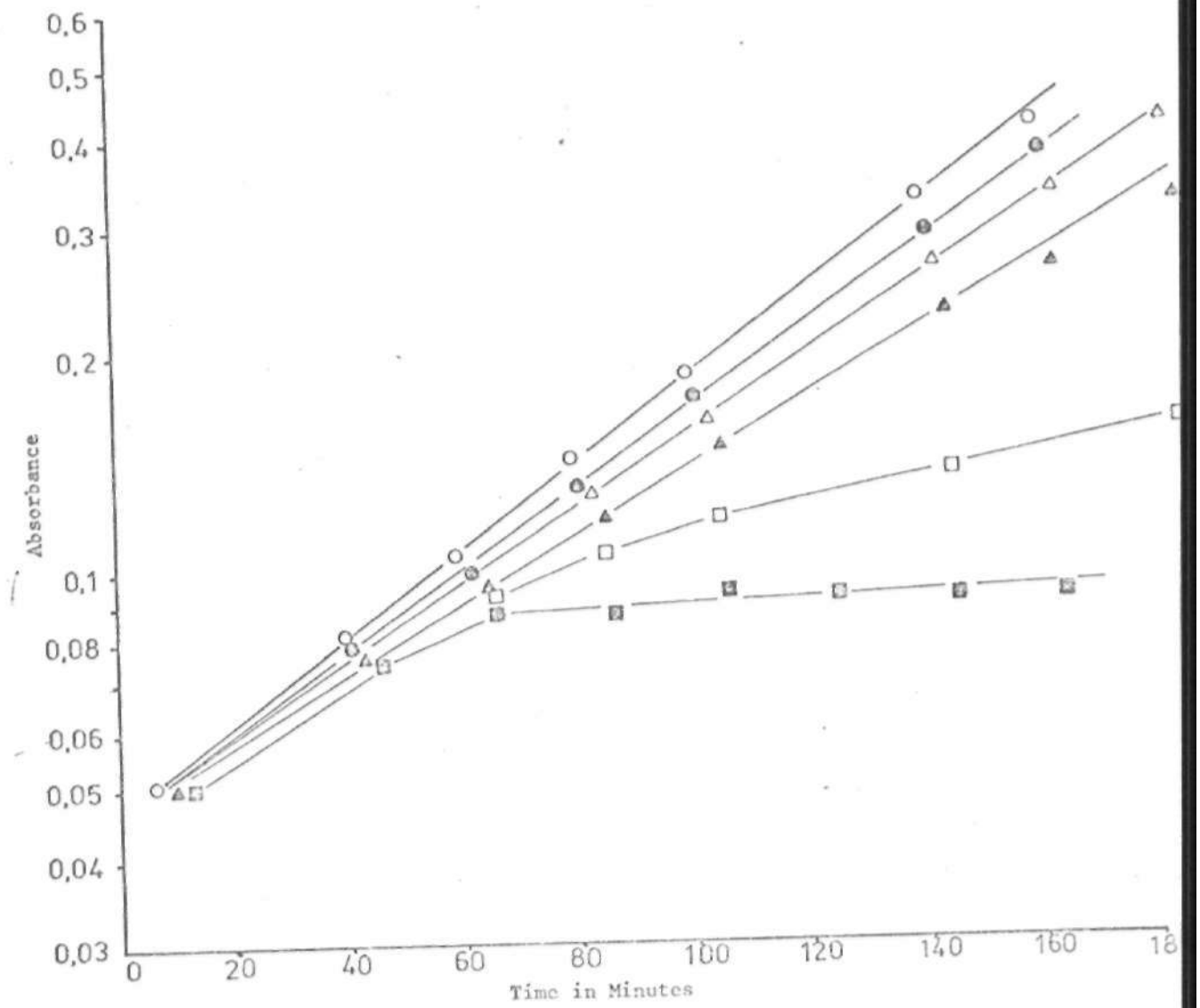


Figure 6.2. Effect of chloroquine on the changes with time of absorbance of E.coli cultures treated with 0.0 (○——), 1.0 (●——), 1.5 (△——), 2.3 (▲——), 2.5 (□——), and 2.8 x 10^{-3}M (■——) chloroquine at pH 6.7.

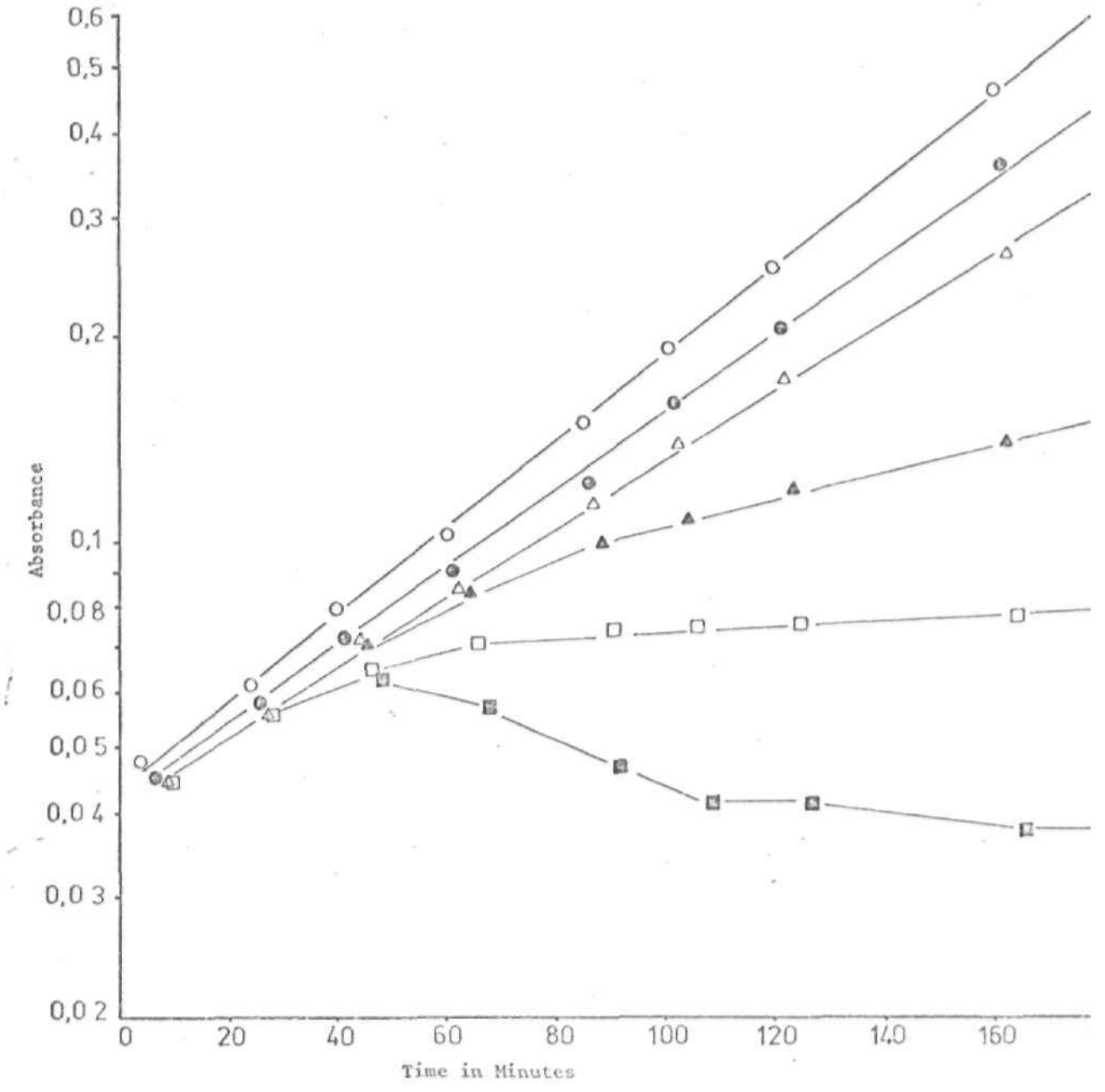


Figure 6.3. Effect of chloroquine on the changes with time of absorbance of E. coli cultures treated with 0.0 (○——), 1.0 (●——), 1.2 (△——), 1.4 (▲——), 1.6 (□——) and 2.0 x 10^{-3}M (■——) chloroquine at pH 7.0.

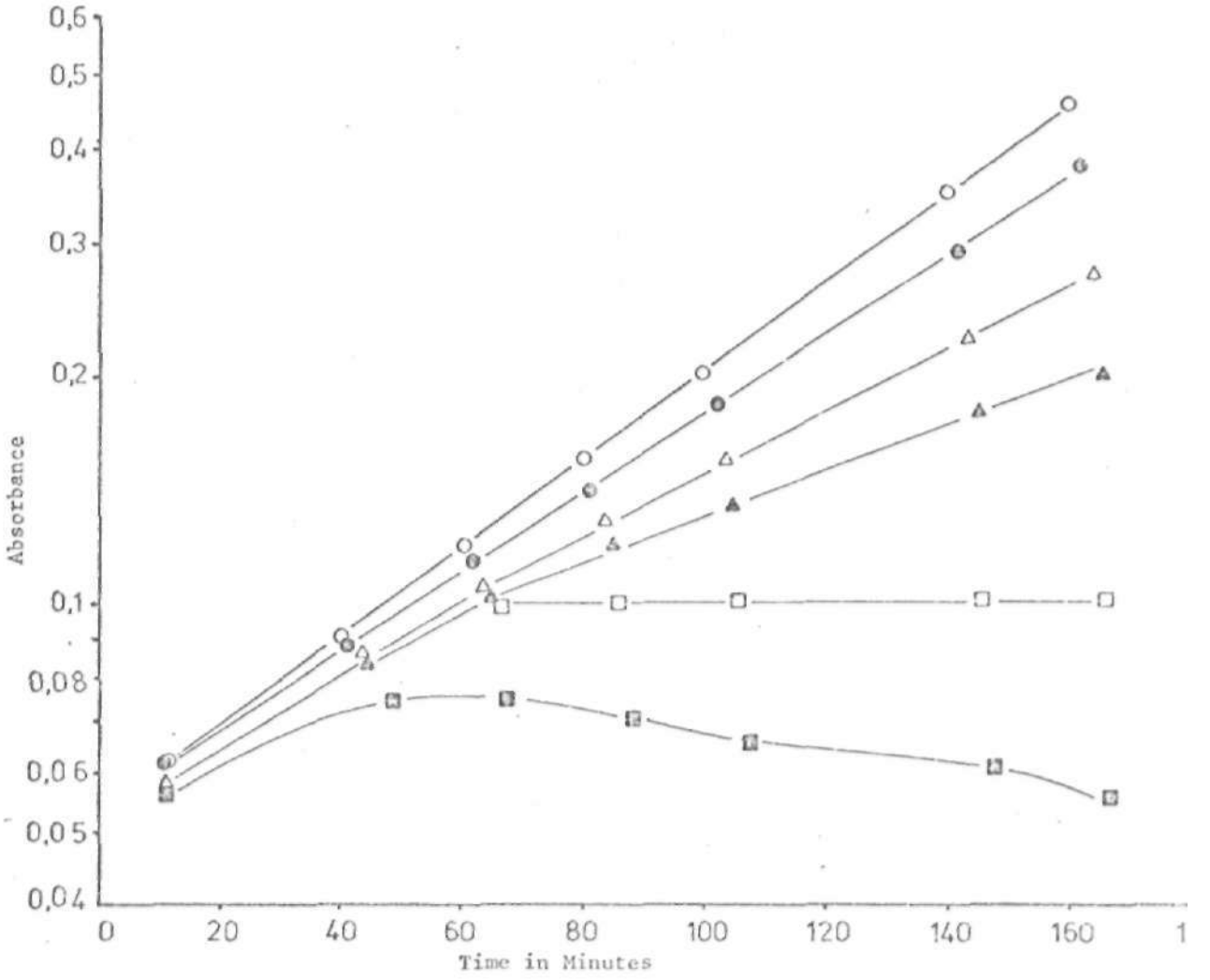


Figure 6.4. Effect of chloroquine on the changes with time of absorbance of E. coli cultures treated with 0.0 (○——), 0.2 (●——), 0.5 (△——), 0.6 (▲——), 0.7 (□——), and 0.8 x 10^{-3} M (■——) chloroquine at pH 7.7.

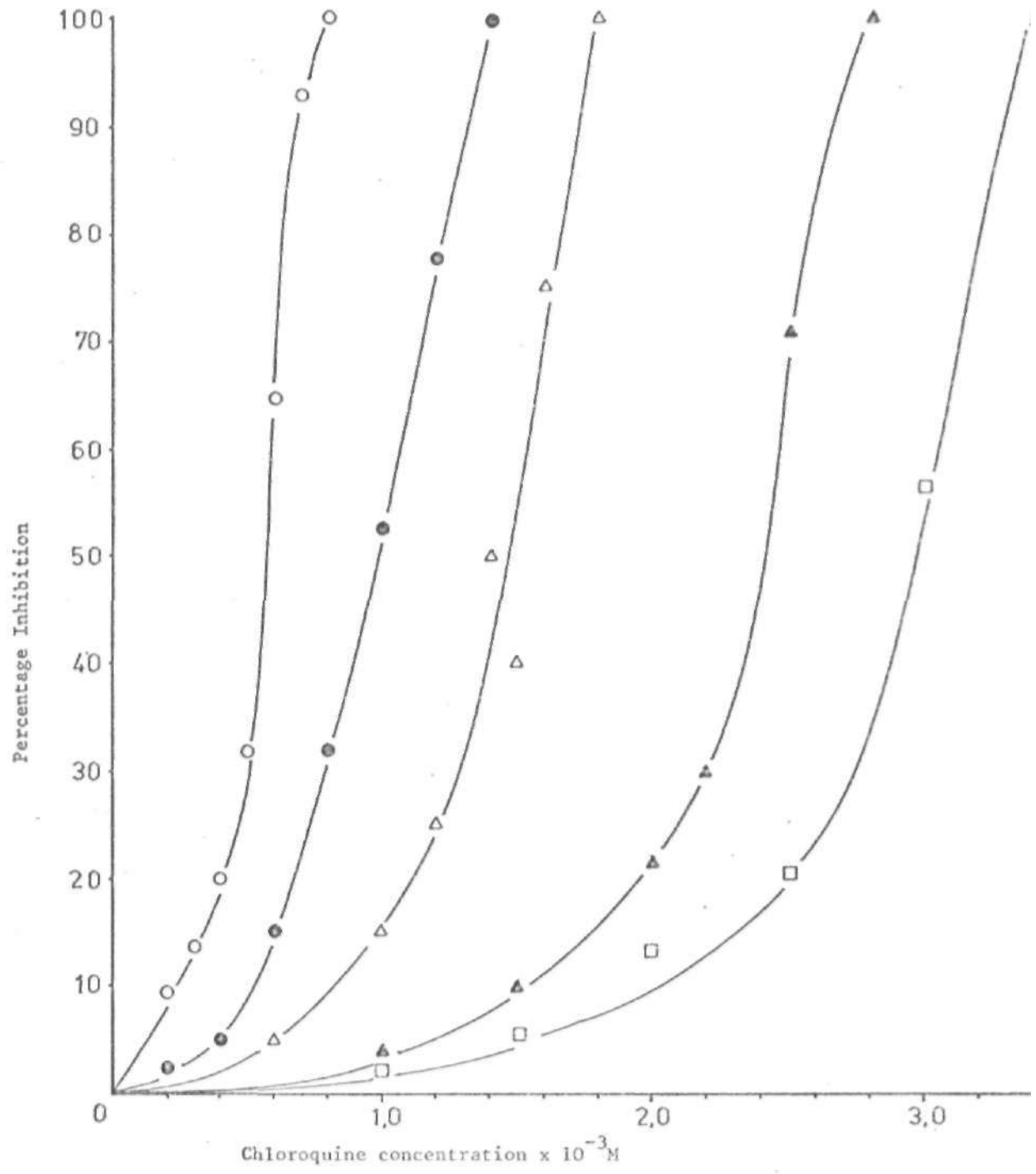


Figure 6.5. Relationship between chloroquine concentration and the percentage inhibition of E. coli cultures in MOPS medium at pH values of 6.4 (□——), 6.7 (▲——), 7.0 (△——), 7.4 (●—) and 7.7 (○—).

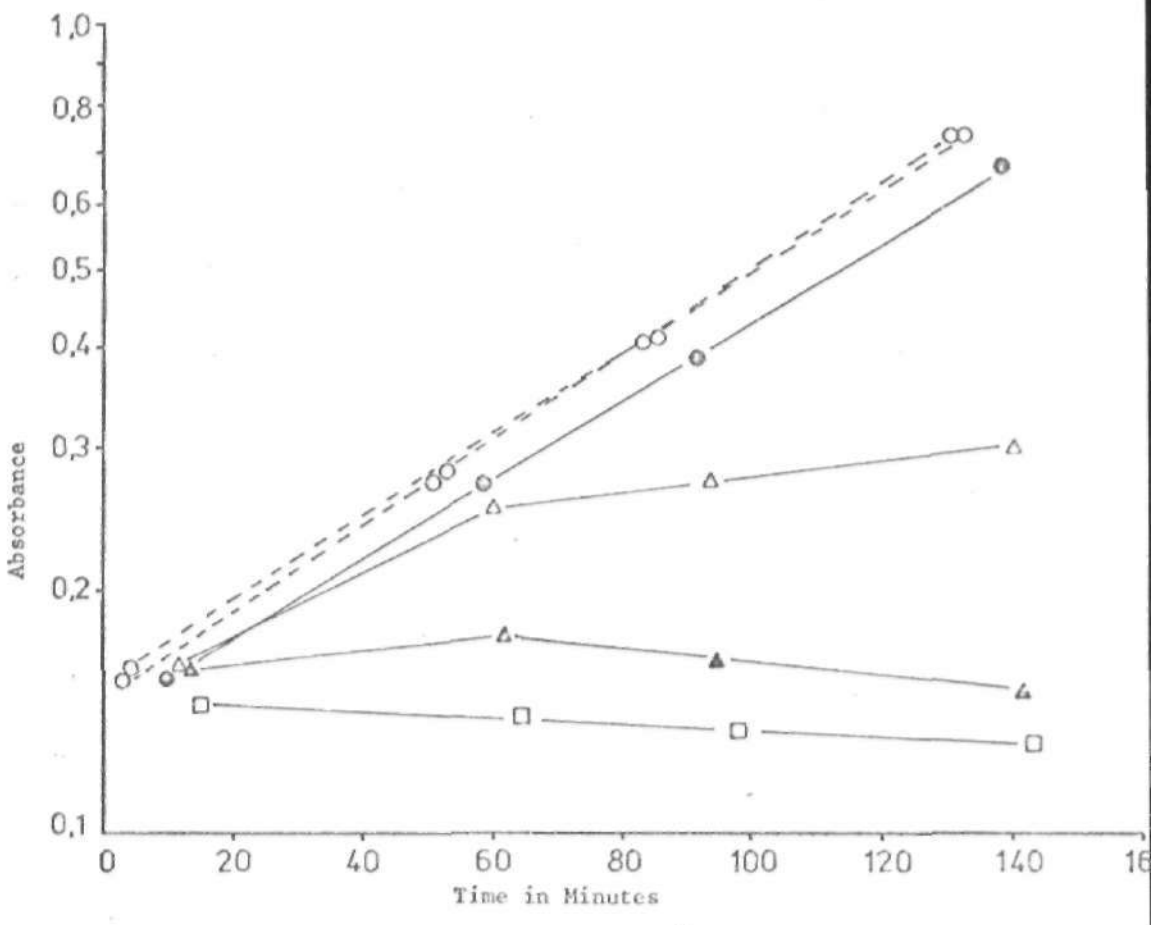
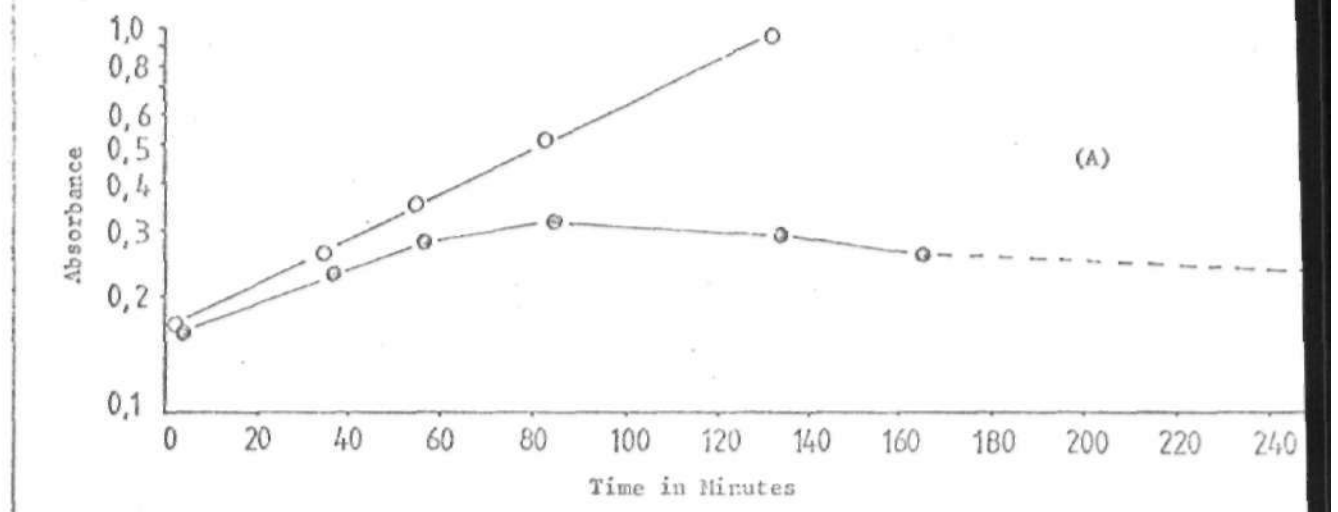


Figure 6.6. Effect of Chloroquine (1.5×10^{-3}M) on the changes with time of absorbance of E. coli cultures treated with 1.5×10^{-3}M chloroquine at pH values of 6.7 (●——), 7.0 (△——), 7.4 (▲—), and 7.7 (□——). (○··;○··) represent control cultures at pH 6.7 and pH 7.0.



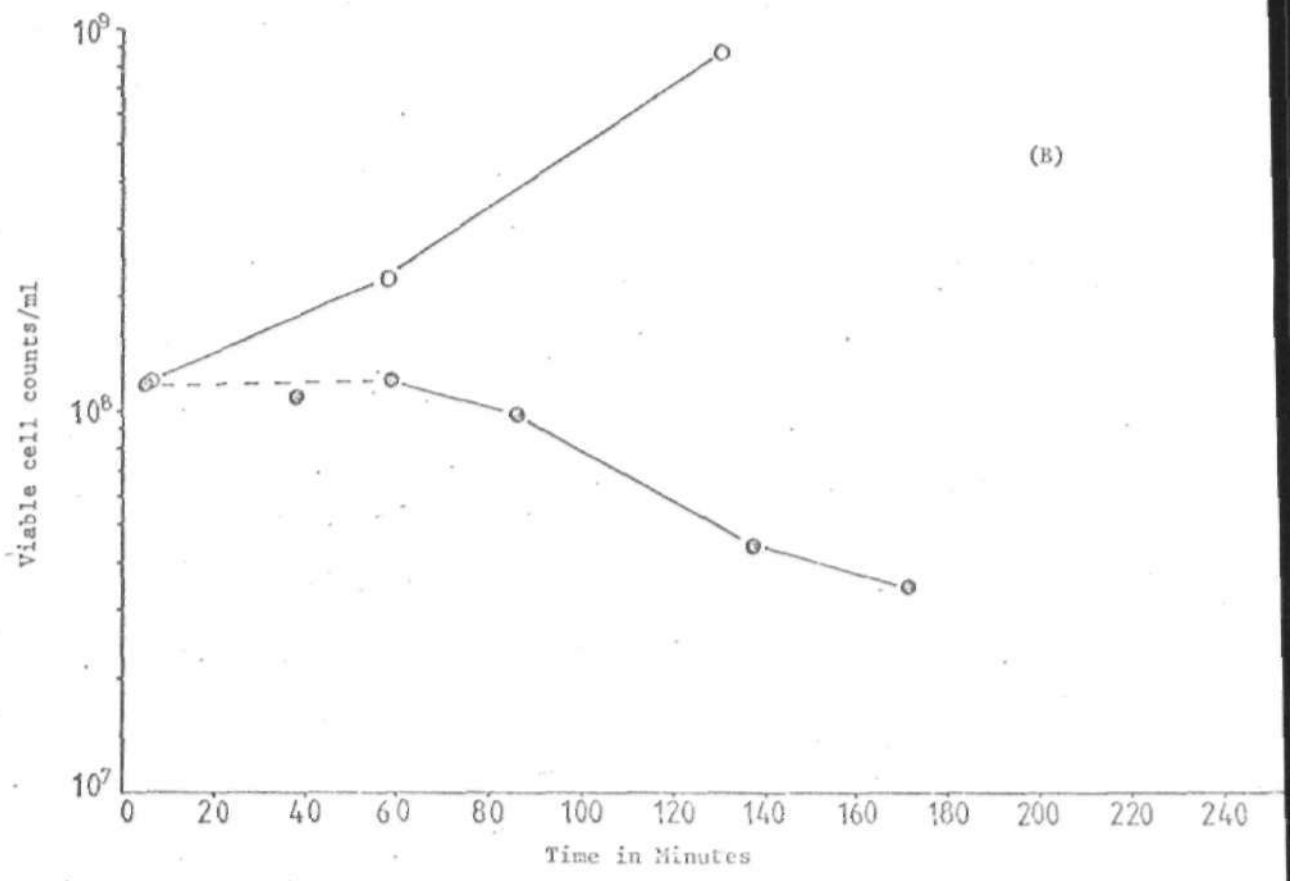
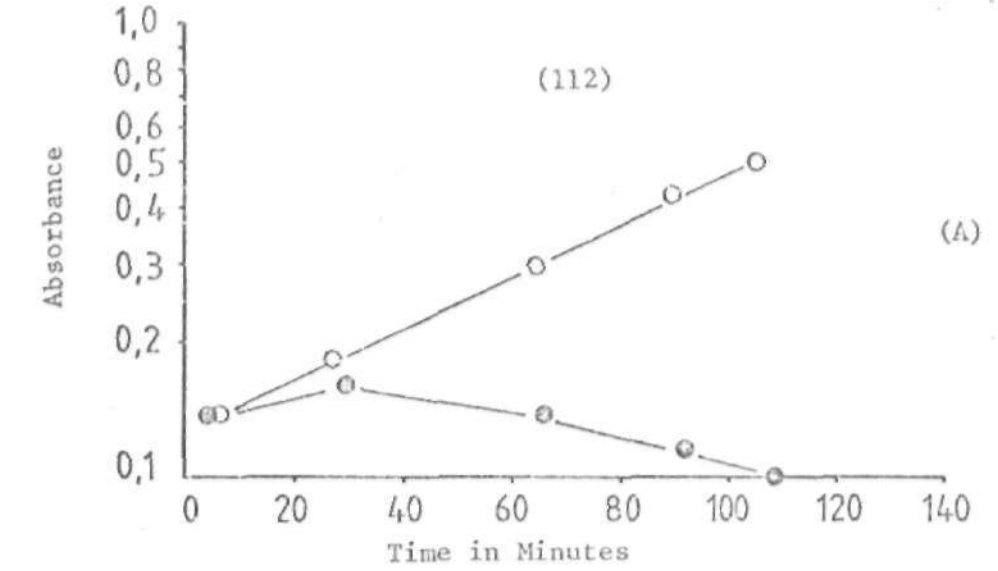


Figure 6.7. Effect of chloroquine on the changes with time of absorbance (A) and viable cell counts per ml (B) of *E. coli* cultures treated with 0.0 (O——) and 2.6×10^{-3}M (●——) chloroquine at pH 6.7.



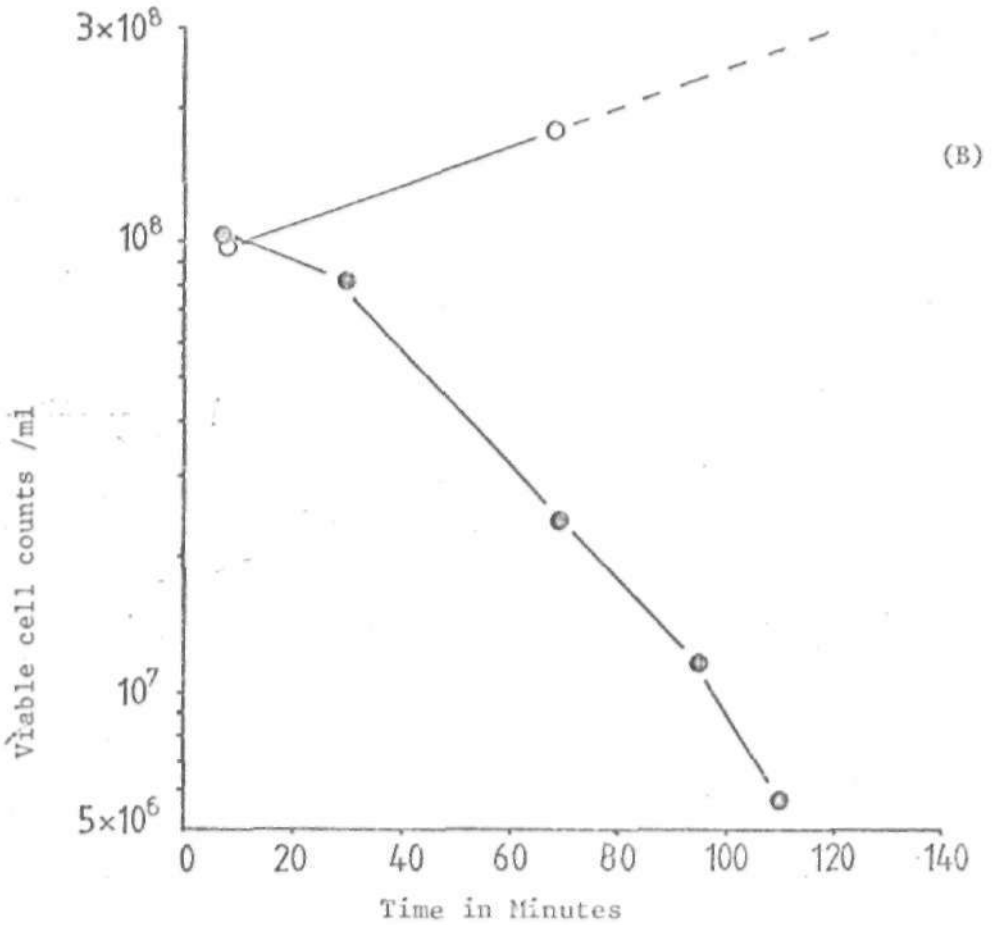


Figure 6.3. Effect of chloroquine on the changes with time of absorbance (A) and viable cell counts per ml (B) of E. coli cultures treated with 0.0 (O——) and 3.0×10^{-3} M (●——) chloroquine at pH 6.7.

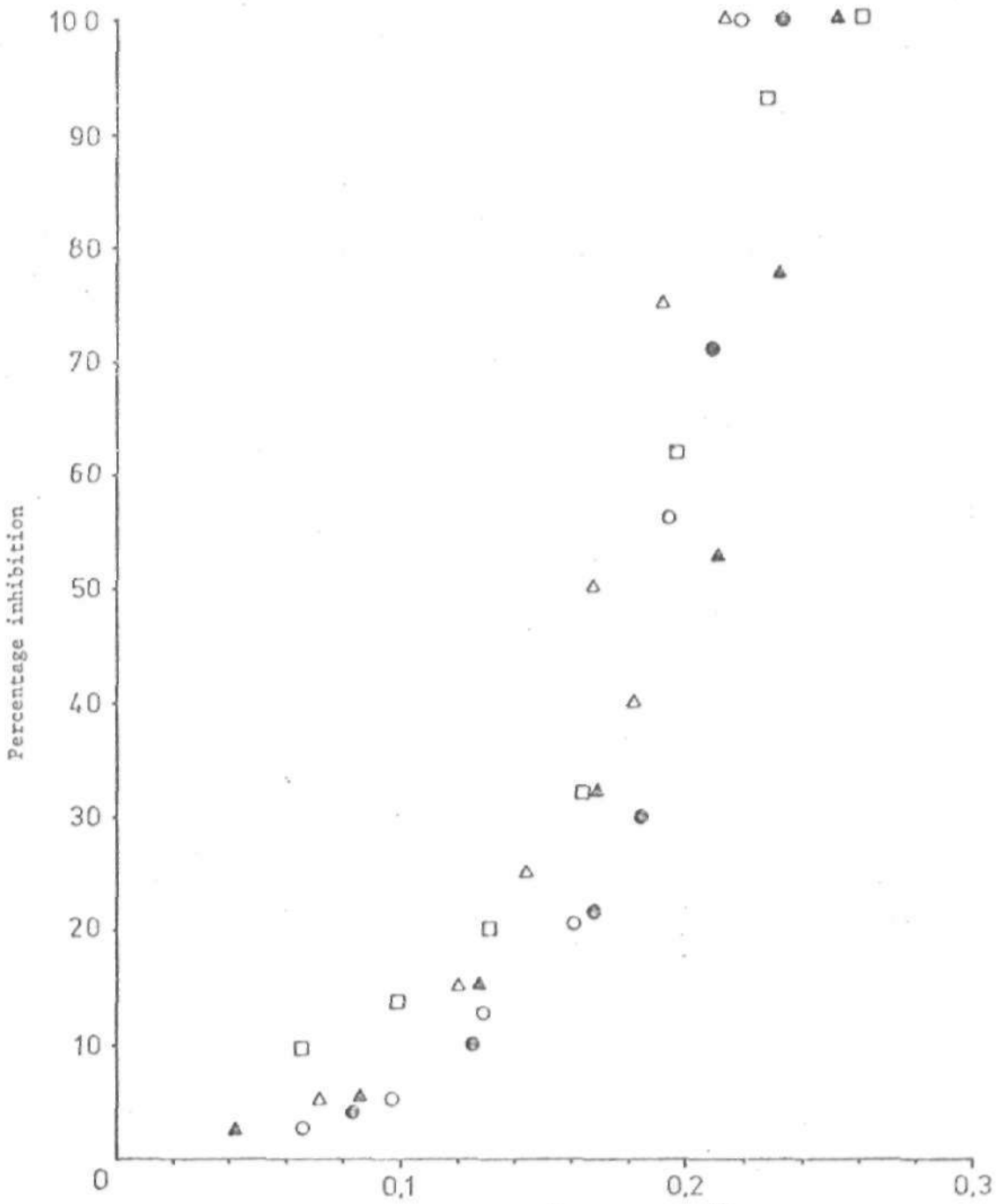


Figure 6.9. The relationship between the "active" concentrations of chloroquine and percentage growth inhibition of E. coli at pH values of 6.4 (○), 6.7 (●), 7.0 (△), 7.4 (▲) and 7.7 (□).

DISCUSSION

pH is one of the environmental factors which affect the activity of antimicrobial agents, especially those that are acidic or basic, where the degree of ionisation influences the potency of the antimicrobial agent (Russell, 1974).

Chloroquine is known to exist in three forms in solution: the unionised base, the monoprotonated and the diprotonated ions, the proportion of each form present being dependent on the pH of the solution (Irvin and Irvin, 1947). When bacterial cells are added to such solutions there is a possibility of differences between the pH of the solution (pHe) and that of the internal part of the cell (pHi). Attempts to study the effect of pH on an antimicrobial agent in such a situation could yield misleading results, as the observable effects are ultimately dependent on the internal pH. However, from the reports of Kashket and Wong (1969), the pHi of the cells used here could be expected to be about the same as the pHe of the solution within the pH range used in these experiments.

The effects of pH on the growth inhibiting action of chloroquine against E. coli have been reported by Wiseman (1972a), and in more detail by Middleton (1976). Wiseman used pH values between 6.3 and 7.7 and reported a difference of about twenty-fold in the activity of chloroquine over this range. Middleton used pH values ranging from 7.0 to 8.0, and the main points of his findings are tabulated in Table 6.1. At pH 7.0 he observed only a small increase in growth inhibition as the concentration of chloroquine was increased from 0.2×10^{-3}M to 1.0×10^{-3}M, which caused 6.5 and 17.5% inhibition respectively. At

pH.7.0		pH.7.4		pH 7.7		pH.8.0	
Conc. in $\times 10^{-3}$M	% Inhibition	Conc. in $\times 10^{-3}$M	% Inhibition	Conc. in $\times 10^{-3}$M	% Inhibition	Conc. in $\times 10^{-3}$M	% Inhibition
0.2	6.5	0.05	20.0	-	-	-	-
1.0	17.5	0.40	40.0	0.01	30.0	-	-
1.5	100.0	0.60	100.0	0.40	100.0	0.025	100.0

Table 6.1.

Effect of pH on Activity of Chloroquine (from Middleton, K.R., 1976).

higher concentrations there was however a rapid rise in the degree of inhibition up to 100% at 1.5×10^{-3}M. Similarly at pH 7.4 his growth inhibition concentration curve showed an initial gradual rise, followed above a concentration of 0.4×10^{-3}M by a steeper rise. At pH 7.7 he obtained an initial rapid rise in activity, followed by a slower roughly linear increase in degree of inhibition with increase in chloroquine concentration. In contrast, at pH 8.0 he obtained a "classical" inhibition/concentration curve, with an initially steep slope which decreased continuously as the concentrations increased.

Thus as the pH was altered there was a qualitative change in the shape of the dose response curve in addition to the quantitative shift in concentrations required for activity, and this was taken by Middleton to be indicative of two independent modes of action of chloroquine with different sensitivity to changes in pH. Such a qualitative effect on the shape of curves was not mentioned by Wiseman (1972a), and no such effect was apparent in the results reported in this thesis. This difference between Middleton's results and those reported here can at present only be attributed to the difference in the nature of the buffering agents used.

Despite the apparent differences in the methods and results of Wiseman and Middleton, both concluded that it is the monoprotonated ionic form that is mainly responsible for the growth inhibitory activity of chloroquine against E. coli. But because of the preliminary nature of Wiseman's work, and the change in shape of the dose response curves with changes in pH in Middleton's experiments, neither set of results could conclusively show that it was the monoprotonated form alone that was active.

The percentages of the different forms of chloroquine in solution at different pH values as calculated from the Laws of Mass Action and Henderson-Hasselbach equation are shown in Table 6.2.

Medium pH	Percentage of QH^{++}	Percentage of QH^{+}	Percentage of Q
6.4	98.0054	1.9942	0.000357
6.7	95.9447	3.9900	0.001386
7.0	92.3162	7.6785	0.005316
7.4	82.6929	17.2770	0.03007
7.7	70.5059	29.3900	0.10227
8.0	54.4212	45.2659	0.3132

Table 6.2.

Percentages of the different ionic species of chloroquine at various medium pH values.

Q = unionised, QH^{+} and QH^{++} = mono- and diprotonated ions respectively.

Using the information from this Table, the inhibition/concentration curve of Figure 6.5 could be redrawn using monoprotonated ion concentrations. If the monocation alone was responsible for the growth inhibiting activity, the curves for the different pH values would then be coincident. That this would not be the case can be seen simply by calculating the concentrations of monocations in the chloroquine solution required to give 100% inhibition at the different pH values. The results of these calculations are shown in Table 6.3.

pH	6.4	6.7	7.0	7.4	7.7
Conc. of CQ (mMolar) for 100% inhibition	3.4	2.8	1.8	1.2	0.8
Equivalent conc. (mMolar) of monocation	0.07	0.11	0.14	0.21	0.24

Table 6.3. Comparison of growth inhibiting activity between total chloroquine concentration and the concentrations of the monocations at various pH values.

From these results it can be seen that the simplistic approach of assuming that only one of the ionic forms of chloroquine is biologically active is untenable and account must be taken of one or both of the remaining forms.

If the assumption is made that both the mono- and diprotonated forms of chloroquine are active and that any contribution from the free base be ignored because of its relatively low concentrations at the pH values studied, then the relative activities of the two ionic forms can be calculated.

If the activity of the monoprotonated form $[QH^{+}]$ is given the value 1, and the activity of the diprotonated form $[QH^{++}]$ the value a, then the total activity in any solution will be $[QH^{+}] + [QH^{++}] \times a$. Comparison of equiactive total chloroquine concentration at different pH values will then allow computation of the value of a, e.g. $[QH^{+}] + [QH^{++}]a$ at pH 6.4 = $[QH^{+}] + [QH^{++}]a$ at pH 6.7. Such calculations were made using pairs of equiactive concentrations at different pH values for 100%, 50% and 20% inhibition levels. The values obtained

ranged between 0.00 and 0.1 with a mean value of about 0.04. Using this mean value, the results shown in Figure 6.5 were redrawn using a concentration scale of $[QH^+] + 0.04\ [QH^{++}]$ and are shown in Figure 6.9.

From this Figure, it can be seen that the results for all the pH values studied are consistent with the growth inhibiting activity of chloroquine being the result of a combined activity of the monoprotonated and diprotonated forms of the drug, the monoprotonated form being apparently about 25 times as effective as the diprotonated form.

Further calculations could have been made to assign a relative activity to the small amounts of the free base form of chloroquine present in solution, but the overall results were not considered accurate enough to justify such a course of action.

The value of the results depicted in Figure 6.6 lies in the fact that the same drug concentration was used against the same population of cells at the same time under the same experimental conditions, except for pH. Hence any differences could only be attributed to differences in pH.

As the threshold bactericidal concentrations of chloroquine were virtually coincident with the concentrations causing 100% inhibition of growth at the different pH values, it can be concluded that bactericidal action also results from a combination of the effects of the monoprotonated and diprotonated forms, with the monocation being the far more active form.

CHAPTER SEVEN

UPTAKE OF CHLOROQUINE BY E.COLI

CHAPTER SEVEN

UPTAKE OF CHLOROQUINE BY ESCHERICHIA COLI

	Page No.
Experimental	122
Radioactive chloroquine	122
Uptake of chloroquine in the presence of glucose	122
Uptake of chloroquine in the absence of glucose	122
Radioactive measurements	123
Results	124
Figures	127
Discussion	134

EXPERIMENTAL

Radioactive Chloroquine

Ring 3-^{14}C chloroquine was obtained from NEN Chemicals GmbH, Dreieich, West Germany, and was mixed with sufficient solution of non-radioactive chloroquine to give a specific activity of 100mCi Mole^{-1}

Uptake of Chloroquine in the Presence of Glucose.

An overnight culture of E. coli grown from a low inoculum was subcultured into fresh medium, and incubated with shaking until well established in the exponential phase of growth. The culture was then filtered through a 0.45µm mean pore size membrane filter, and the retained cells washed with and resuspended at 37°C in fresh medium containing twice the normal concentration of glucose. The absorbance of the resulting suspension was adjusted to 0.15 with more medium, and incubation with shaking resumed.

When the absorbance of the culture had reached about 0.5, 10ml portions were added to flasks containing 10ml volumes of either glucose free medium alone, or of a solution in glucose free medium of ^{14}C labelled chloroquine at twice the finally required concentrations. Incubation with shaking was continued, and growth of the cells followed by taking absorbance measurements at intervals up to 4 hours. 1.0ml samples were taken from the chloroquine treated cultures at 10 to 20 minute intervals for radioactive measurements as described below.

Uptake of Chloroquine in the Absence of Glucose.

Cells of E.coli from exponentially growing cultures with an absorbance of between 0.4 and 0.6 were harvested by membrane filtration,

washed and resuspended in glucose free medium at 37°C, and the absorbance adjusted to 0.500. After incubation with shaking for about 60 minutes, 10.0ml volumes of this suspension were added to flasks containing 10ml of glucose free medium or of solutions in glucose free medium of chloroquine at twice the finally required concentration, and incubation with shaking continued. Absorbance measurements were taken and 1.0ml samples removed for radioactivity measurements at about 10 minute intervals. After about 90 minutes glucose was added to the suspensions to give a final concentration of 2mg/ml and measurements continued for up to a further 2 hours.

Radioactive Measurements.

Samples were filtered through 30mm diameter 0.45μm pore size membrane filters with gentle suction, and the retained cells rapidly washed (<10 seconds) with about 20ml of distilled water. The membrane filters holding the ^{14}C labelled cells were attached to flat aluminium planchets with 'Durofix' adhesive, and dried under an infra-red lamp.

The radioactivity of the labelled cells was measured using a Beckman Lowbetta II Automatic Geiger Planchet Counter, fitted with an ultra-thin ($80\mu g/cm^2$) window. The prepared planchets were counted for periods of time which allowed a count of at least 2,000 to be attained.

0.1ml volumes of the chloroquine treated cultures, cells and medium combined, were dried on to planchets and also counted to enable the conversion of radioactivity measurements into quantities of chloroquine.

All counts were corrected for background.

RESULTS

Figure 7.1(a) illustrates the changes in absorbance with time of cultures of E. coli treated with 0.0 and 0.5 x 10^{-3}M ^{14}C labelled chloroquine. In both cases, the cells were growing, the chloroquine treated ones at the slower rate with a mean generation time of 68 minutes compared to 59 minutes for the control. Uptake of ^{14}C chloroquine by these cells is shown in Figure 7.1(b). There was an initial rapid uptake followed by a slower uptake which continued throughout the period of the experiment at a rate apparently proportional to the rate of growth, so that the uptake per unit weight of cells remained constant at about 15μMoles Gm^{-1}. The amount of chloroquine taken up by the cells was small compared to the amount in the medium, the uptake at the end of the experiment corresponding to only 1% of the available drug.

In cultures treated with 1.0 x 10^{-3}M chloroquine (Figure 7.2), the absorbance rose slowly for about 50 minutes, remained almost constant for the next 20 minutes and then gradually decreased. The uptake again showed an initial rapid phase, followed by a slow increase with time, which was not related to the increase in absorbance, up to about 100 minutes, after which the uptake first ceased and then started to decrease. The maximum uptake was about 55μMoles Gm^{-1}, corresponding to approximately 1% of the total present.

For the cell suspensions treated with 4.0 x 10^{-3}M chloroquine (Figure 7.3), the absorbance was fairly constant for about 40 minutes after which it started decreasing gradually, and continued to do so throughout the experiment. The drug uptake was again rapid initially,

followed by a slowing of the rate until about 40 minutes. Uptake levelled off at 120μMoles Gm^{-1}, corresponding to about 0.45% of the total present.

The uptakes with time, and the relationship between uptake and absorbance of the cells treated with the three chloroquine concentrations used are compared in Figures 7.4 and 7.5.

The uptake of chloroquine with time by cells of E. coli treated with 1.0 and 4.0 x 10^{-3}M concentrations of the drug in the absence of glucose and after its subsequent addition are shown in Figures 7.6 and 7.7, together with changes in absorbance of the suspensions. The absorbance remained virtually constant in the control and treated suspensions throughout the period of glucose starvation. After the addition of glucose the absorbance of the controls increased as the cells began to grow; the absorbance of the suspension treated with 1.0 x 10^{-3}M chloroquine increased only slightly, and that of the suspension treated with 4.0 x 10^{-3}M began to decrease slowly.

In the suspension treated with 1.0 x 10^{-3}M chloroquine, uptake of the drug was initially at least as rapid as it had been in the presence of glucose (cf. Figure 7.2); in the absence of glucose, however, uptake was complete within 20 minutes, levelling off at a value of about 35μMoles Gm^{-1}. After the addition of glucose to this suspension there was an apparent rapid loss of some chloroquine from the cells, followed by a further uptake at a rate similar to the slow secondary uptake by cells treated with this concentration of chloroquine in the presence of glucose from the beginning.

In the suspensions treated with 4.0×10^{-3}M chloroquine there was again a rapid uptake which was complete within 10 minutes, after which there was no evidence of further uptake, even after the addition of glucose. Uptake in this experiment corresponded to about 70µMoles Gm^{-1}, compared with the 120µMoles Gm^{-1} observed when glucose was present from the beginning (cf. Figure 7.3).

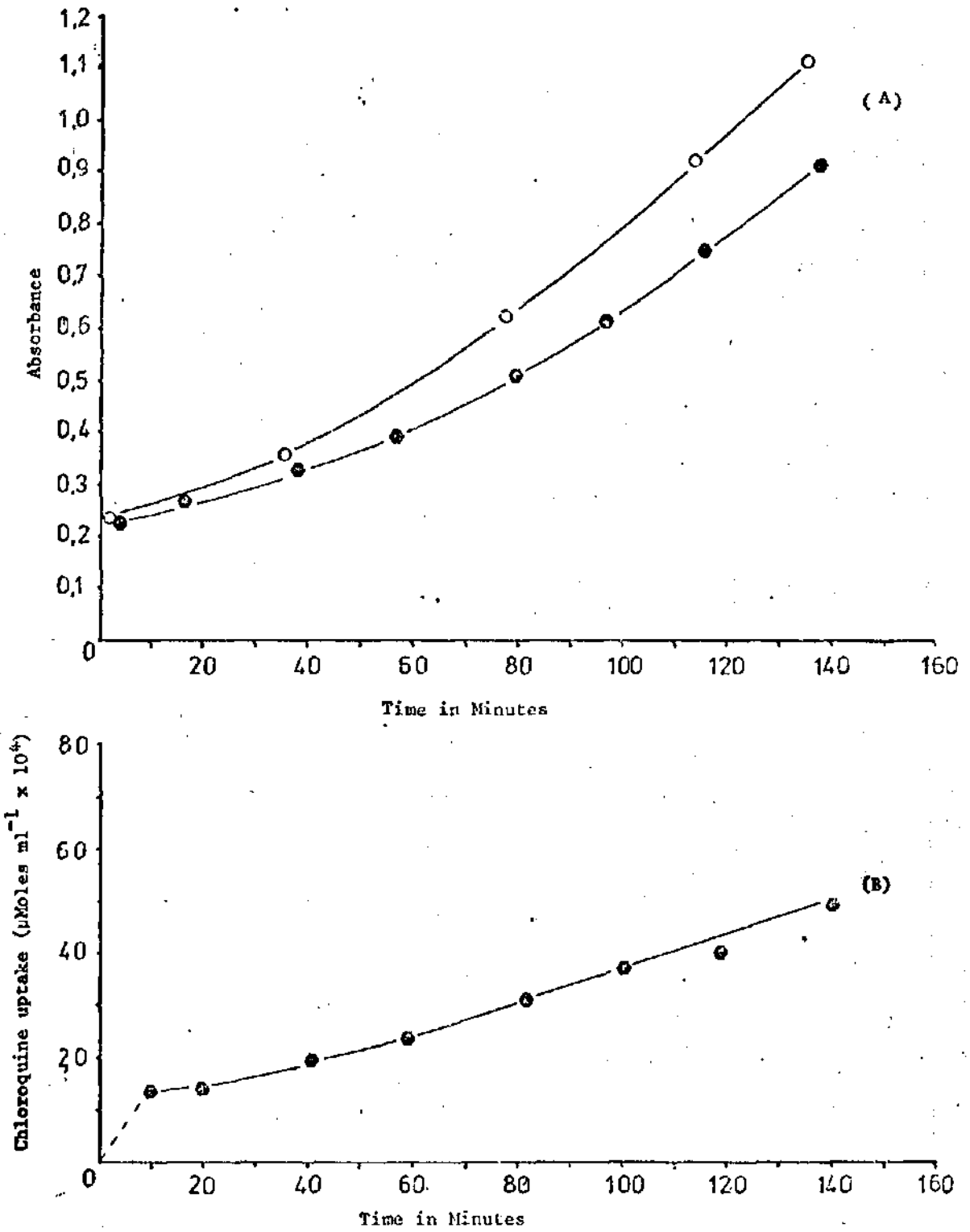


Figure 7.1. Uptake of chloroquine (B) by E. coli cells treated with 0.5×10^{-3}M chloroquine, in the presence of glucose. The absorbance curves of the control (O——), and the chloroquine treated (✪——) cells are shown in (A).

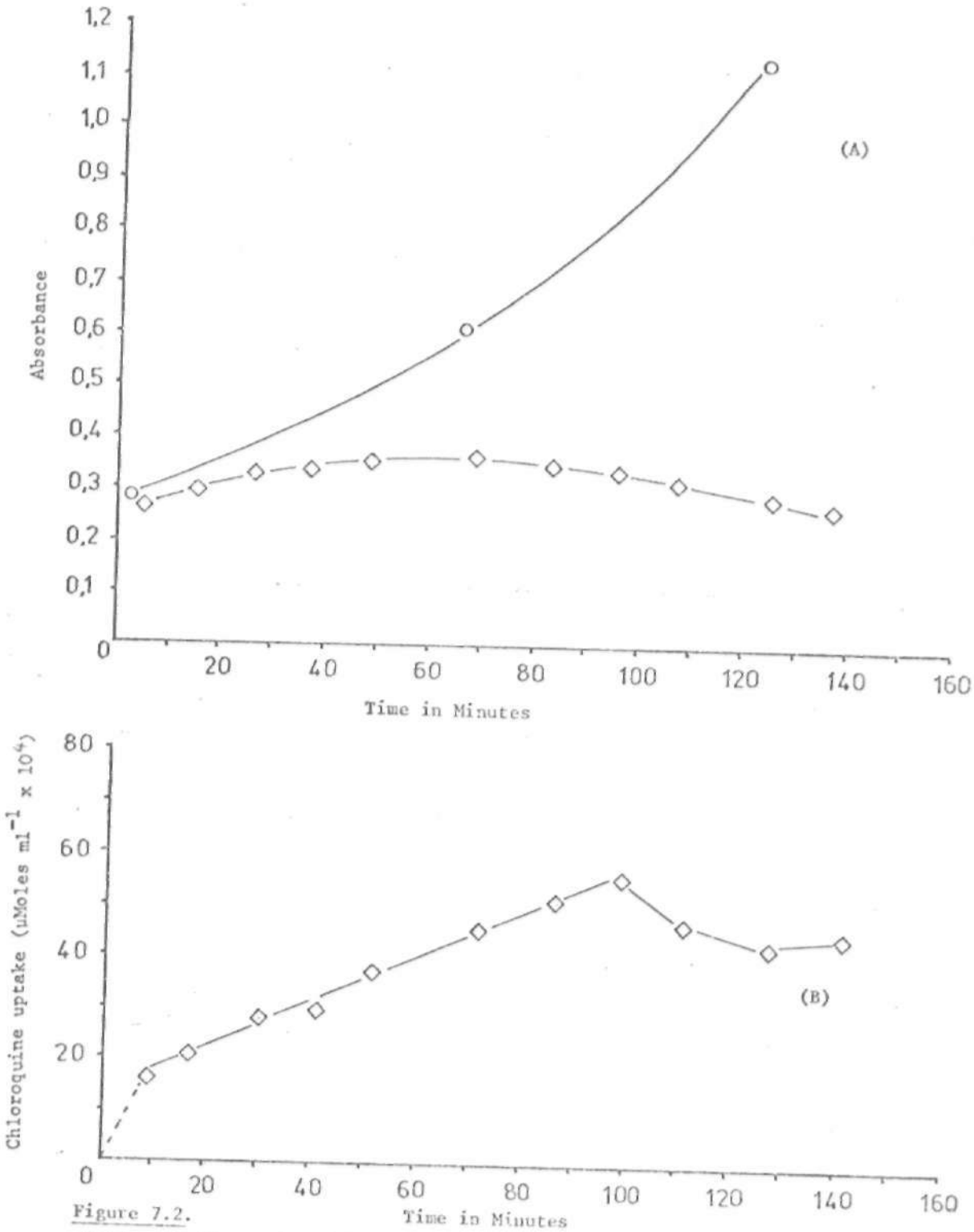


Figure 7.2.

Uptake of chloroquine (B) by E. coli cells treated with 1.0×10^{-3} M chloroquine, in the presence of glucose. The absorbance curves for the control (O——), and the chloroquine treated (◇——) cells are shown in (A).

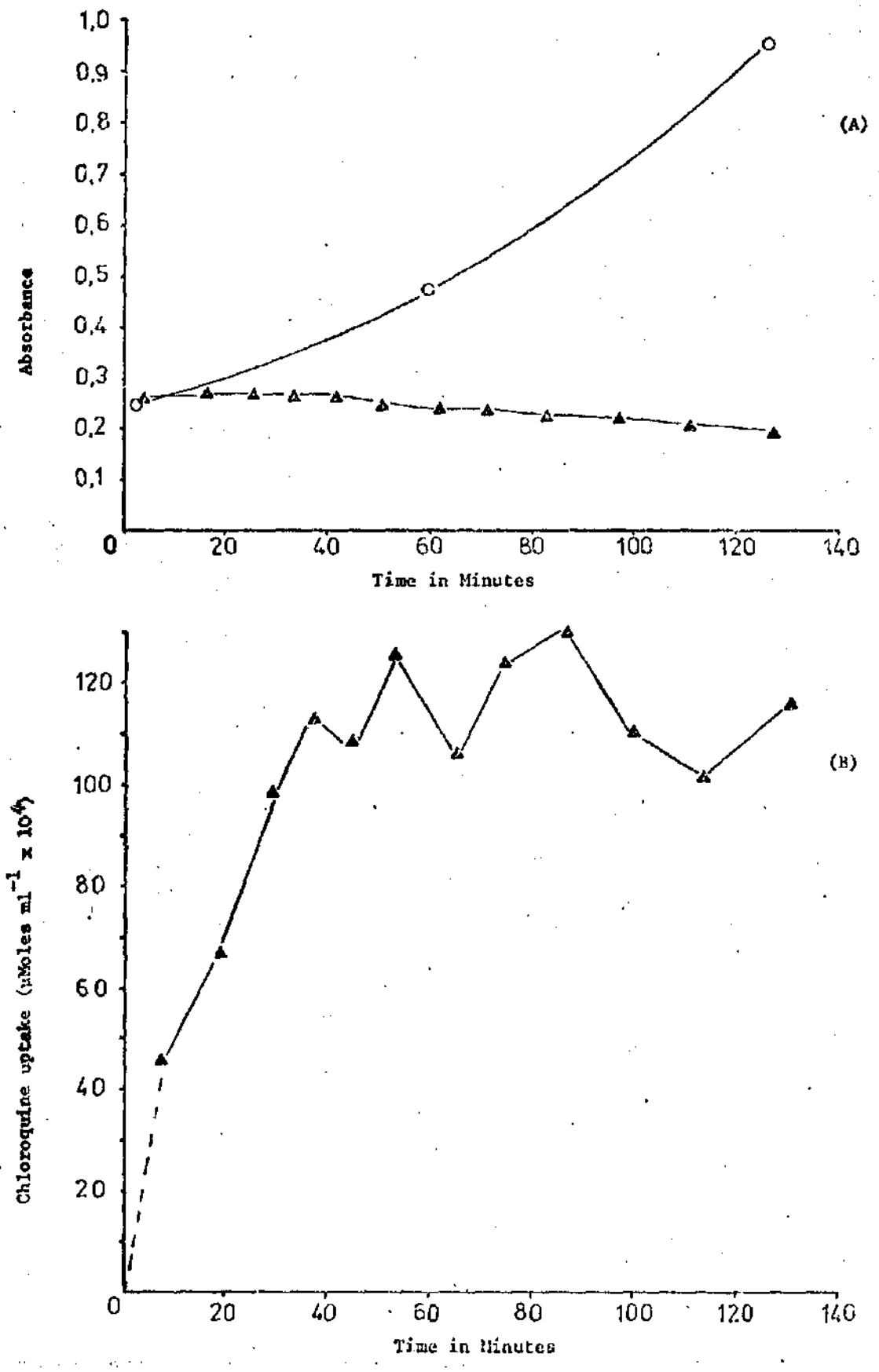


Figure 7.3. Uptake of chloroquine (B) by E. coli cells treated with 4.0 x 10^{-3}M chloroquine, in the presence of glucose. The absorbance curves for the control (O—), and the chloroquine treated (▲——) cells are shown in (A).

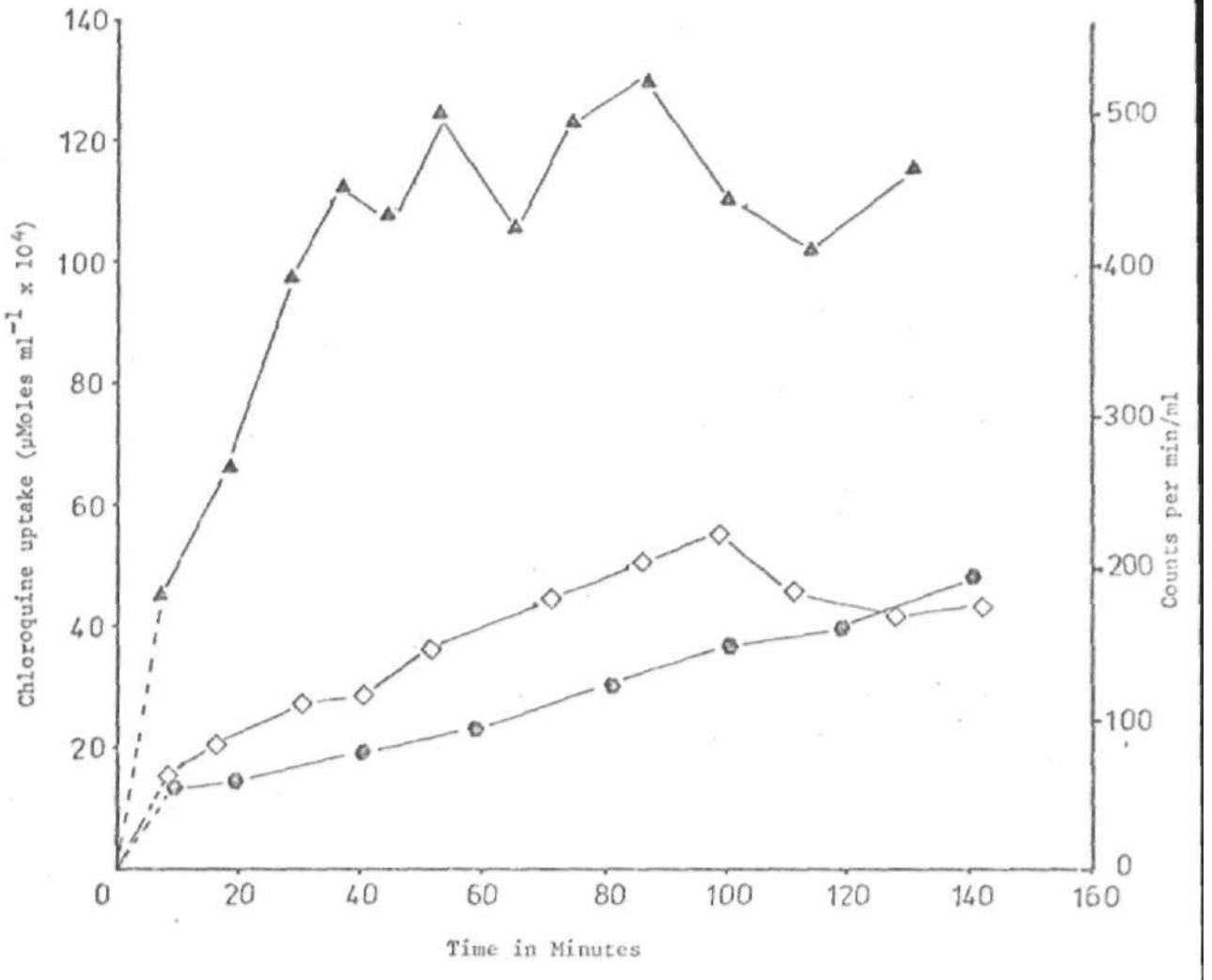


Figure 7.4. Comparison of uptake of chloroquine by E. coli cells treated with 0.5 (●—), 1.0 (◇——), and 4.0 x 10^{-3}M (▲——) chloroquine, in the presence of glucose.

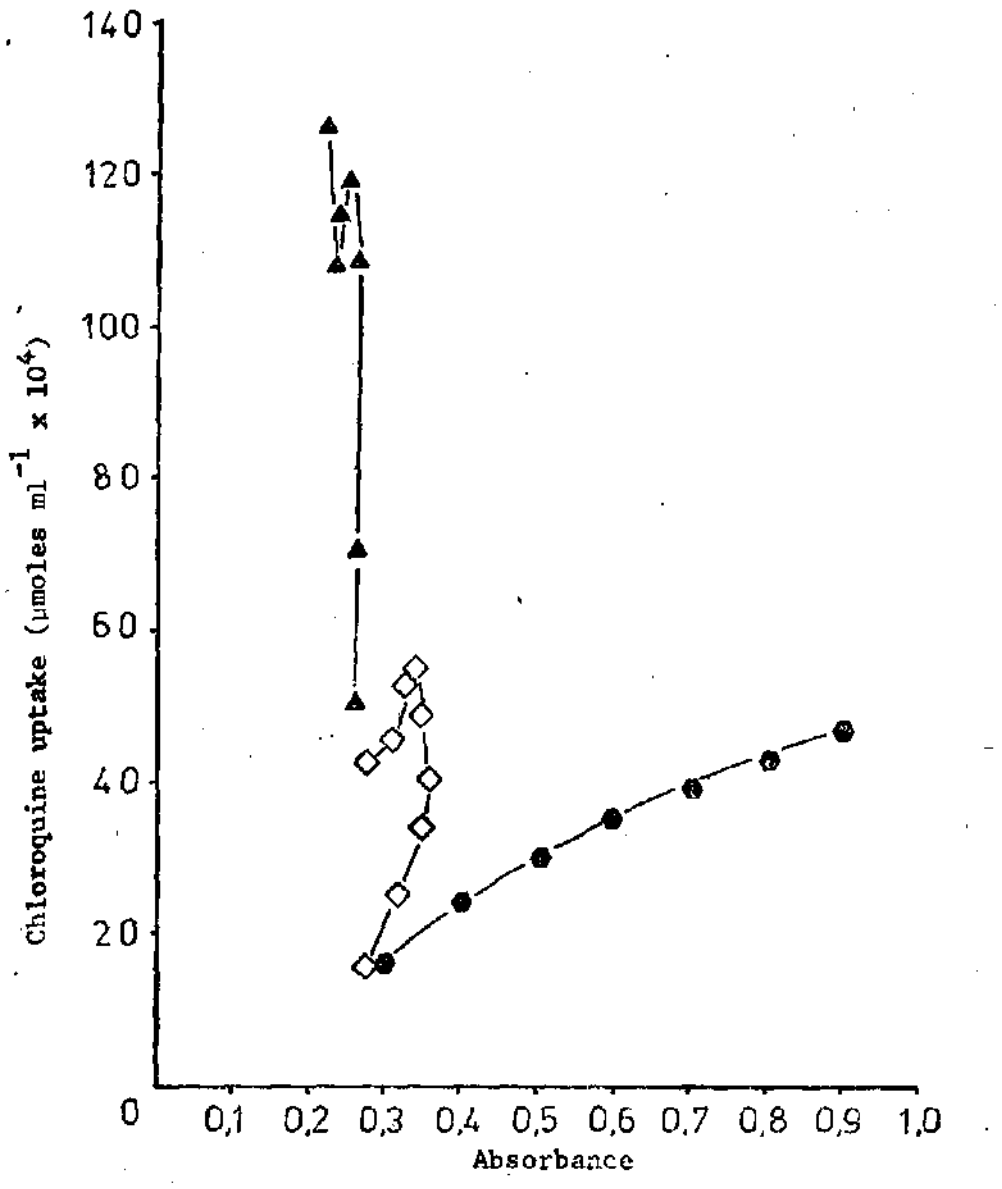


Figure 7.5. The relationship between the absorbance of, and the uptake of chloroquine by cells of E. coli treated with 0.5 (●——), 1.0 (◇——) and 4.0 x 10^{-3}M (▲——) chloroquine, in the presence of glucose.

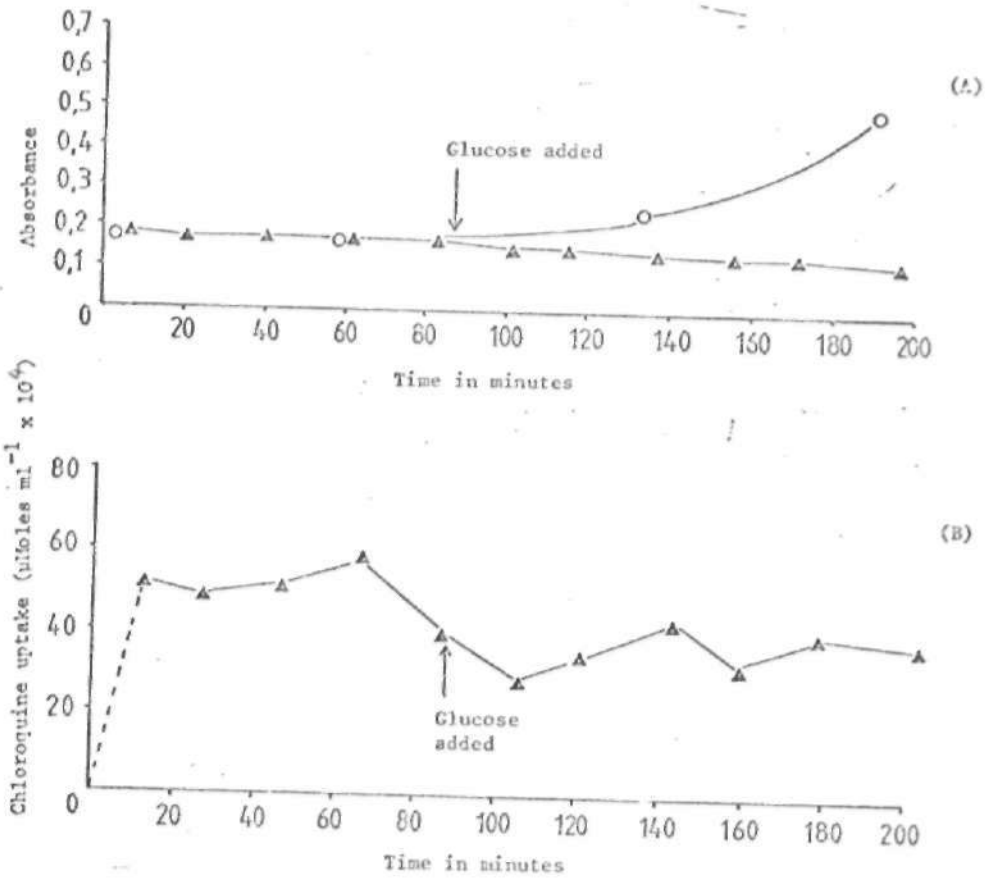


Figure 7.7. Uptake of chloroquine (B) by E. coli cells treated with 4.0×10^{-3}M chloroquine, in the absence of glucose. The absorbance curves of the control (O——), and the chloroquine treated (Δ——) cells are shown in (A).

DISCUSSION

The results of previous studies of the uptake of chloroquine by cells of *E. coli* have been reported by Wiseman (1972b) and by Middleton (1976). Both of these workers determined uptake indirectly by measuring the residual concentration of the drug in the supernatant fluids from centrifuged chloroquine treated suspensions, and calculating uptake by difference. Because of the relatively low affinity of chloroquine for *E. coli*, and the basic inaccuracy of the difference method, this technique required the use of very thick cell suspensions many times more concentrated than those used in the study of antibacterial activity. In addition to this use of 'thick' suspensions both these workers also chose to study uptake only in the absence of glucose.

The direct method of measuring the uptake of chloroquine used in the work described in this thesis was inherently more sensitive than the method used by Wiseman and Middleton, and allowed the use of cell concentrations similar to those used in the study of bactericidal action. With this direct method, however, the membrane filters and the collected cells had to be washed free from chloroquine solution trapped between the cells or within the membrane, and this washing, even though rapid, might result in the loss of chloroquine loosely bound to the cells.

Despite the differences in methodology, it is interesting and perhaps surprising to note that the highest uptakes per gram of cells treated with the same concentration of chloroquine in glucose free conditions observed in the work described in this thesis are

similar to the maximum uptake levels reported by Middleton. For example, the maximum uptake level for unwashed cells of E. coli treated with 1.0×10^{-3}M chloroquine in a Tris buffered medium as reported by Middleton was about 70μMole Gm^{-1} of cells, while that obtained by the present method for washed and dried cells of E. coli treated with the same drug concentration in a MOPS buffered medium was about 40μMoles Gm^{-1}.

In the presence of glucose the study of uptake from partially inhibiting concentrations of chloroquine, such as 0.5×10^{-3}M, is complicated by growth of the cells and it seems likely that in such cultures the overall pattern results from an equilibrium set up between the rate of uptake and the rate of growth.

With higher concentrations of chloroquine, the complicating effect of substantial changes in cell concentrations is absent, and from these results it is obvious that uptake in the presence of glucose is ultimately higher than in its absence, suggesting perhaps some active transport mechanism for uptake.

In the absence of glucose, however, although the final uptake level is lower, since no drug uptake was obtained after the initial 20 minutes or so, it does appear that the initial uptake level is relatively higher.

From the results obtained using 4×10^{-3}M chloroquine, the level of uptake required for ultimate bactericidal effect can be attained in the absence of glucose, even though the manifestation of this effect is not seen until after the addition of glucose.

It seems likely that the uptake of chloroquine by E. coli cells is of two types, one, an initial rapid uptake by a passive mechanism, and the other, a slower uptake by an active process, and that the active uptake can only take place in the presence of a carbon and energy source, such as glucose. Both the passive and active uptake mechanisms may contribute their quota to attain bactericidal levels of the drug under different conditions, but it seems that in high concentrations of chloroquine passive uptake alone allows the uptake of potentially bactericidal amounts of the drug.

Polet and Barr (1969), working with erythrocytes parasitised by chloroquine sensitive Plasmodium knowlesi, demonstrated that ^{3}H-labelled chloroquine uptake was characterised by two phases, an initial phase of rapid uptake lasting 5 to 15 minutes, during which the red cells concentrated an amount of 30 to 50% of the total amount taken up, followed by a slower uptake reaching a saturation level after 3 hours. They reported that increase in extracellular drug concentration gave increased drug uptake, which was directly proportional to the extracellular concentrations at medium levels ranging from 10^{-8} to 2.0 x 10^{-7}M; but that at higher molar concentrations, the parasites concentrated a smaller percentage. In the absence of glucose, uptake was reduced to about 50%. The active uptake was reported to be energy-dependent, and the chloroquine concentrating mechanism independent of the growth inhibitory effect of the drug.

The effects of substrates like glucose, glycerol, fructose and pyruvate on the accumulation of chloroquine and amodiaquine by Plasmodium berghei (CQ-sensitive) have also been demonstrated by Fitch

et al., (1974, 1975). They reported that the above-mentioned substrates stimulated the accumulation of the drug in the parasite drug receptors, and proposed that such substrates were involved in the accumulation process.

These reports, especially that of Poiet and Barr, may thus offer some explanations for two of the observations made from the work reported in this Chapter. (1) The relatively high initial uptake level by chloroquine treated cells in the absence of glucose may be due to the fact that the initial passive uptake was allowed to go on until completed, whereas in the presence of glucose, the initial passive uptake may be prematurely terminated by the slow active uptake. (2) The 'surprise' continuation of active uptake by cells treated with bactericidal concentrations of chloroquine in the presence of glucose, when their total cell mass was decreasing, may be due to the independence of the uptake mechanism on the growth inhibitory effect of the drug.

The results of the work described here have demonstrated the biphasic nature of chloroquine uptake by E. coli cells, and the reduced uptake, which is mainly passive, in the absence of glucose, and show that what applies in plasmodia can obtain in bacterial cells.

It may be valuable to develop the direct method employed here with bacterial cells, and use it for further uptake studies. Uptake can be determined at different pH values, and the results compared with those of plasmodia reported by Fitch et al. (1974). The proportions of the different ionic species taken up, and possibly their destinations in the cells, could also be determined.

CHAPTER EIGHT

EFFECT OF CHLOROQUINE ON THE UTILISATION OF THYMIDINE

CHAPTER EIGHT

EFFECT OF CHLOROQUINE ON THE UTILISATION OF THYMIDINE

	Page No.
Experimental	141
Radioactive growth medium	141
Uptake and incorporation of thymidine	141
Methods A to F	141
Results	146
Figures	152
Discussion	160

Thymine is one of the basic components of deoxyribonucleic acid (DNA), and if a compound interferes with the uptake of this pyrimidine into a tissue cell or micro-organism, or prevents its incorporation into cell DNA, synthesis of the latter is inhibited. The results of early viability experiments suggested some similarities between the death curves obtained with chloroquine treated cells and those characteristic of "Thymineless Death", and the work described in this Chapter was undertaken to investigate the effects of chloroquine on the uptake and utilisation of thymine by E. coli.

Strains of E. coli not requiring thymine as a growth factor do not take up exogenous thymine (Crawford, 1958); they do, however, utilise its nucleoside, thymidine, to some extent (Rachmeler et al., (1961), and take it up quite extensively in the presence of deoxyadenosine (Boyce and Setlow, 1962). In the following experiments, deoxyadenosine was added at the same time as thymidine to obtain good uptake.

Unlabelled thymidine and deoxyadenosine were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A. 2-^{14}C -thymidine was obtained from the Radiochemical Centre Ltd., Amersham. Trichloroacetic acid was from the British Drug Houses Ltd., Poole, Dorset, and Ampicillin Sodium (Penbritin) was obtained from Beecham Research Laboratories, Brentford, England.

EXPERIMENTAL

Radioactive Growth Medium

200µg/ml of deoxyadenosine was mixed with 5µg/ml of unlabelled thymidine and a sufficient quantity of [2-^{14}C]-thymidine (50µCi/ml) specific activity of 56 Ci/M to give an activity of 0.02µCi/ml in the final solution (Middleton, 1976). In practice, this worked out as 5.0ml of a 4mg/ml deoxyadenosine solution mixed with 1.0ml of a 0.5 mg/ml solution of unlabelled thymidine and 0.04ml of a 50µCi/ml solution of [2-^{14}C]-thymidine, made up to 100ml with growth medium. In some of the experiments, the proportion of ^{14}C-labelled thymidine was reduced as the stock solution dwindled. This was not considered a serious drawback since the underlying objective was to observe differences in the uptake patterns between the control and chloroquine treated cells, and with the smaller proportion of [2-^{14}C]-thymidine, acceptable radioactive counts were still obtainable.

Uptake and Incorporation of Thymidine.

METHOD A. Addition of Chloroquine to Growing Cultures at the Same Time as [2-^{14}C]-thymidine.

An overnight culture of E. coli was filtered through a 0.45µm mean pore size membrane filter, and the retained cells washed with and resuspended at 37^{o}C in fresh medium containing 5µg/ml of unlabelled thymidine, 200µg/ml of deoxyadenosine and twice the normally required concentration of glucose. The absorbance of the resulting suspension was adjusted to 0.10 with more medium, and incubation with shaking resumed.

Meanwhile, 24.5ml of growth medium containing 5μg/ml unlabelled thymidine, 200μg/ml deoxyadenosine and sufficient [2-^{14}C]-thymidine to give 0.04μCi/ml was added to each of two 250ml conical flasks. 0.5ml of a 0.4M chloroquine solution was added to one flask, and 0.5 ml of glucose-free medium to the other. The two flasks were placed in the shaking water bath to attain bath temperature.

When the absorbance of the growing culture had reached 0.3, 25ml of it was measured into each of the above flasks, and incubation with shaking continued. Growth was followed by taking absorbance measurements at intervals for up to 3 hours.

At 10 to 20 minute intervals 1.0ml samples of both the control and treated cultures were filtered through separate 30mm 0.45μm mean pore size membrane filters and the collected cells rapidly (<10 seconds) washed with 20ml distilled water. The membranes together with their retained cells were then mounted on flat aluminium discs, using Durofix adhesive, dried under an infra-red lamp, and the radioactivity in the samples measured in a Beckman Lowbeta II automatic geiger planchet counter fitted with an ultra-thin (80μg/cm^2) window. Filtration of cell free solutions of radioactive thymidine in glucose free medium showed that no significant amount of thymidine was adsorbed on to the membrane material.

1.0ml samples were also taken at intervals from each of the cultures, pipetted into 1.0ml volumes of ice-cold 10% Trichloroacetic acid (TCA), and stored at 4^{o}C for 30 minutes. The cells and any TCA-insoluble precipitates formed were then collected on prewetted membrane filters, washed with 5ml ice-cold TCA, and 10ml distilled water, and the membranes prepared for radioactive counting as above.

In order to enable radioactive counts to be converted into amounts of thymidine 0.1ml samples of cultures, both cells and medium, were dried on to planchets and the radioactivity counted.

METHOD B. Addition of Chloroquine to Growing Cultures about 60 minutes after the Addition of $[2\text{-}^{14}C]$-thymidine.

The method used was the same as Method A, except that the addition of chloroquine was delayed until about 60 minutes after commencing incubation of the cells in the presence of ^{14}C-labelled thymidine.

METHOD C. Addition of Chloroquine and ^{14}C labelled Thymidine at the Same Time to Glucose Free Cell Suspensions, Followed after 90 Minutes by the Addition of Glucose.

The procedure was as described in Method A, except that the growing culture was replaced by a suspension of cells harvested from an exponentially growing culture by membrane filtration, washed with and resuspended in glucose free medium to give an absorbance of 0.300, and incubated at 37°C with shaking for 40 minutes. Glucose was added to the suspensions about 90 minutes after commencing incubation in the presence of chloroquine.

METHOD D. Addition of Chloroquine to Growing Cells Previously Labelled with $[2\text{-}^{14}C]$-thymidine.

Medium containing the normal concentrations of glucose, 5µg/ml of unlabelled thymidine, 200µg/ml of deoxyadenosine and 0.02µCi/ml of $[2\text{-}^{14}C]$-thymidine, was incubated with cells from exponentially growing cultures to produce an absorbance of about 0.08, and the

resulting suspension incubated at 37°C in the shaking water bath.

After at least 3 hours the culture was filtered through a 0.45μm mean pore size membrane filter, and the retained cells washed with and resuspended at 37°C in fresh medium containing twice the normally required glucose concentration, 5μg/ml unlabelled thymidine, and 200μg/ml deoxyadenosine. The absorbance of the resulting suspension was adjusted to about 0.15, and incubation with shaking continued. 24.5ml of glucose free medium containing the same concentration of unlabelled thymidine and deoxyadenosine as above was measured into each of two 250ml conical flasks. 0.5ml of a 0.4M chloroquine solution was added to one flask, and 0.5ml of glucose free medium to the other, and the flasks placed in the shaking water bath at 37°C.

When the absorbance of the pre-labelled culture had reached 0.3, 25.0ml of it was measured into each of the above flasks, and measurements of absorbance, and of radioactivity in cells, and in TCA-insoluble fractions were made as in Method A.

METHOD E. Addition of Chloroquine to Glucose Free Suspensions of Cells Previously Labelled with [2-^{14}C]-thymidine Followed After 90 Minutes by Addition of Glucose.

The procedure was as described in D above, except that the cells pre-labelled with ^{14}C thymidine were filtered, resuspended in glucose-free medium and incubated for 40 minutes before their addition to the chloroquine solutions. Glucose was added about 90 minutes after the commencement of incubation in the presence of chloroquine.

METHOD F. Effect of Addition of Ampicillin to Growing Cells Previously Labelled with [2-^{14}C]-thymidine.

The procedure was as described in Method D, except that 200µg/ml of ampicillin sodium was added in place of the chloroquine.

RESULTS

The various methods applied to the study of thymidine utilisation in E. coli cells were undertaken to determine the effects of chloroquine on (1) the uptake of this nucleoside and its incorporation into the macromolecules of the cells, and (2) the fate of thymidine which had been utilised by cells before the addition of chloroquine. Figures 8.1 to 8.5 illustrate the effects of chloroquine on the uptake and incorporation of thymidine into E. coli cells, while Figures 8.6 to 8.8 illustrate the effects of the drug on previously utilised precursor.

Uptake of a macromolecule precursor by a cell does not necessarily mean that it has been incorporated into macromolecules. A method of demonstrating incorporation of such a precursor is treatment of the cells with ice-cold trichloroacetic acid (TCA). This causes the release of low molecular weight materials from the metabolic pool, and precipitates macromolecules. If the precursor is radioactively labelled, then the amount of radioactivity in the washed and dried TCA-insoluble precipitates is proportional to the amount of the precursor incorporated into macromolecules.

It was generally observed that the cells grew at slower exponential rates than normally, when fed with deoxyadenosine and unlabelled thymidine. Growing the cells overnight with these nucleic acid precursors did not fully correct this defect, but there was sufficient difference between control cells and chloroquine treated ones to justify their use in this investigation.

Figure 8.1 illustrates the growth, through changes in absorbance with time, and the uptake and incorporation of ^{14}C-thymidine by cells

of E. coli fed with this DNA precursor and treated with 4.0×10^{-3}M chloroquine. The absorbance curves show that the control cells were growing exponentially with a mean generation time of 80 minutes, while the chloroquine treated cells exhibited a gradual decrease in absorbance throughout the experiment, indicating shrinkage or lysing of the cells. The uptake of ^{14}C-thymidine by the control cells was proportional to the rate of growth until the cell concentration reached an absorbance value of about 0.4 at 600nm (i.e. about 4×10^{8} cells), after which it began to slow down relative to the growth rate. The incorporation of thymidine into TCA insoluble material was almost co-incidental with uptake, indicating that almost all the ^{14}C-thymidine taken up was incorporated into the cells' DNA with very little free thymidine remaining in the metabolic pool.

The results for the cells treated with 4×10^{-3}M chloroquine show that during the first few minutes thymidine is taken up and incorporated into TCA insoluble material by the cells at about the same rate as in the untreated cells. After this initial period of relatively rapid uptake the total amount of radioactive thymidine within the chloroquine treated cells remained constant for about 50 minutes and then began to decrease. The amount of radioactivity in the TCA insoluble material however increased slightly after the initial rapid uptake, and then remained constant until about 90 minutes after the addition of chloroquine before it began to decrease.

These results suggest that uptake of thymidine and its incorporation into high molecular weight material, presumably DNA, can continue for a short time after the addition of bactericidal concentrations of chloroquine to growing cells of E. coli, that the treated cells later

leak both low and high molecular weight materials back into the growth medium, and that finally the high molecular weight materials into which thymidine has been incorporated are degraded back to low molecular weight non-TCA precipitable compounds.

Single experiments were carried out using 1.0 and 2.0 x 10^{-3}M chloroquine. The results (not illustrated) showed that the control cells behaved similarly to those described above; cells treated with 1.0 x 10^{-3}M chloroquine took up about twice as much thymidine as those treated with 4.0 x 10^{-3}M, but maintained a similar uptake pattern. Cells treated with 2.0 x 10^{-3}M behaved as those treated with 4.0 x 10^{-3}M.

The relationship between absorbance and uptake of ^{14}C-thymidine is illustrated in Figure 8.2. With the control cells there was a clear linear relationship between absorbance and uptake of labelled thymidine up to an absorbance value of about 0.38, showing that the rate of uptake was proportional to cell concentration during this "linear" period.

Figure 8.3 shows the effect of the addition of 4.0 x 10^{-3}M chloroquine to cells already growing in the presence of labelled thymidine. In this experiment both the absorbance of the suspension and the amount of labelled thymidine in the cells began to decrease almost immediately after the addition of the chloroquine, but the radioactivity in the TCA insoluble material increased slightly for about 30 minutes and then remained constant for a further 30 minutes before it began to decrease.

Figure 8.4 shows the results of an experiment designed to observe the effect of treating cells with chloroquine in glucose free medium on

the uptake of thymidine after the subsequent addition of glucose. The Figure shows that there was some increase in absorbance of the control culture prior to the addition of glucose, suggesting that the cells were able to make some use of the added thymidine and/or deoxyadenosine as carbon sources. The use of thymidine is confirmed by the substantial incorporation of radioactivity into the control cells during this glucose free period. The cells treated with 4.0×10^{-3}M chloroquine showed some slight uptake and incorporation of thymidine during the first 60 minutes prior to the addition of glucose followed by a decrease in the amount of non-TCA precipitable radioactivity in the cells. No further change in the amount of radioactivity in the cells or in the TCA-insoluble material was observed after the addition of glucose.

Figure 8.6 illustrates the effect of chloroquine on thymidine which had previously been taken up and incorporated into the DNA of normally growing cells of E. coli. By the time the experiment illustrated in the Figure was commenced ^{14}C-thymidine had been removed from the growth medium, although unlabelled thymidine and deoxyadenosine were still present. The cells had been "fed" for at least three hours previously with labelled thymidine. Therefore, although the control cells were growing, as shown by the increase in absorbance, the radioactivity level could not be expected to rise.

The results for the control cells show a fairly constant level of radioactivity for the whole period of the experiment, indicating that the previously incorporated ^{14}C-thymidine was being retained by succeeding generations of cells. In the cultures treated with 4.0×10^{-3}M chloroquine the radioactivity in the cells began to decrease

almost immediately after addition of the drug, 50% having been lost after about 90 minutes, and 90% after about 230 minutes. The amount of radioactivity in the form of TCA-insoluble material remained constant for more than 30 minutes, but then also began to decrease.

Experiments using 1.0×10^{-3}M chloroquine gave a similar pattern of results except that the percentage of radioactivity lost from the cells was lower.

Figure 8.7 shows the results obtained when E. coli cells which had been fed with ^{14}C-thymidine for 3 hours, followed by removal of unused radioactivity from the medium,were incubated in the absence of glucose for about an hour and then treated with 4.0×10^{-3}M chloroquine while still glucose free. These glucose free cells again apparently utilised part of the unlabelled thymidine and/or deoxyadenosine as a carbon source and grew slightly before the addition of glucose. In the control suspensions, the radioactivity within the cells and in the TCA-insoluble material remained constant for at least four hours, indicating that no breakdown of the prelabelled macromolecules was occurring.

In the suspensions treated with chloroquine in the absence of glucose, the amount of radioactivity in the cells and in the TCA insoluble material remained constant for at least 80 minutes. After the addition of glucose to these suspensions, however, the radioactivity within the cells began to decrease almost immediately, followed after about 50 minutes by decrease in the radioactivity in the TCA-insoluble material.

The addition of bactericidal concentrations of ampicillin to growing cultures of E. coli causes lysis of the cells and release of

their contents. Figure 8.8 shows the results obtained after the addition of such a lethal concentration of ampicillin to growing cultures of E. coli in which the cells had been prelabelled by growth for 3 hours in the presence of ^{14}C-thymidine.

After the addition of ampicillin both the absorbance and the radioactivity within the cells decreased sharply; the radioactivity in the TCA insoluble material however remained constant for at least 3½ hours, showing that under these conditions, breakdown of the prelabelled cell macromolecules did not occur.

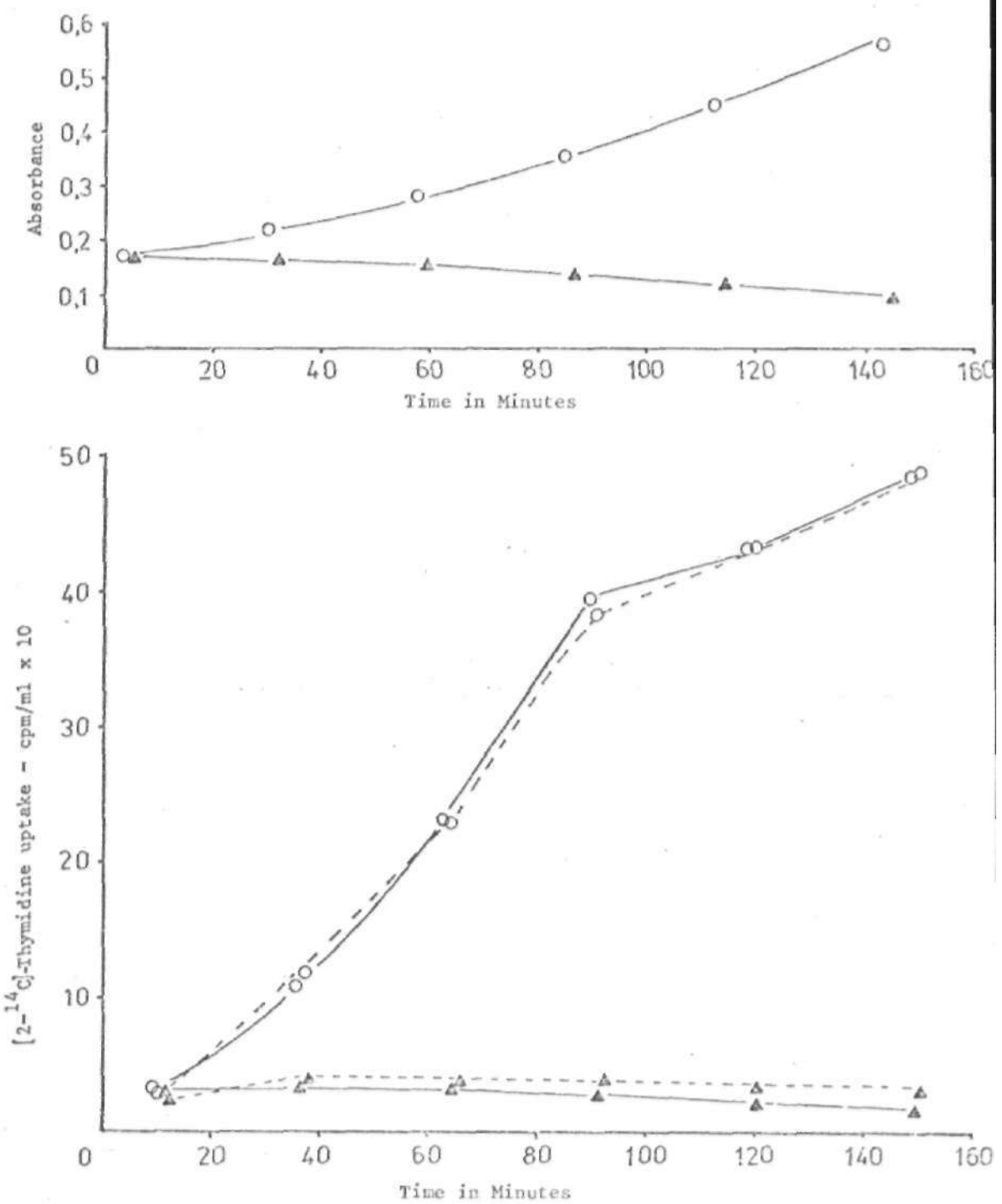


Figure 8.1. The effect of chloroquine on the changes with time of absorbance (A) (O-,Δ-), and uptake (O—,Δ—) and incorporation into cold TCA-insoluble precipitates (O-,Δ--) of [2-^{14}C]-thymidine (B) by E.coli cells. Chloroquine was added at the same time as [2-^{14}C]-thymidine.

Control O—

Chloroquine treated (4.0×10^{-3}M) Δ---

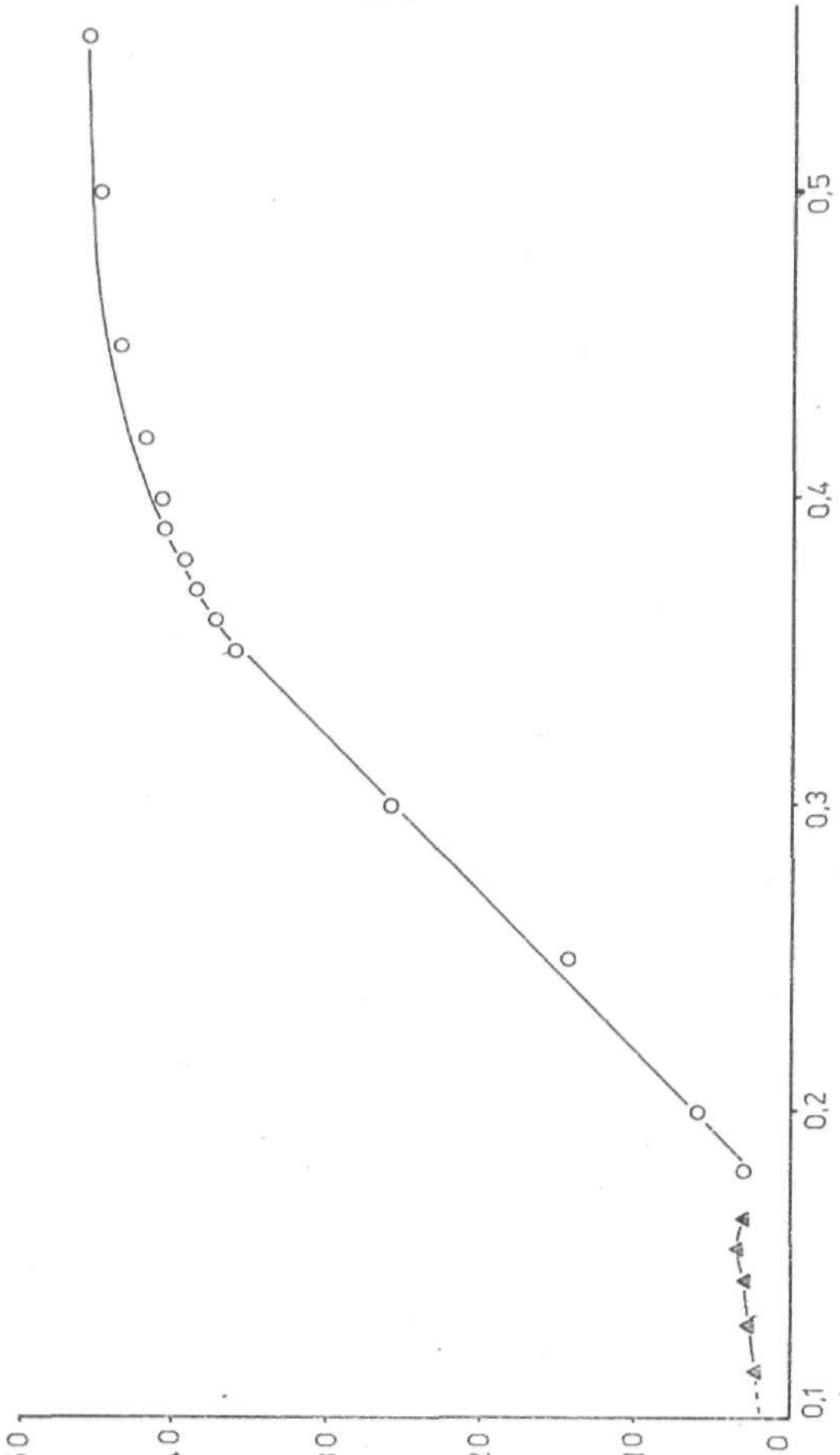


Figure 8.2. The relationship between absorbance and uptake of $2\text{-}^{14}C$ -thymidine by E. coli cells treated with 0.0 (O——) and 4.0×10^{-3}M (▲——) chloroquine. Chloroquine was added at the same time as [2-^{14}C]-thymidine.

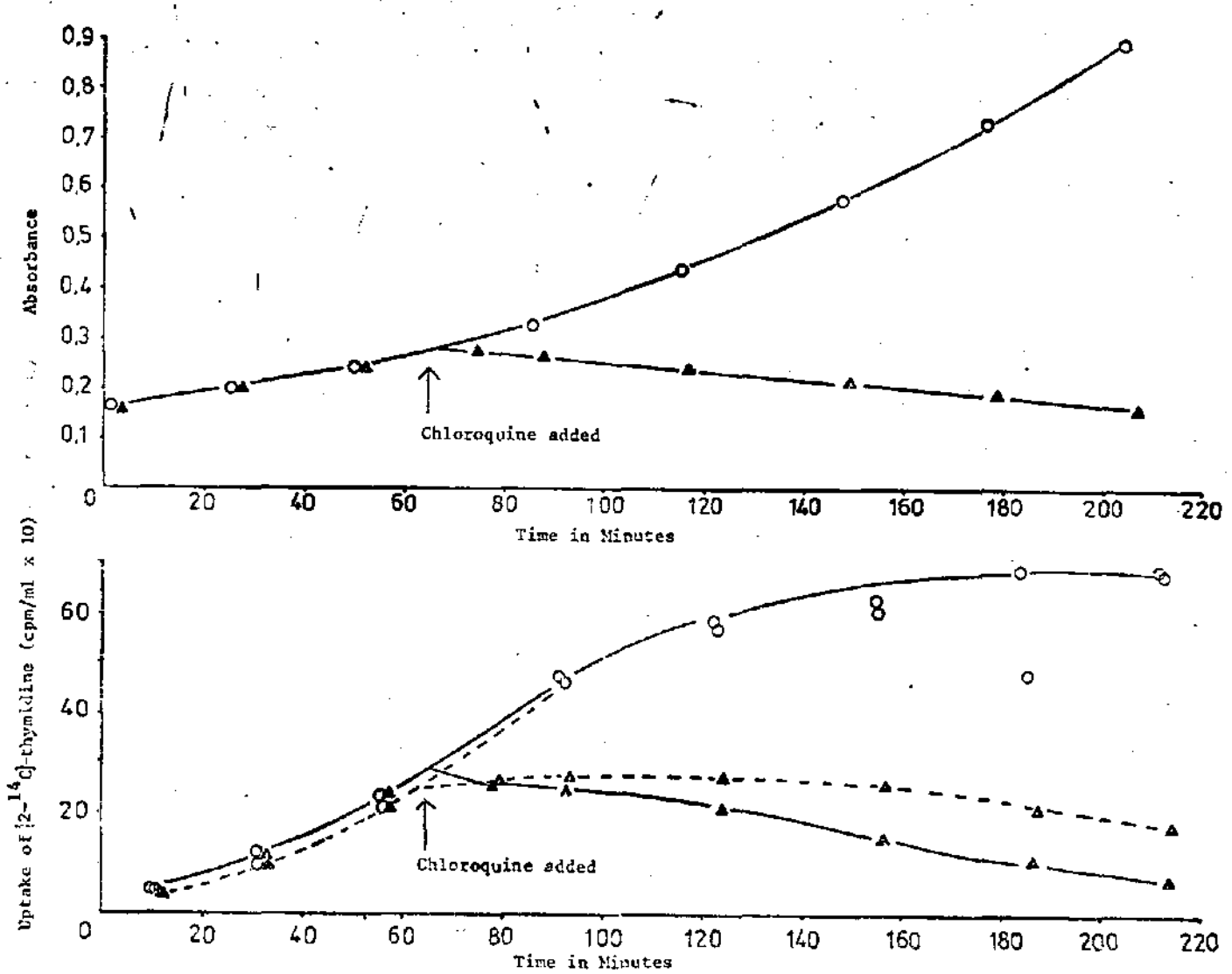
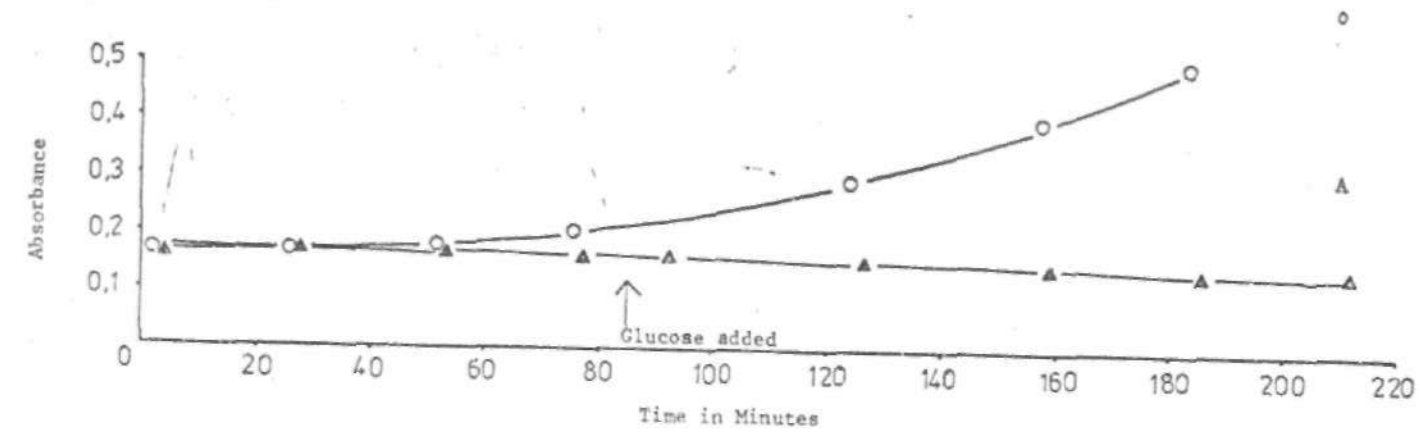


Figure 6.3. The effect of chloroquine on the changes with time of absorbance (A) (○–▲–), and uptake (○–▲–), and incorporation into cold TCA-insoluble precipitates (○-▲-) of [2-^{14}C]-thymidine (B) by E. coli cells. Chloroquine was added about 60 minutes after the addition of 2-^{14}C -thymidine.



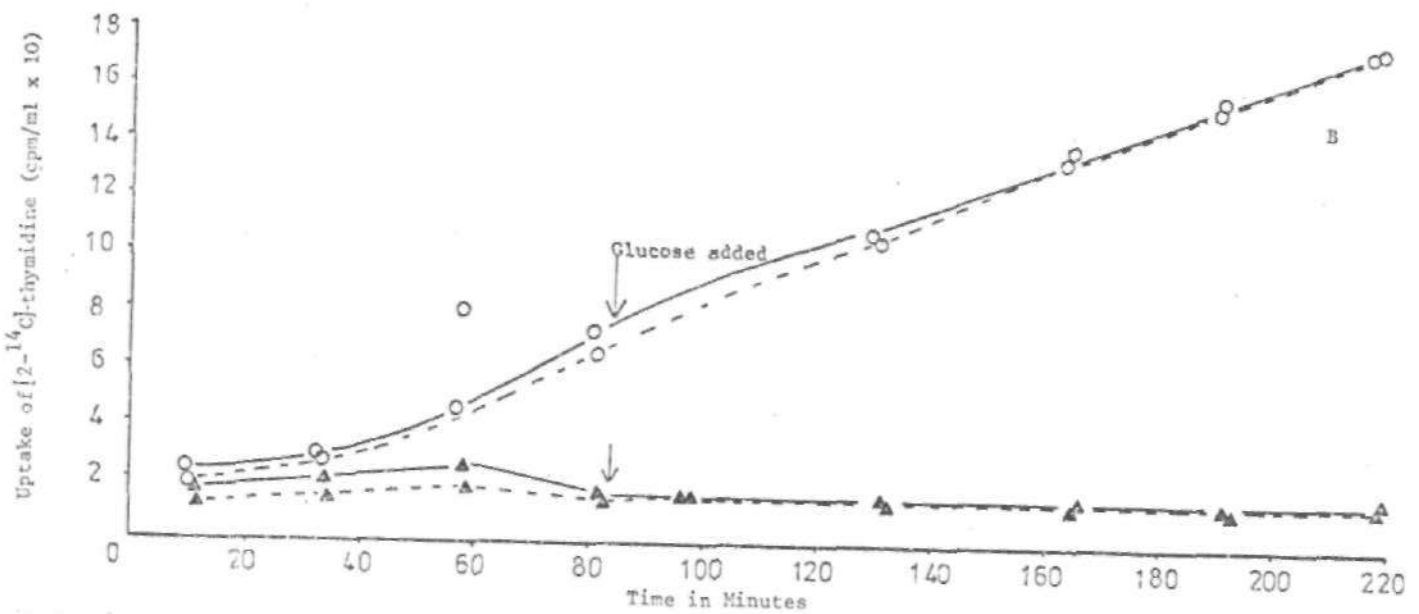


Figure 8.4. The effect of chloroquine on changes with time of absorbance (A) (O–▲–), and uptake (O¬▲–) and incorporation into cold TCA-insoluble precipitates (O--▲--) of [2-^{14}C]-thymidine (B) by E. coli cells before and after the addition of glucose. Chloroquine was added at the same time as 2-^{14}C -thymidine.
Control O———
Chloroquine treated (4.0×10^{-3}M) ▲———

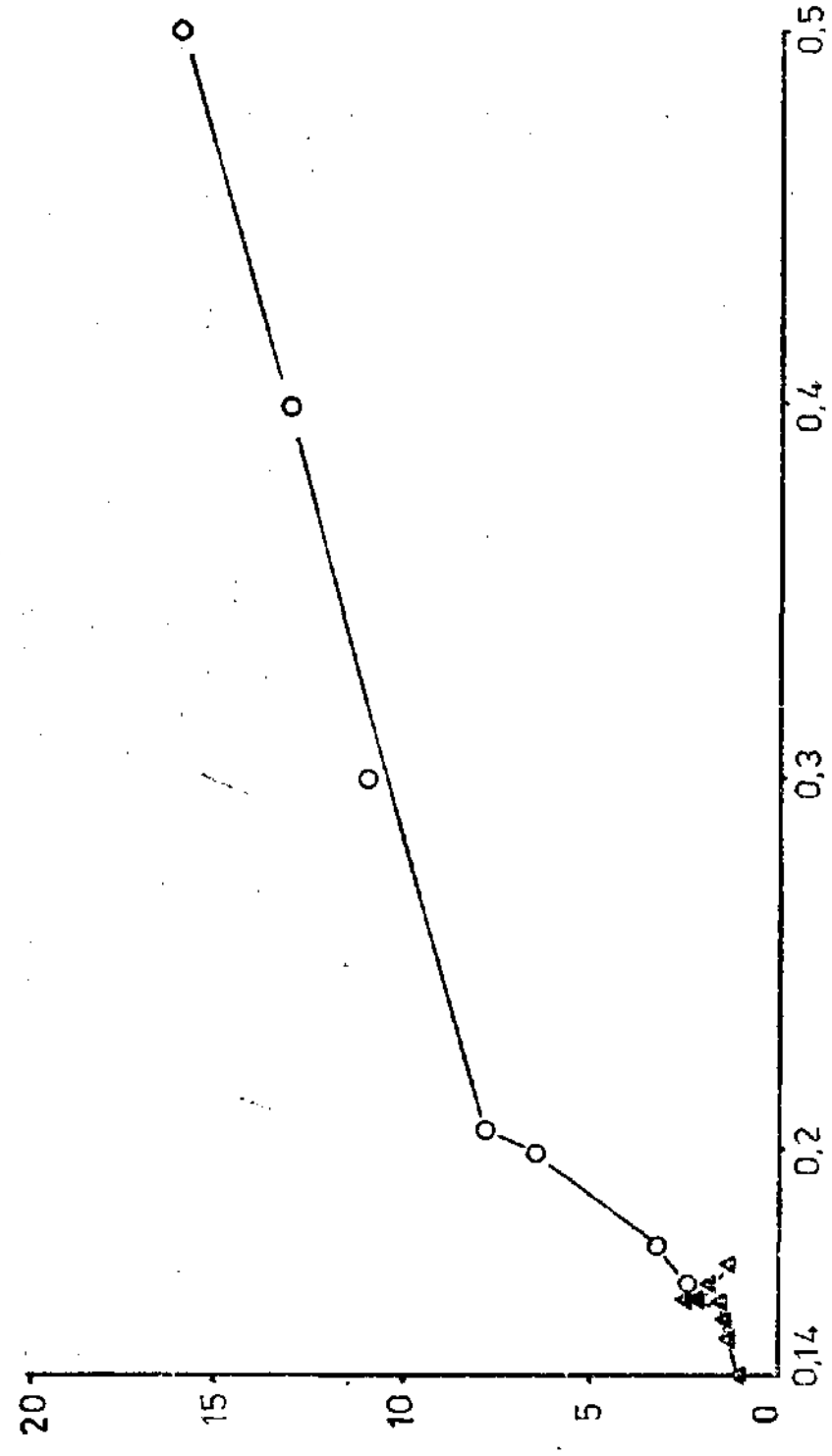


Figure 8.5. The relationship between absorbance and uptake of 2-^{14}C -thymidine by E. coli cells before and after the addition of glucose. Chloroquine was added at the same time as 2-^{14}C -thymidine.

Control O—

Chloroquine treated (4.0×10^{-3}M) ▲---

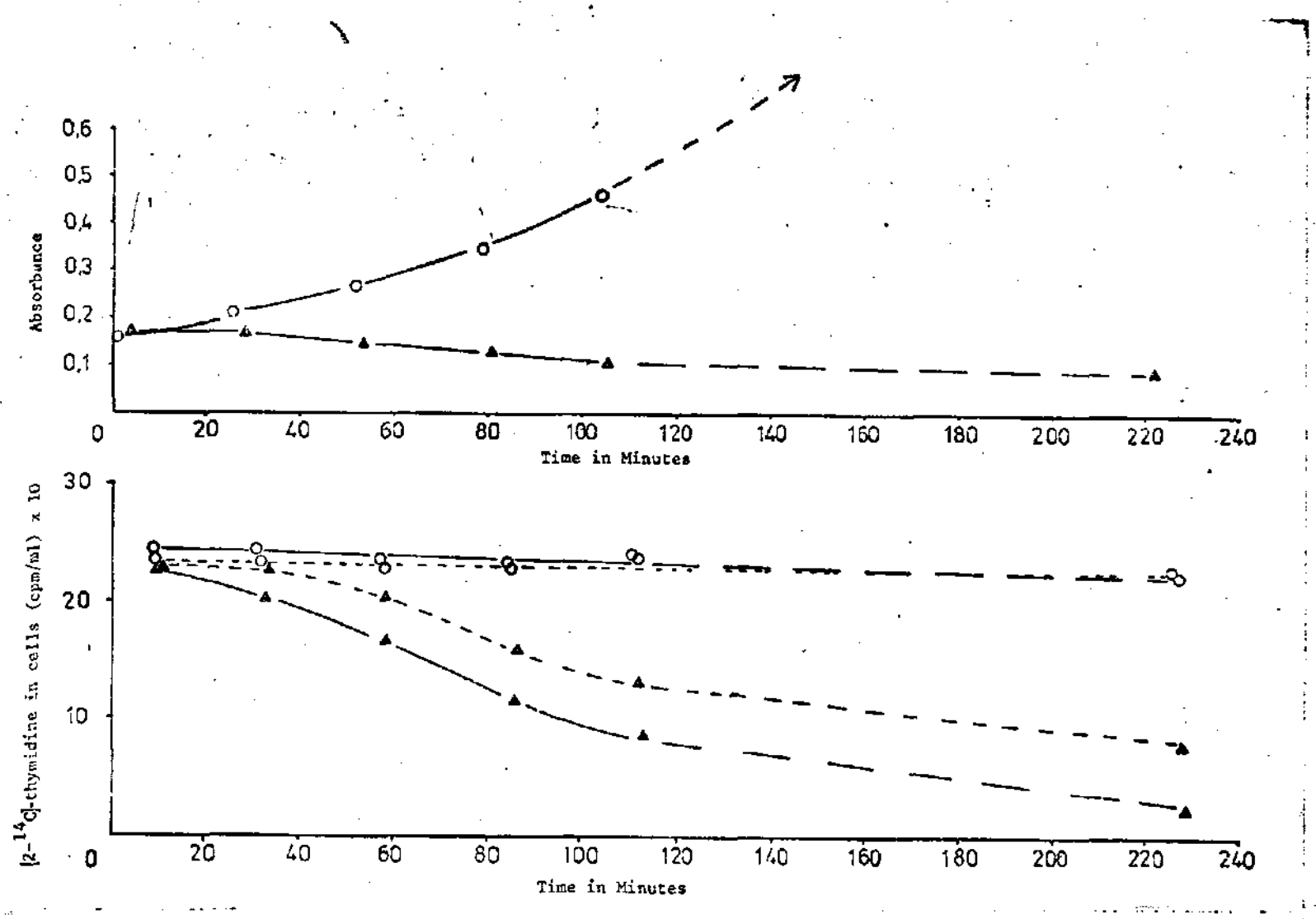


Figure 8.6. The effect of chloroquine on changes with time of absorbance (A), and the fate of already incorporated [2-^{14}C]-thymidine (B) in E. coli cells.

Absorbance: Control (O——)

Chloroquine (4.0×10^{-3}M) treated (▲——)

Level of radioactivity in cells: Control (O——)

Chloroquine (4.0×10^{-3}M) treated (▲——)

Level of radioactivity in cold TCA-insoluble precipitates: Control (O- - -)

Chloroquine (4.0×10^{-3}M) treated (▲- - -)

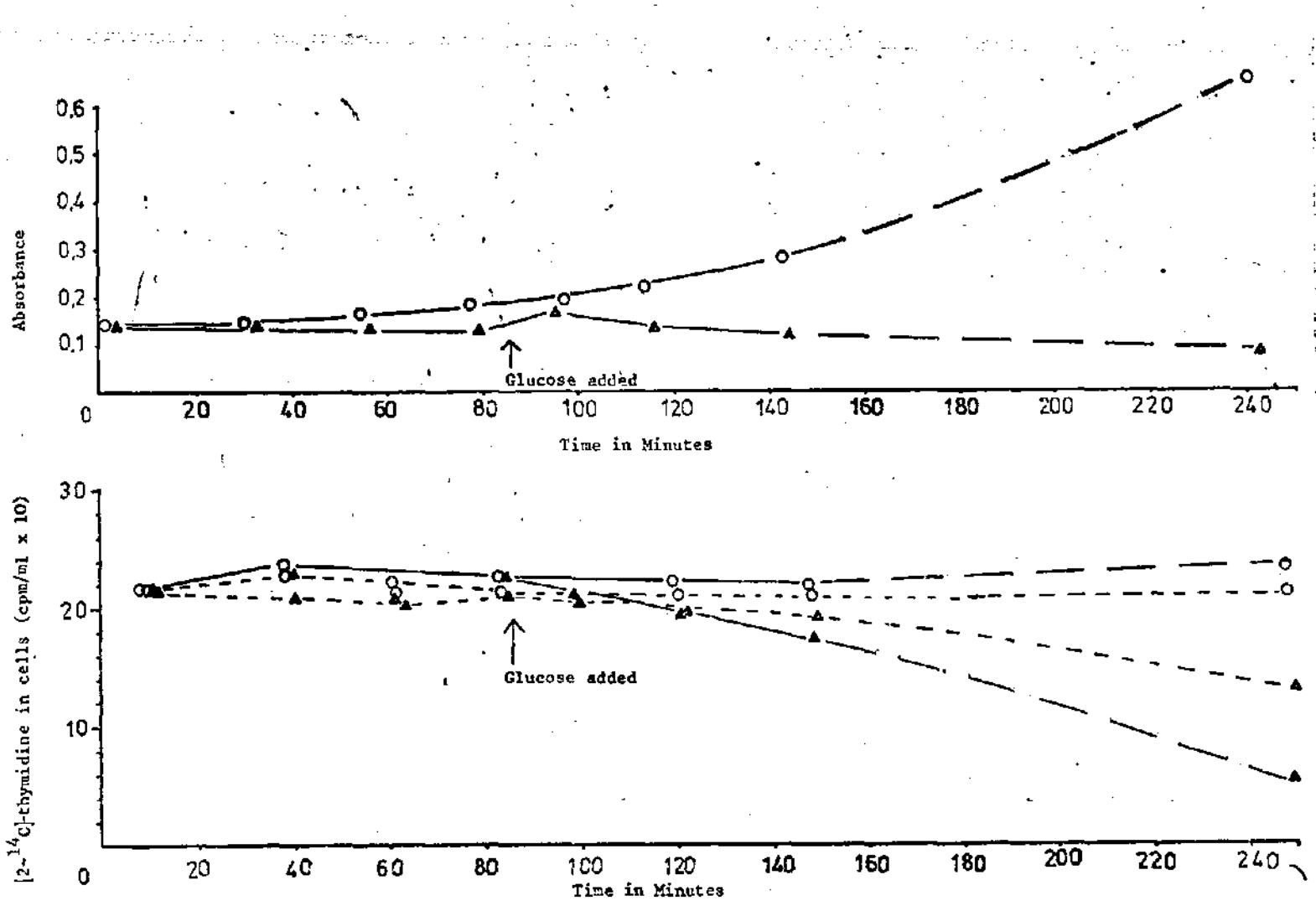


Figure 8.7. The effect of chloroquine on the changes with time of absorbance (A), and the fate of already incorporated [2-^{14}C]-thymidine (B) in *E. coli* cells before and after the addition of glucose.

Absorbance: Control (○——)
Chloroquine (4.0×10^{-3}M) treated (▲——)

Level of radioactivity in cells: Control (○——)
Chloroquine (4.0×10^{-3}M) treated (▲——)

Level of radioactivity in cold TCA-insoluble precipitates:
Control (○- - -)
Chloroquine (4.0×10^{-3}M) treated (▲- - -)

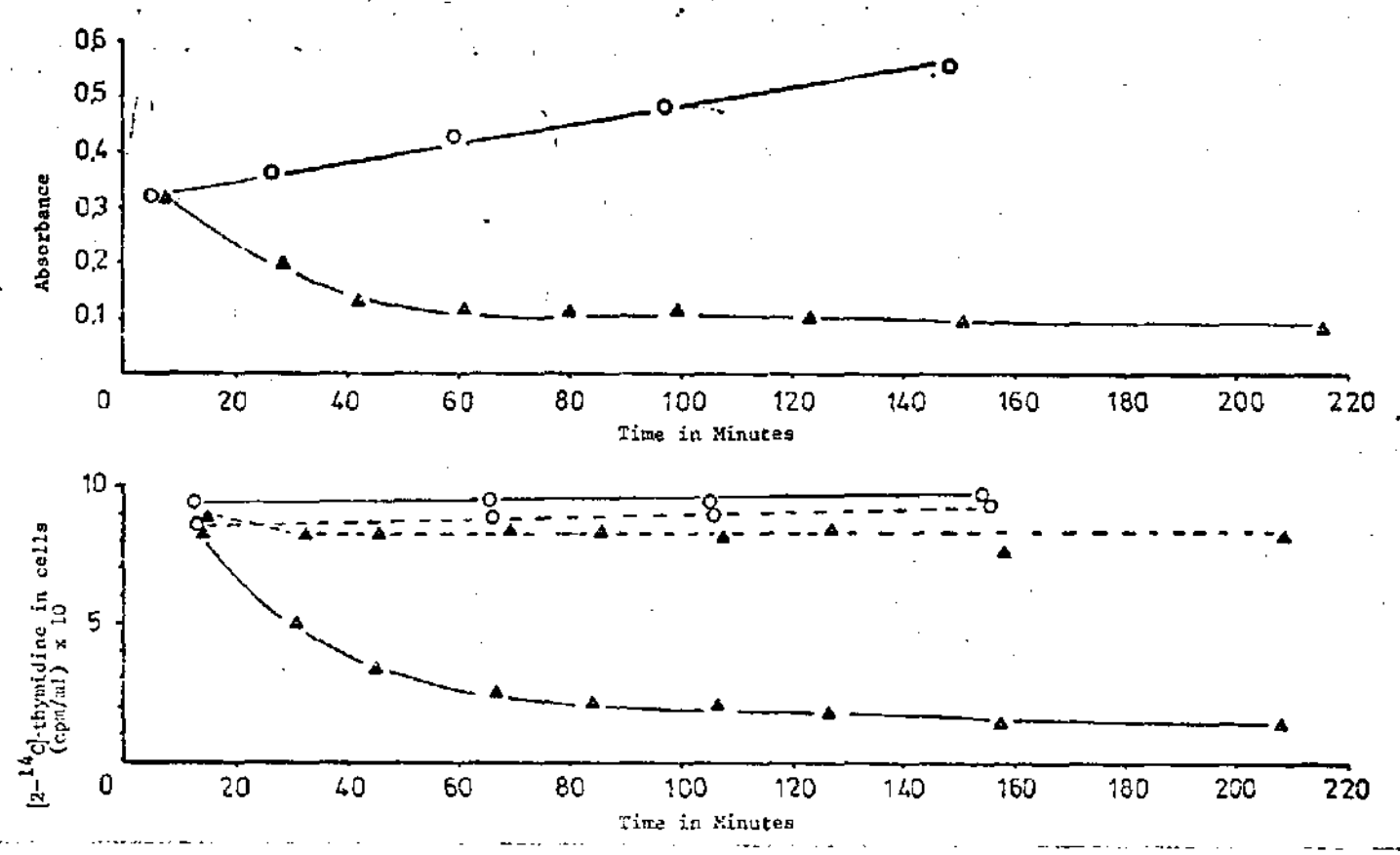


Figure 8.8. The effect of Ampicillin Sodium on the changes with time of absorbance (A), and the fate of already incorporated [2-^{14}C]-thymidine (B) in E. coli cells.

Absorbance: Control (O—)

Amp.Sod. (200µg/ml) treated (▲——)

Level of radioactivity in cells: Control (O——)

Amp.Sod. (200µg/ml) treated (▲—)

Level of radioactivity in cold TCA-insoluble precipitates:

Control (O- - -)

Amp.Sod. (200µg/ml) treated (▲- - -)

DISCUSSION

Chloroquine is known to form molecular complexes with the nucleic acids of various cells (Hahn and associates, and many other workers as reported in Chapter One), and to interfere with the syntheses of DNA and RNA. It has also been shown by Conklin and Chou (1972), and Conklin, Heu and Chou (1973) to inhibit the uptake of thymidine, uridine and amino acids by Tetrahymena pyriformis, and to inhibit markedly both DNA and RNA syntheses by solubilised DNA and RNA polymerases, and DNA synthesis by nuclei from the same organism. More recently, however, Middleton (1976) studied the effects of chloroquine on the utilisation of thymidine, uracil and phenylalanine by E. coli. He reported that DNA synthesis was less sensitive to the drug than RNA and protein synthesis, the uptake of thymidine and its incorporation into macromolecules continuing for some time in cultures treated with inhibiting concentrations of chloroquine even after increase in absorbance of the cultures had ceased.

The results reported in this Chapter suggest that thymidine uptake and incorporation continue for only a few minutes after the addition of bactericidal concentrations of chloroquine to growing cultures of E. coli.

Under the conditions of cultivation used, untreated control cells utilised thymidine at a rate proportional to the rate of growth for about 90 minutes, after which the rates of uptake and incorporation decreased progressively, possibly as a result of the conversion of thymidine to thymine by the inducible enzyme, thymidine phosphorylase (Rachmeler et al., 1961).

The results obtained with growing cells of E. coli which had been prelabelled by growth in the presence of ^{14}C-thymidine before the addition of chloroquine show that DNA is released from treated cells and that the released DNA is then degraded to low molecular weight compounds.

In contrast, the DNA released from prelabelled cells by growth in the presence of ampicillin shows no evidence of degradation even after 3½ hours.

The ^{14}C-thymidine prelabelled cells treated with chloroquine in the absence of glucose did not leak DNA into the medium until after the addition of glucose, confirming the inactivity of the drug against non-growing cells.

Inhibition of the uptake and incorporation of thymidine by chloroquine treated cells, as shown in this study, suggest an explanation for the thymineless death type curves obtained from the viability experiments of Chapter 4. Even though this strain of E. coli can synthesise its own thymine, it is probable that chloroquine can still block its incorporation into the cells' DNA.

Thymineless Death is the terminology given to the dying of thymine-requiring strains of bacteria when they are deprived of this pyrimidine. Although RNA and protein syntheses continue for a while, DNA synthesis is blocked; this leads to unbalanced growth, which in turn leads to irreversible loss of viability (Barner and Cohen, 1954; Cohen and Barner, 1954, and Cohen, 1971). Other occurrences which accompany this phenomenon include increased turbidity of cell cultures and elongation in some strains of bacteria, induction of colicin and prophage,

and degradation of DNA. The latter process was shown by Reiter and Ramareddy (1970) to be initiated at the DNA replicating point and to continue back along the chromosome. Newly synthesised strands are first degraded, and then both the new strands and the template are degraded to double strand segments. Cell death is said to be coincident with loss of DNA. However, it was pointed out that a carbon and energy source was essential for death of the cells (Cohen and Barner, 1954); in the absence of glucose death was completely prevented in some strains and greatly retarded in others (Nakayama and Hanawalt, 1974).

The results described in this Chapter indicate the ability of chloroquine to block the uptake and incorporation of thymidine in this strain of E. coli, and to cause the leakage and degradation of both newly formed and preformed DNA in growing cultures, but not in glucose-free cultures. It can therefore be proposed that simulation of the Thymineless Death phenomenon might be a mode of antibacterial action of chloroquine.

GENERAL DISCUSSION AND EPILOGUE

GENERAL DISCUSSION AND EPILOGUE

	Page No.
General Discussion	165
Epilogue	171
Attack on the vector, the Anopheles mosquito	171
Malaria vaccines	172
Improving the knowledge on current antimalarials	173
Development of new antimalarials	174

GENERAL DISCUSSION

The results of the studies undertaken in this project have confirmed the growth inhibiting activity of chloroquine against cells of E. coli, first reported by Wiseman (1972a) and later by Middleton (1976), and have shown that increasing the concentration of the drug produces bactericidal effects. In this thesis, the bulk of the work lays emphasis on the effects of chloroquine on this organism at bactericidal concentrations, and some of the factors that affect these effects.

The approximate minimum bactericidal concentration at 37°C and pH 7.4 for growing cells of E. coli in the MOPS buffered minimal salts medium used is about 1.0×10^{-3}M, and increasing the concentration beyond this up to ten-fold, does not appear to have any significant effect on the rate of kill, although the time of maintenance of this rate of kill is increased. The similarity of bactericidal effects on growing cells of E. coli obtained with such a wide range of drug concentration is rather extraordinary, and may be pointing out a peculiarity in the drug uptake by the cells.

The presence of glucose in the growth medium, at least for a period, seems to be a necessity for the bactericidal action of chloroquine, since in its absence the drug is rendered inactive for concentrations up to 8.0×10^{-3}M. This indicates that the drug is ineffective against resting non-growing cells, and that an important mode of its antimicrobial action is connected with growth and/or cell division. However, the presence of some killing effect in the absence of glucose with 16.0×10^{-3}M chloroquine suggests that there may be at least a secondary mode of anti-bacterial action which is independent of cell

growth, and which becomes manifest at this high concentration.

The effect of changes in pH on the antibacterial action of chloroquine has also been demonstrated, and shows how sensitive the drug is to pH changes. Both the growth inhibiting and the bactericidal actions of chloroquine increase with increase in pH. Thus the approximate minimum bactericidal concentration at pH 6.7 is 2.6×10^{-3}M, whilst at pH 7.7 it is about 0.7×10^{-3}M, showing about a four-fold increase in potency for unit rise in pH. This finding is rather interesting, as it suggests that the theoretical DNA intercalator, the double-protonated species, is less active bactericidally than the mono-protonated one. For example, at pH 6.7 the percentage of drug as dications in solution is 95.94 while that in the form of monocations is 3.99; and at pH 7.7 the percentage of dications is about 65 while that of the monocations is about 34. The drug becomes more effective as the percentage in the dication form decreases. If the double protonated cation is indeed the DNA intercalating species as reported by Cohen and Yielding (1965a), Victor et al. (1971) and Bolte et al. (1977), then important as this reaction with DNA may be, it appears not to be the primary mechanism of antibacterial action of chloroquine. Another recent finding on the effect of pH on the biological activity of chloroquine is that of Mego and Chung (1979), who reported increase in inhibition of cathepsin B. with increase in pH.

The results obtained from uptake studies suggest that there is an initial passive uptake, perhaps similar to that described by Polet and Barr (1969), which is energy independent, and does not need active transport. In E. coli cells that are deprived of their sole carbon and energy source, glucose, there is little rise of uptake above this

initial passive uptake level. However, since the level of this initial uptake is to some extent concentration dependent, higher uptake levels are obtainable with higher drug concentrations, thus making possible potentially bactericidal levels of uptake in the absence of glucose at high drug concentrations.

In the absence of glucose, initial passive uptake is apparently terminated, and followed by a slower rate of active uptake, which can continue until well after the total cell mass has stopped increasing.

Another finding, which may offer an explanation for the similarity of the bactericidal effects obtained when growing cells of E. coli are treated with different drug concentrations is that only a small amount of the chloroquine in the growth medium is taken up by the cells, the percentage being taken up at the lower concentrations being higher than at higher concentrations, suggesting that receptors can only take up a limited amount, so that once this level is reached, increasing the drug concentration in the medium has little or no increased effect on the cells.

The experiments on thymidine uptake indicate that bactericidal concentrations of chloroquine strongly inhibit the uptake and incorporation of thymidine by growing cells of E. coli, and cause degradation of DNA and release of already incorporated thymidine from the cells. In the absence of glucose, there is hardly any observable uptake or incorporation of thymidine by the chloroquine treated cells, neither is there any degradation of preformed DNA until the re-addition of glucose to the growth medium, again confirming the inactivity of chloroquine on resting non-dividing cells of E. coli.

A possible deduction from these thymidine experiments is that chloroquine is capable of depriving or starving E. coli cells of thymidine, and although the strain used here does not require exogenous thymine or thymidine, it is apparent that chloroquine could prevent the utilisation of any thymine synthesised by the cells. It is therefore not too surprising that the death curves obtained from the results of the viability experiments have some similarities to thymineless death curves, especially at the lower bactericidal concentrations. As occurs in thymineless death, there is an increase in cell turbidity initially even though cell viability is decreasing; there is an initial lag in the death curve followed by rapid exponential kill, and the percentage loss in viability is over 99% after 2 hours of exposure to chloroquine (cf. 99.5 after 150 minutes obtained by Ohkawa, 1975). Also, during conditions for thymineless death in the absence of a carbon and energy source, especially glucose, death is completely prevented in some strains, or greatly retarded (Nakayama and Hanawalt, 1974), a situation which also occurs in the glucose-starved chloroquine treated cells reported here. It can therefore be proposed that a partial simulation of the thymineless death phenomenon is a mode of antibacterial action of chloroquine. However, the points of departure of the death curves for chloroquine treated cells from those of thymineless death, namely, the reduced increase in cell turbidity, or absence of it at higher concentrations of the drug, the shorter lag period, or its disappearance with higher concentrations, suggest that there is another mode of bactericidal action of the drug, which is perhaps independent of DNA inhibition.

The main findings from this study of the bactericidal effects of

chloroquine on E. coli can be summarised as follows:-

1. Chloroquine can now be described as bactericidal for E. coli, and this effect does not seem to vary significantly with variations in drug concentrations above the threshold for bactericidal activity.
2. The bactericidal action of chloroquine is very sensitive to changes in pH, a four-fold increase in potency being obtained for unit rise in pH.
3. Chloroquine is relatively inactive against resting, non-growing cells, a carbon and energy source such as glucose present in the growth medium or cell nutrient being a necessity for its activity.
4. The uptake of the drug into E. coli cells is relatively small, and is dependent to some extent on drug concentration in the growth medium, and on the physiological state of the cells, so that increase in the drug concentration does not necessarily mean a corresponding increase in drug uptake. The mechanism of the latter is not known.
5. There is a strong indication from this study that chloroquine rapidly inhibits the uptake and incorporation of thymidine into cell DNA in E. coli, and causes the breakdown of preformed DNA. The methods employed, however, could not confirm the mechanism by which these actions are effected, but from the works of Hahn, Cohen and others it might be assumed that the degradation process is brought about by the binding or intercalation of the drug with the cell DNA.

No other specific mechanism of antibacterial action is obvious from the results reported in this thesis, although there are indications

that other such mechanisms exist, especially from the results obtained from the pH experiments which demonstrated that increased activity with increase in pH was due to increase in the monocations in the growth medium. Middleton (1976) reported that chloroquine inhibited the uptake of oxygen by E. coli cells, thus interfering with oxidative processes, and this effect increased with increase in concentration. Inhibition of some oxidative processes in plasmodia by the drug had earlier been reported by Skelton et al. (1968), Turnbull et al. (1971) and Cho and Aviado (1968). This action of chloroquine is distinct and clearly separate from binding with the nucleic acids and constitutes a different mechanism of action. These show that there may be several mechanisms of action for the antimicrobial effect of chloroquine. On an individual basis they may not be lethal, but the summation of these effects may mean death to the microorganism.

The findings from the studies reported here may help to suggest further work on chloroquine, using the same test organism, which is "simple", better understood, and far easier to grow than plasmodia. This may enable future workers to establish its route and manner of uptake, and its site or sites of accumulation; the ionic forms in which it is present within the cell; and the effects it is exerting on different parts of the cell. The knowledge gained from such studies may be helpful in leading mankind to a better use of this well worn old hand, chloroquine.

EPILOGUE

The war against malaria has been on for a long time, and although well fought, it is apparently not yet over, especially with increase in the drug-resistant plasmodia. Several tactics have been employed, a number of which are still in progress.

1. Attack on the Vector, the Anopheles Mosquito.

The World Health Organisation has contributed much in the attempts to eradicate the anopheline vectors of malaria. Since the prevalence of DDT-resistant strains of these mosquitoes, thousands of compounds have been screened for insecticidal activity. The first successes, such as propoxur (a carbamate) and malathion (a phosphothioate) became front line insecticides against DDT- and dieldrin-resistant strains in Africa and Central America for some time (WHO, 1971). But, like their predecessors, resistance against them developed (Breeland et al., 1970; Georghiou et al.,1972). Out of about 2000 compounds evaluated for insecticidal activity by 1974, only 30 were sufficiently promising to justify large scale field trials (WHO, 1975). None was as persistent in action, or as generally safe, as DDT, and all were considerably more expensive (Service, 1977). A new race of pyrethroids, especially "Permethrin" and its derivatives, are proving a success, as they are effective against the insects, have long residual activity on inactive surfaces, and are biodegradable (Elliott et al., 1973, 1975; Service, 1977). Entomophagous parasites and predators, over a thousand of which had been found to feed on anopheline mosquitoes, have been and are still being tried, but so far not with much success.

The success of vector control will depend on a well-planned multidirectional approach, including combination of all the various methods that have killing effects on mosquitoes, well trained field personnel, and of course, better education and higher hygienic standards for human populations in the malarious areas of the world.

2. Malaria Vaccines.

Another approach to the eradication of malaria is through the development of malaria vaccines, a programme which is of great potential value. The difficulties lie in the complicated life cycles of plasmodia, which make it difficult to produce the organism in large quantities, and their antigenic products in pure forms, and the very many strains which occur within the same species, giving rise to many distinct antigenic variants. However, some success has been attained, though vaccine development is still in its early stages. Ducklings have been protected against P. lophurae malaria by immunisation with unconcentrated plasma from acutely infected birds (Corwin, McGhee and Sloan, 1965). Rats immunised with P. knowlesi antigen developed only very low parasitaemias when challenged with P. berghei as compared to unvaccinated controls (Cox, Milar and Patterson, 1968). X-irradiation as a useful means of attenuation has been tried with some success. Mice vaccinated with x-irradiated sporozoites of P. berghei were protected against sporozoite and erythrocytic challenges (Nussenzweig, Vanderberg, and Most, 1969). Through the use of killed vaccines young chickens have been partially protected from P. gallinaceum malaria by immunisation with killed free parasites (Richards, 1966). Mice vaccinated with cell-free extracts of P. berghei also showed partial immunity to subsequent challenge (Zuckerman et al., 1967). More recently,

attempts have been made on human immunisation against malaria. Clyde et al.,(1975), demonstrated through a human volunteer, protection for up to 3 and 6 months respectively against falciparum and vivax sporozoites. Species specificity of antigen and antibody was demonstrated.

Progress is also being made in the preparation of antigens from P. falciparum. For example, Siddiqui, Kramer and Richard-Crum (1978) prepared an erythrocytic late stage P. falciparum antigen which successfully protected Aotus monkeys against subsequent challenge with a homologous strain of P. falciparum. Rieckmann, Mrema and Campbell have also demonstrated that Plasmodium falciparum obtained from continuous culture were capable of inducing a pronounced immunity to malaria in Aotus monkeys.

The production of antigenic preparations in pure forms to meet the current ethical standard is still a problem, but at least the prospect for a successful human malaria vaccine is apparently improving.

3. Improving the knowledge on Current Antimalarials.

In spite of the fact that many antimalarial drugs have been in use for decades, or even over a century as in the case of quinine, much is still to be known about them. So research workers are still investigating modes of action, or their effects on various microbial and mammalian systems. Through these, it is hoped that better routes or methods of administration could be developed, or the structural formula of the drugs altered to synthesise new antimalarials. Thus new derivatives of Primaquine have been synthesised by La Montagne, Markovac and Menke (1977); Tanabe et al.(1978), and Shetty and Blanton (1978), which have been effective against P. cynomolgi and P. berghei malarias.

4. Development of New Antimalarials.

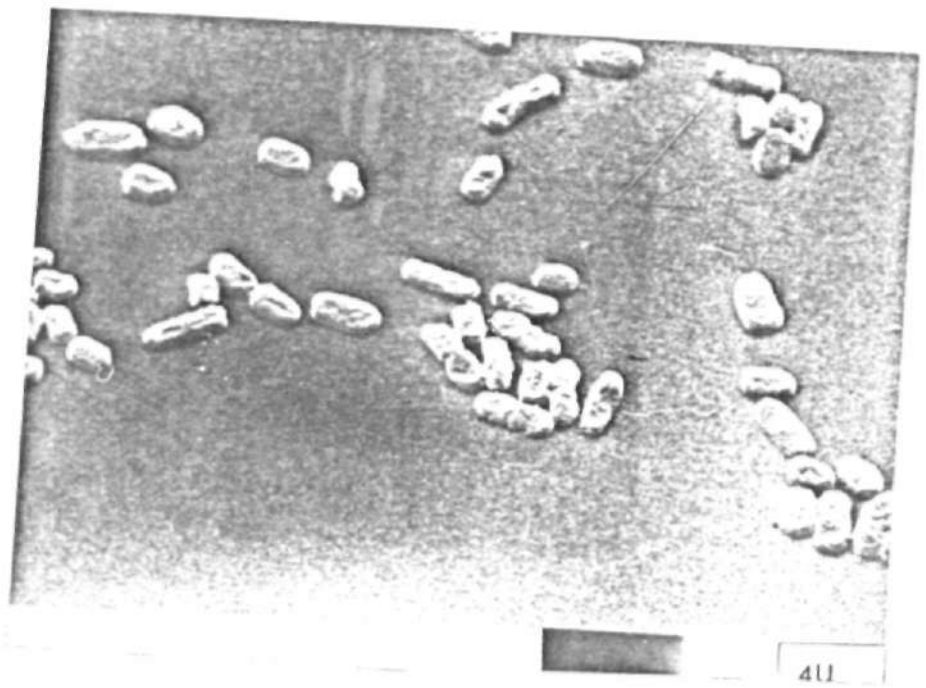
Through the knowledge of the general structures and modes of action of current antimalarials, potential new ones of entirely new structures are being synthesised. Thus new folate antagonist antimalarials of quinazolinediamines and related compounds have been synthesised by Eslager et al. (1978), some of which showed antimalarial activity against drug-resistant P. berghei in mice and P. falciparum in owl monkeys, as well as bactericidal antifolate activity against Streptococcus faecalis. Razdan et al.(1978) have synthesised new derivatives of Benzothiopyrans which were curative against P. berghei in mice, and Shamblee and Gillespie (1979), new trichloronaphthalene amino alcohols which have been found effective against P. berghei in mice. Although these new developments may still be in their infant stages, they offer some promise for the near future.

By 1975, the United States Army Antimalarial Drug Development Programme, perhaps the world's largest of its type, had screened over 200,000 compounds for antimalarial activity (Kinnamon and Rothe, 1975), and for every 3,000 of such tested compounds, only one was considered suitable for use in the final test for clinical toxicological evaluations. Mefloquine, a quinolinemethanolamine, is one such compound, which is proving promising. It has been found effective against both chloroquine sensitive and chloroquine-resistant malarias in humans, and, in addition to its therapeutic efficacy, exerted prolonged activity after a single dose (Rieckmann et al., 1974; Davidson et al., 1977). It cannot be predicted, however, how good or long lasting this 'promise' would be, since it too may eventually succumb to drug-resistant plasmodia like its predecessors.

It is likely that the final solution, from the human side of malaria, lies with the success of antimalarial vaccines. Until these are satisfactory, the best use of the available current and new antimalarials will have to be made.

APPENDIX

Electron micrographs of control and chloroquine treated cells of *Escherichia coli* in the presence and absence of glucose.



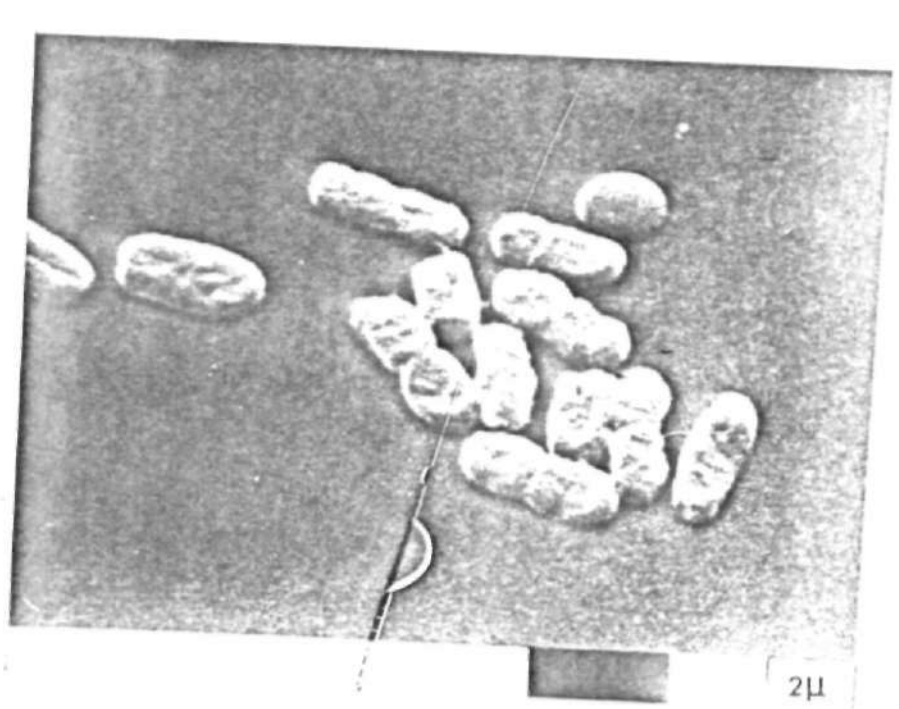
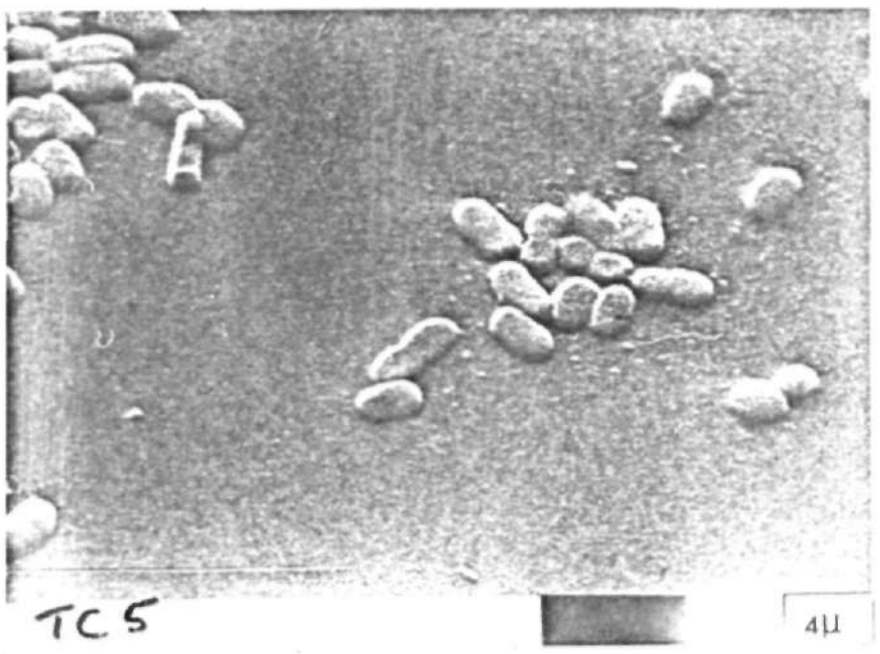


PLATE 1. Control cells of E. coli in the presence of glucose, 40 minutes after commencement of experiment.



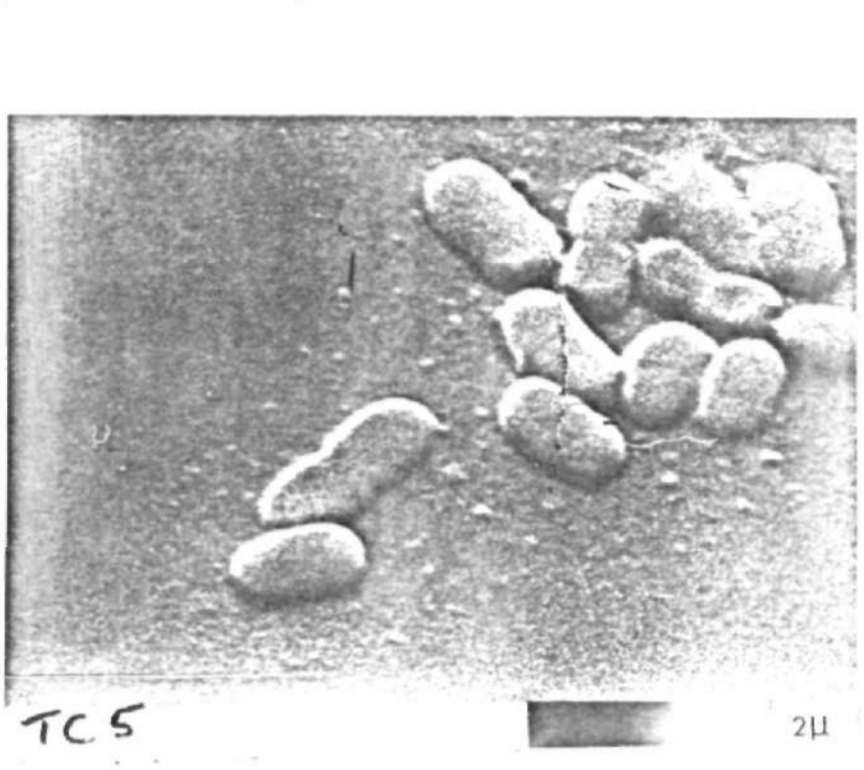
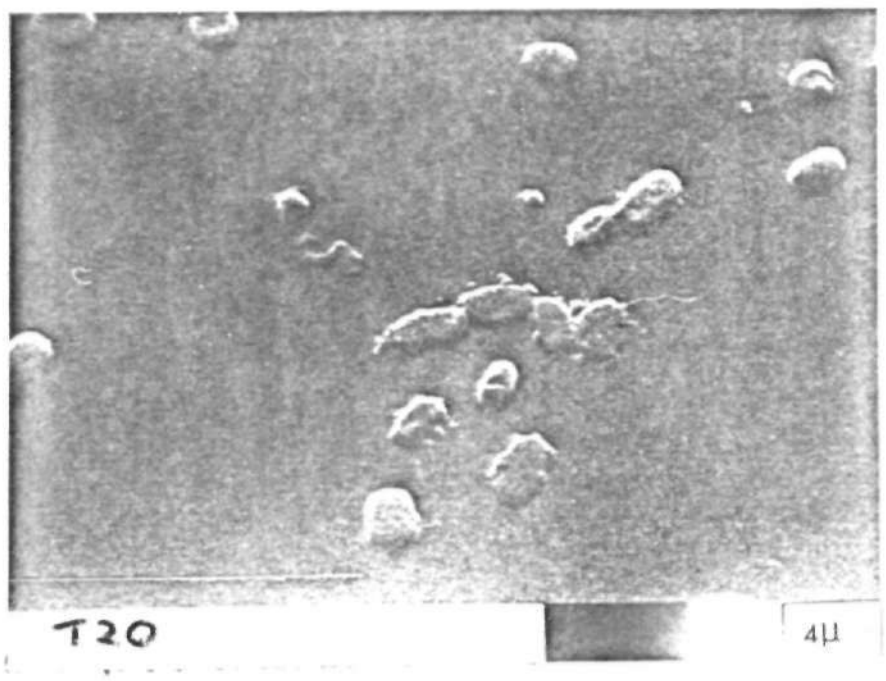


PLATE 2. Cells of E. coli, 5 minutes after the addition of 4.0×10^{-3}M chloroquine, in the presence of glucose.



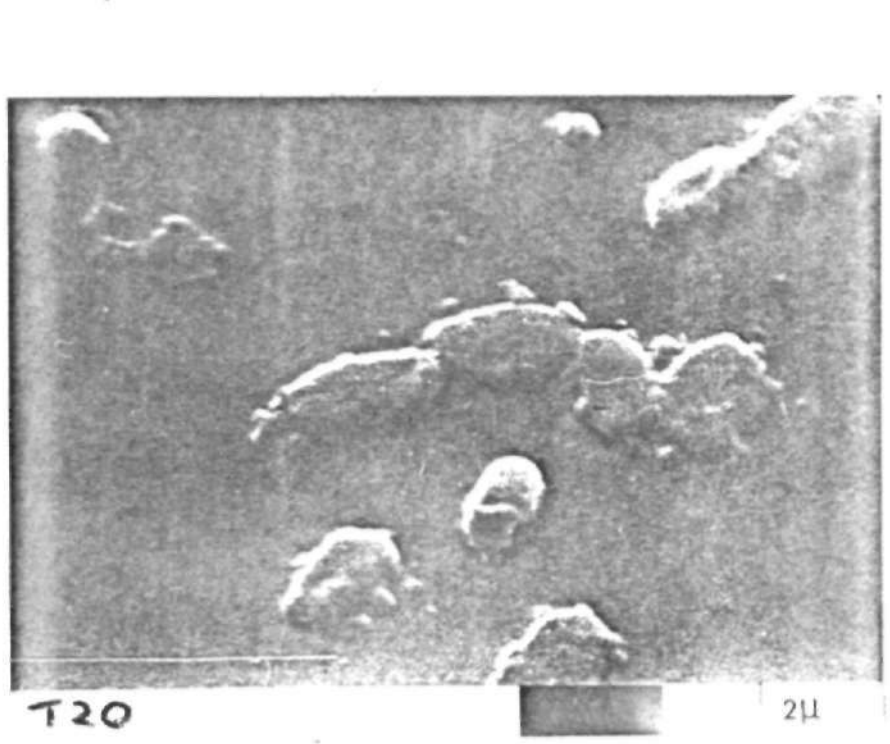
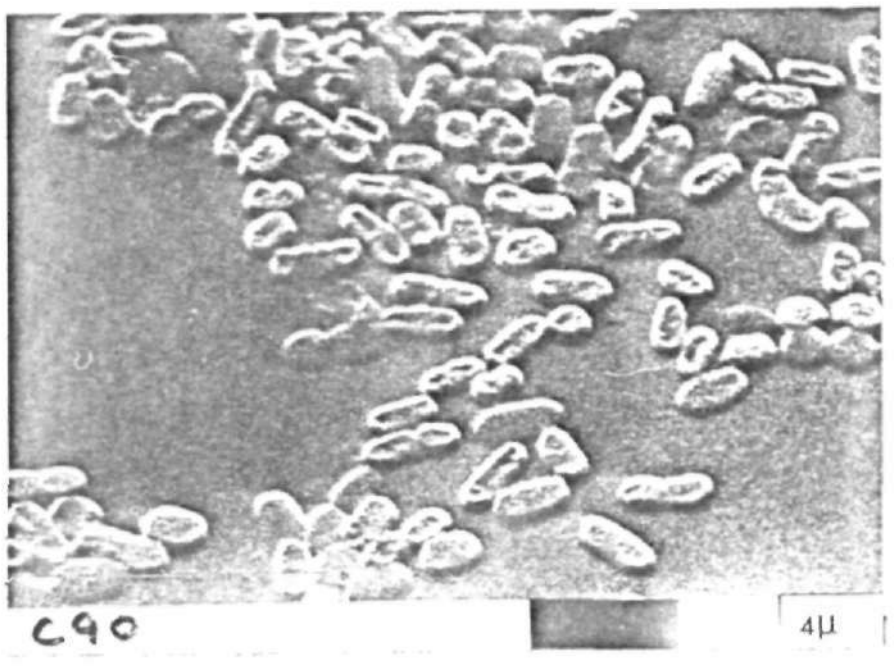


PLATE 3. Cells of E. coli, 20 minutes after the addition of 4.0×10^{-3} M chloroquine in the presence of glucose.



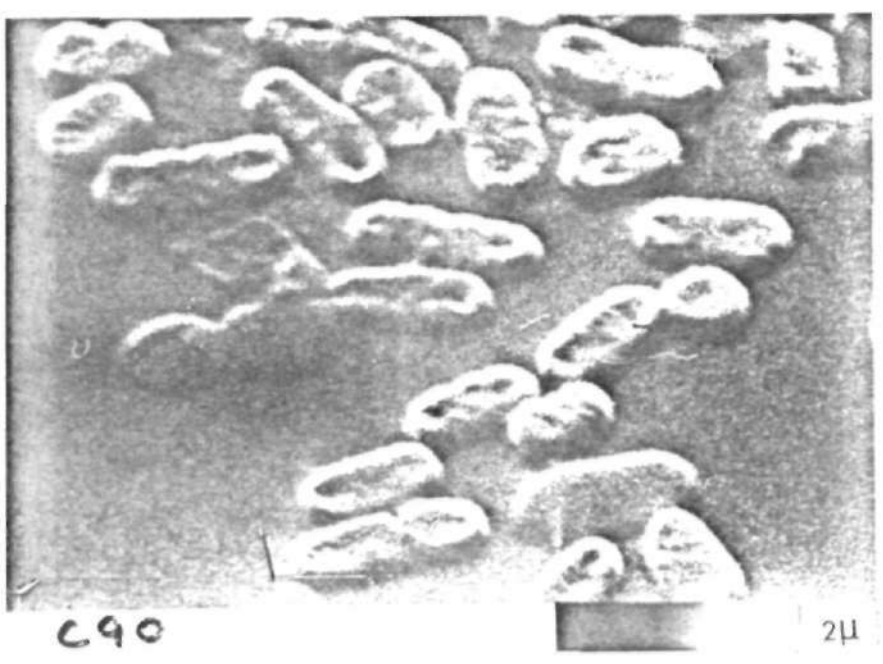
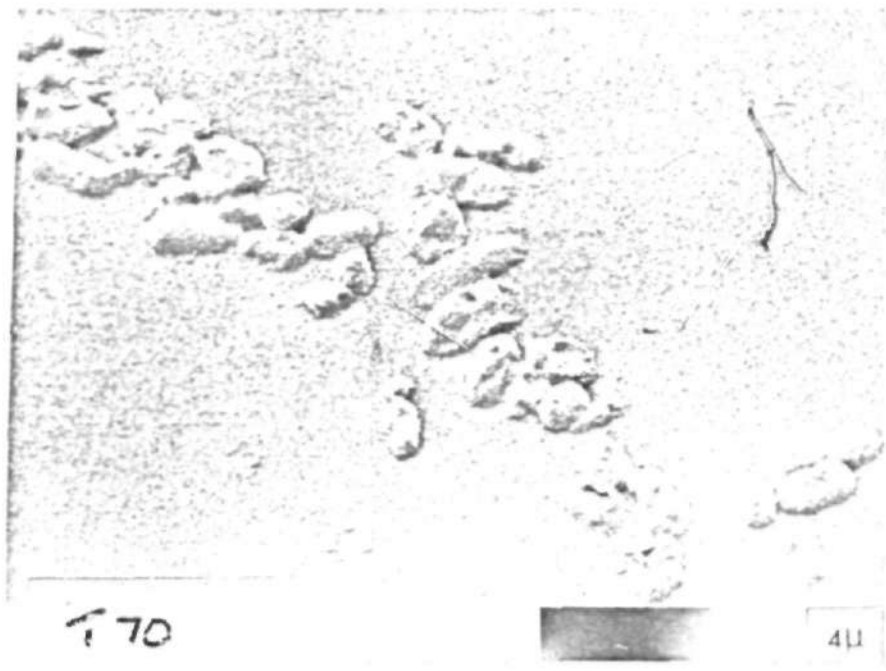


PLATE 4. Control cells of E. coli in the presence of glucose, 90 minutes after commencement of experiment.



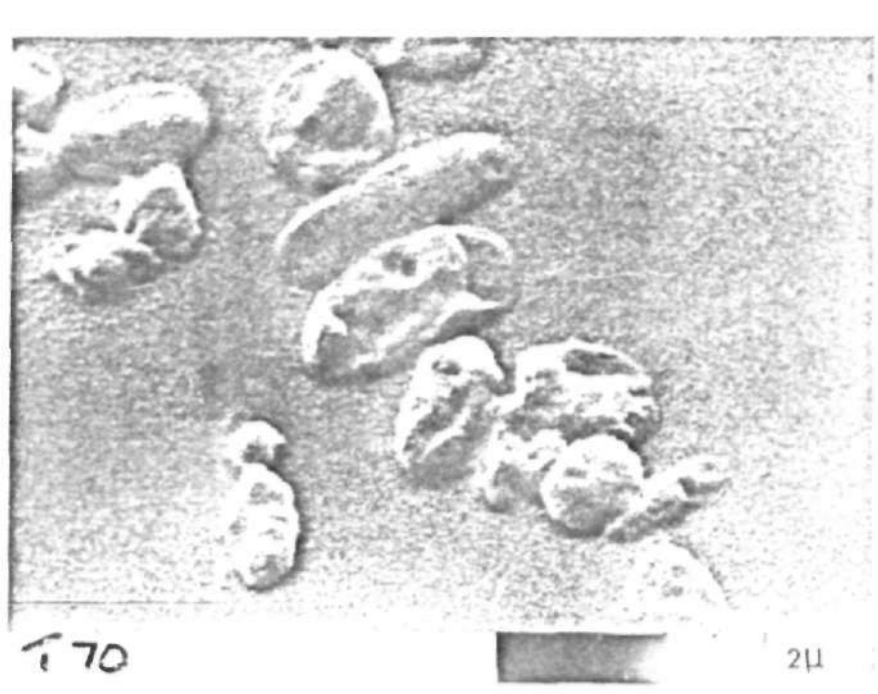
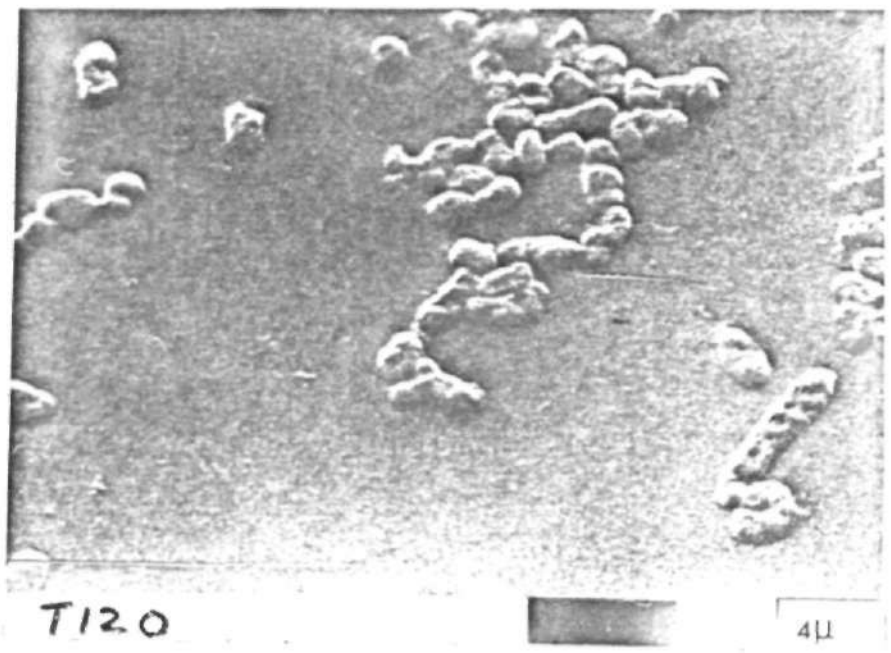


PLATE 5. Cells of E. coli, 70 minutes after the addition of 4.0×10^{-3}M chloroquine in the presence of glucose.



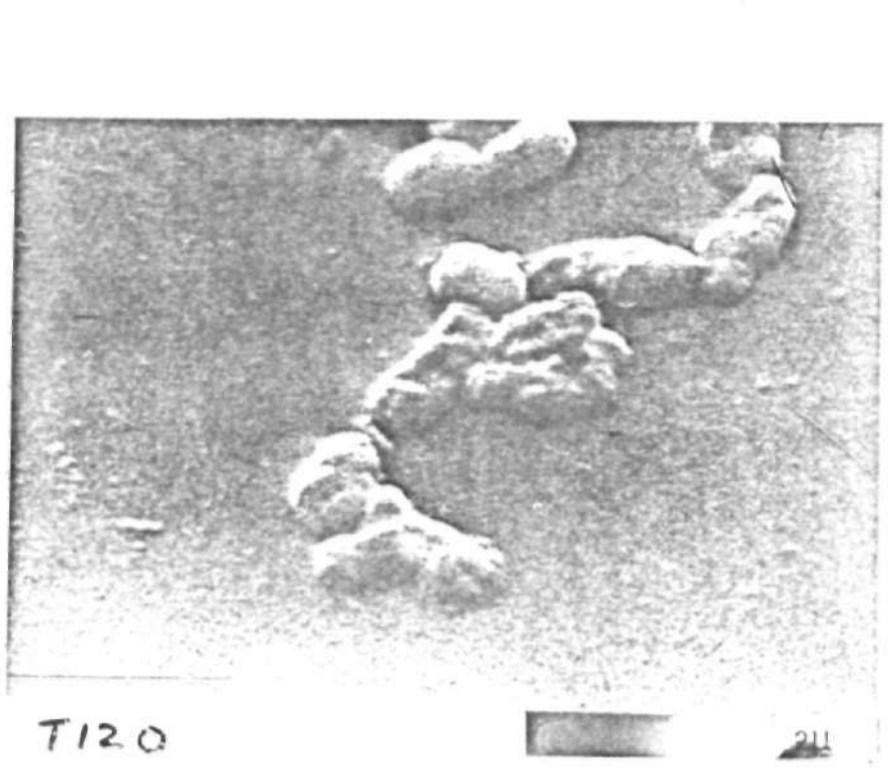
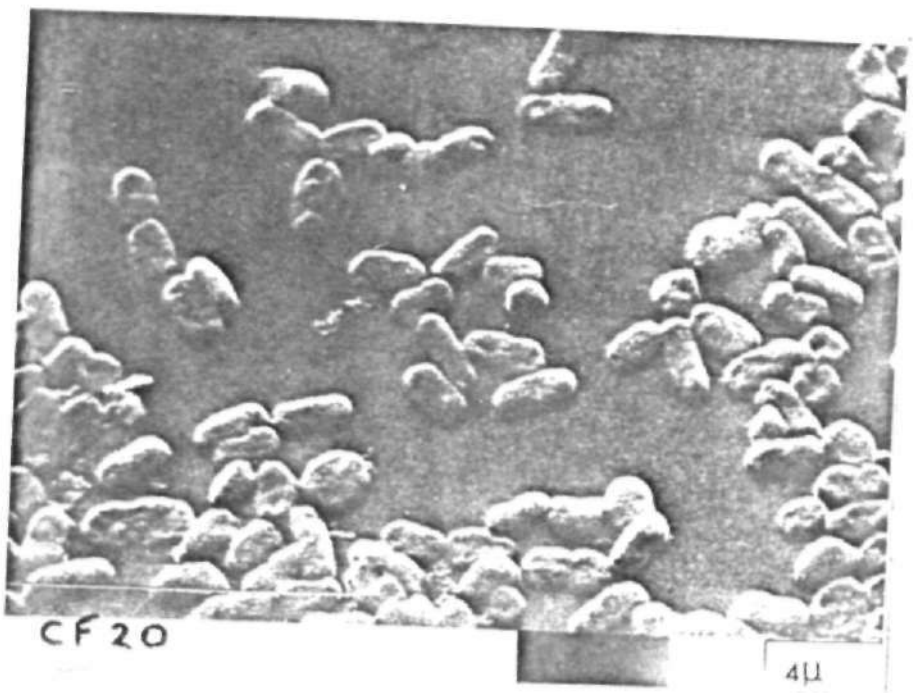


PLATE 6. Cells of E. coli, 120 minutes after the addition of 4.0×10^{-3} M chloroquine in the presence of glucose.



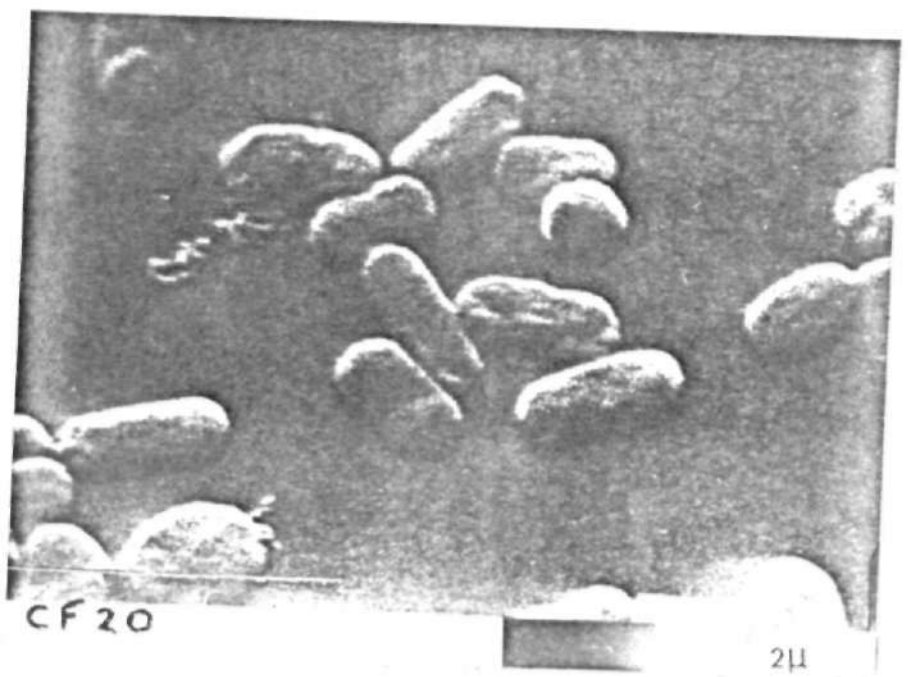
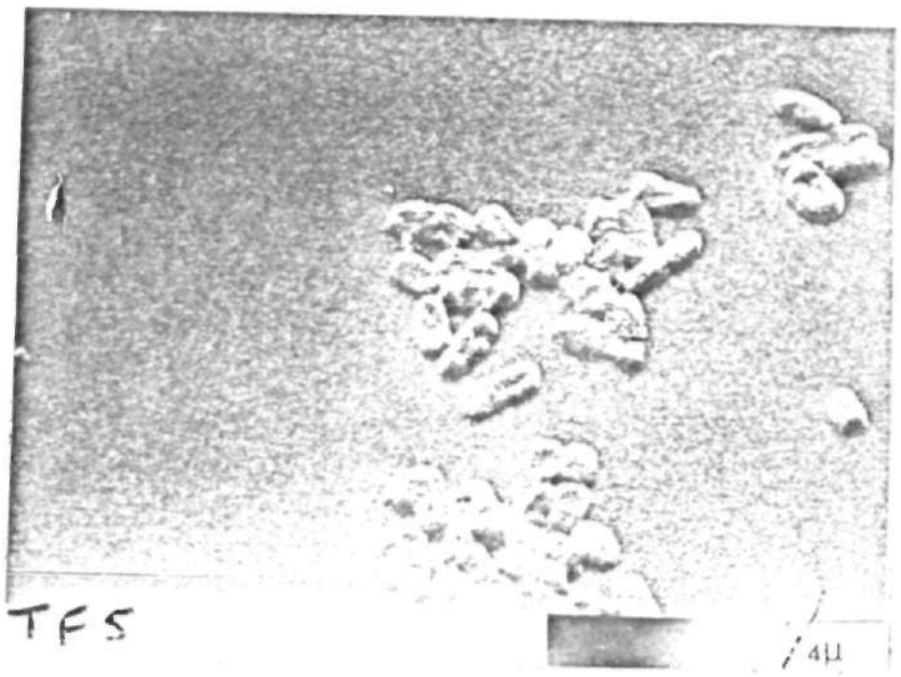


PLATE 7. Control cells of E. coli in the absence of glucose 20 minutes after commencement of experiment.



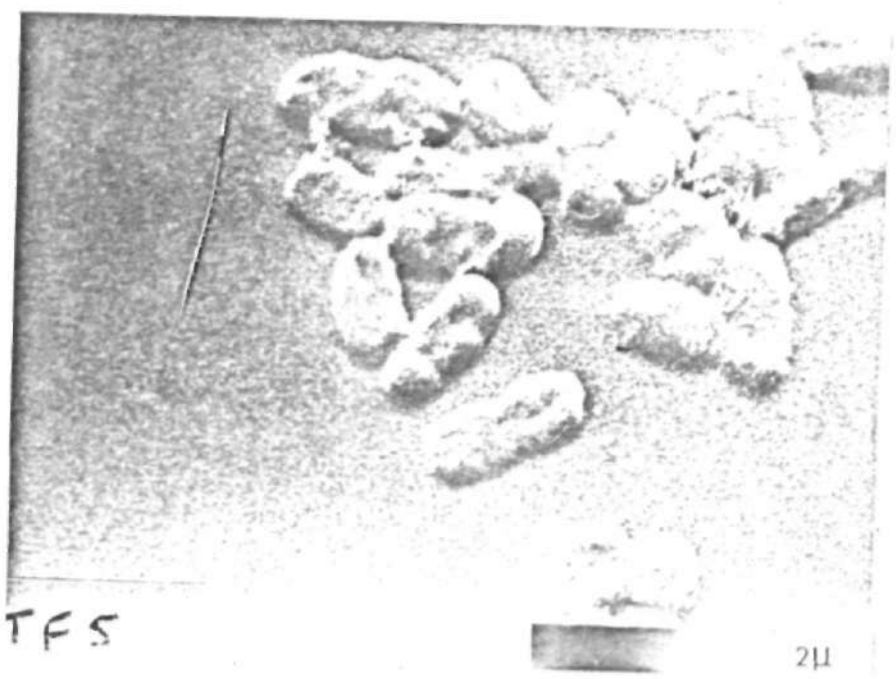


PLATE 8. Cells of E. coli, 5 minutes after the addition of 4.0×10^{-3}M chloroquine in the absence of glucose.

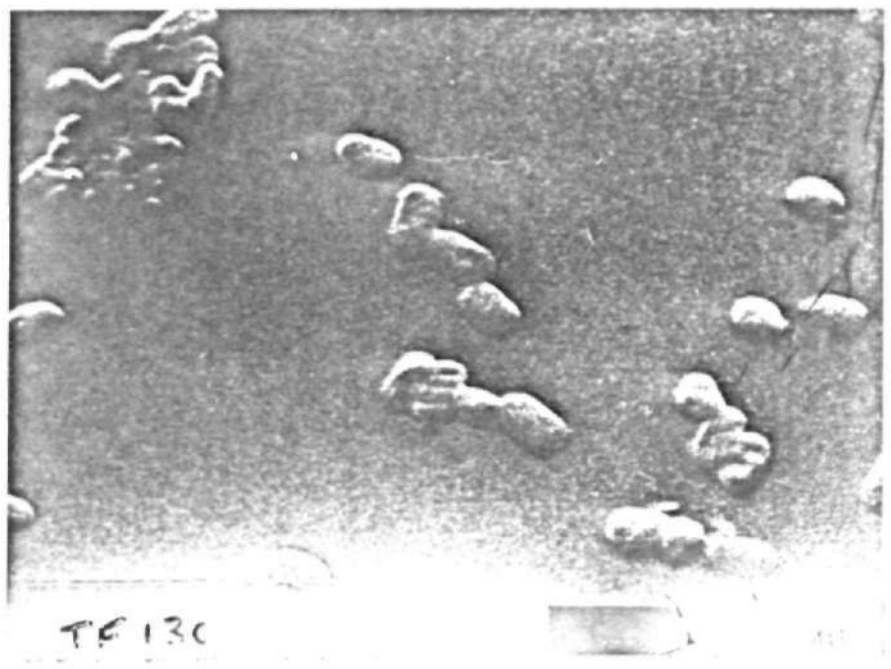


PLATE 9. Cells of E. coli, 130 minutes after the addition of 4.0×10^{-3} M chloroquine in the absence of glucose.

REFERENCES

ALI, S.N., FLETCHER, K.A. and MAEGRAITH, B.G. (1969). Effect of antimalarials on the carbohydrate metabolism of malaria parasites. Trans.R.Soc.Trop.Med.Hyg., 63, 3-4.

ANDERSAG, H., JUNG, S.B.H. (1939). Verfahren zur Darstellung von in 4-Stellung basisch substituerte Aminogruppen enthaltenden Chinolinverbindungen. Germ.Pat. 683-692, as quoted by Hahn, F.E. (1975).

AYITEY-SMITH, E. and BOYE, G.L. (1974). Effect of chloroquine on histamine-induced bronchial asthma in the guinea-pig. J.Pharm. Pharmac., 26, 208-209.

BARNER, H.D. and COHEN, S.S. (1954). The induction of thymine synthesis by T_2 infection of a Thymine requiring mutant of Escherichia coli. J. Bacteriol., 68, 80-88.

BARUFFA, G. (1966). Clinical trials in Plasmodium falciparum malaria with a long-acting sulphonamide. Trans.R.Soc.Trop.Med.Hyg., 60 222-224.

BERLINER, R.W., EARLE, D.P., TAGGART, J.V., ZUBROD, C.G., WELCH, W.J., CONAN, N.J., BAUMANN, E., SCUDDER, S.T. and SHANNON, J.A. (1948). VI. The physiological disposition, antimalarial activity, and toxicity of several derivatives of 4-aminoquinoline. J.Clin. Invest., 27, 98-107.

BOCK, E. (1939). The morphological changes in human malaria parasites through the action of atebrin. Arch.Schiffs-U. Trophen Lyg., 43, 209-214.

BOLTE, J., DEMUYNCK, C. and LHOMME, J. (1977). Synthetic models of Deoxyribonucleic Acid Complexes with Anti-malarial Compounds. Forces involved in the stacking interaction between Aminoquinoline

and the nucleotide bases. J.Med.Chem., 20 (12), 1607-1611.

BOURKE, A.T.C., PUHOMCHAREON, S., CADIGAN, F.C., GOULD, D.J., and PINSWASDI, K. (1966). Prevalence of malaria exhibiting reduced sensitivity to chloroquine in Southern Thailand. Trans.R.Soc. Trop.Med.Hyg., 60, 225-230.

BOUVIER, F. and SICARD, N. (1975). Interference of dna ts Mutations of Escherichia coli with Thymineless Death. J.Bacteriol. 214 (3), 1198-1204.

BOYCE, R.P. and SETLOW, R.B. (1962). A simple method of increasing the incorporation of thymidine into the deoxyribonucleic acid of Escherichia coli. Biochim.Biophys.Acta, 61, 618-620.

BREELAND, S.G., KLIEWER, J.W., AUSTIN, J.R. and MILLER, C.W. (1970). Observation on malathion-resistant adults of Anopheles albimanus Wiedemann in coastal El Salvador. Bull.Wld.Hlth.Org., 43, 627-631.

BRITISH NATIONAL FORMULARY (1976-78). Pharmaceutical Press, London.

BRITISH PHARMACEUTICAL CODEX (1973). Pharmaceutical Press, London.

CARLO, M. De and RUSSO, V. (1970). Applicazioni Terapeutiche Della Chlorochina.Arch.Ital.Sci.Med.Trop.Parasitol., 51 (7-12), 97-121.

CHANDRA, H. (1978). Chemical occlusion of rhesus monkey oviducts with chloroquine. Contraception, 18 (3). 233-238.

CHEMICAL ABSTRACTS (1972-1978). American Chemical Society, Columbus, Ohio.

CHO, Y.W. and AVIADO, D.M. (1968). Pathologic Physiology and Chemotherapy of Plasmodium berghei.IV. Influence of chloroquine on oxygen uptake

of Red Blood Cells infected with sensitive or resistant strains. Exptl.Parasitol. 23, 143-150.

CIAK, J. and HAHN, F.E. (1966). Chloroquine: Mode of Action. Sci., 151, 347-349.

CLIFTON, C.E. (1966). Aging of Escherichia coli. J.Bacteriol. 92, (4), 905-912.

CLYDE, D.F., McCARTHY, V.C., MILLER, R.M. and WOODWARD, W.E. (1975). Immunization of Man against falciparum and vivax Malaria by use of Attenuated Sporozoites. Am.J.Trop.Med.Hyg., 24(3), 397-401.

COATNEY, G.R. (1963). Pitfalls in a discovery: the chronicle of Chloroquine. Am.J.Trop.Med.Hyg. 12, 121-128.

COATNEY, G.R., COOPER, W.C., EDDY, N.B. and GREENBERG, J. (1953). "Survey of Antimalarial Agents." Pub.Health Monogr. No.9, U.S.A. Govt. Printing Office, Washington, D.C.

COHEN, H.G. and REYNOLDS, T.B. (1975). Comparison of Metronidazole and Chloroquine for the treatment of Amoebic Liver Abscess. Gastroent., 69, 35-41.

COHEN, S.N. and YIELDING, K.L. (1965a). Inhibition of DNA and RNA polymerase reactions by Chloroquine. Proc.Natl.Acad.Sci. USA., 54, 521-527.

COHEN, S.N. and YIELDING, K.L. (1965b). Spectrophotometric studies of the interaction of Chloroquine with Deoxyribonucleic Acid. J.Biol.Chem. 240, 3123-3131.

COHEN, S.S. (1971). On the Nature of Thymineless Death. Ann.N.Y. Acad.Sci., 186, 292-301.

COHEN, S.S. and BARNER, H.D. (1954). Studies on unbalanced growth in Escherichia coli. Proc.Natl.Acad.Sci. USA., 40, 885-893.

COLWELL, E.J., PHINTUYOTHIN, P., SADUDEE, N., BENJAPONG, W., NEOYPATI-MANONDH, S. (1972). Evaluation of an in vitro technique for detecting chloroquine-resistant falciparum malaria in Thailand. Am.J.Trop.Med.Hyg., 31, 6-12.

COMER, R.D., YOUNG, M.D., PORTER, J.A., GAULD, J.R. and MERRITT, W. (1968). Chloroquine resistance in Plasmodium falciparum malaria on the Pacific Coast of Colombia. Am.J.Trop.Med.Hyg., 17, 795-799.

CONKLIN, K.A. and CHOU, S.C. (1970). Antimalarials: Effects on in vivo and in vitro Protein Synthesis. Sci., N.Y., 170, 1213-1214.

CONKLIN, K.A. and CHOU, S.C. (1972). The effects of Antimalarial drugs on uptake and incorporation of macromolecular precursors by Tetrahymena pyriformis. J.Pharm.Exptl.Ther., 180, 158-166.

CONKLIN, K.A., HEU, P. and CHOU, S.C. (1973). The Effects of Anti-malarial Drugs on Nucleic Acid Synthesis in vitro in Tetrahymena pyriformis. Mol.Pharmacol., 9, 304-310.

CORWIN, R.M., McGHEE, R.B. and SLOAN, B.L. (1965). J.Parasit., 51, suppl. 36., as quoted by Pinder, 1973.

COVELL, G., COATNEY, G.R., FIELD, J.W., and SINGH, J. (1955). "Chemo-therapy of Malaria", World Health Organisation, Geneva.

COX, H.W., MILAR, R. and PATTERSON, S. (1968). Serologic cross-reactions of serum antigens associated with acute Plasmodium and Babesia infections. Am.J.Trop.Med.Hyg., 17, 13-18.

CRAWFORD, L.V. (1958). Thymine metabolism in strains of Escherichia coli. Biochim.Biophys.Acta, 30, 428-429.

DAVIDSON, M.W., GRIGGS, B.G., BOYKIN, D.W. and WILSON, W.D. (1977). Molecular structural Effects Involved in the Interaction of Quinolinemethanolamines with DNA. Implications for Antimalarial Action. J.Med.Chem., 20 (9), 1117-1122.

DAWES, E.A. and RIBBONS, D.W. (1962). Endogenous metabolism of Escherichia coli. Biochem.J. 82, 49p.

DOUGHERTY, P.F., YOTTER, D.L., and MATTHEWS, T.R. (1976). Chemically defined medium for susceptibility testing of antibacterial agents. Antimicrob.Agents Chemother. 10 (6), 923-925.

ECKER, R.E. and LOCKHART, W.R. (1961). Specific Effect of Limiting Nutrients on Physiological Events During Culture Growth. J.Bacteriol. 82, 511-516.

ELLIOTT, M., FARNHAM, A.W., JANES, N.F., NEEDHAM, P.H. and PULMAN, D.A. (1975). Insecticidal activity of pyrethrins and related compounds. VII. Insecticidal dihalovinyl analogues of cis and trans chrysanthemates. Pesticide Science 6, 537-542.

ELLIOTT, M., FARNHAM, A.W., JANES, N.F., NEEDHAM, P.H., PULMAN, D.A. and STEVENSON, J.H. (1973). A photostable pyrethroid. Nature, Lond., 246, 169-170.

ESLAGER, E.F., JACOB, P., JOHNSON, J., WERBEL, L.M., WORTH, D.F., RANE, L. (1978). Antimalarial drugs. 39. Folate Antagonists.13. J.Med.Chem. 21 (10¼, 1059-1070.

ESSIEN, E.E. (1978). Metabolism of chloroquine: N-Oxidation, an important

metabolic route in man and its significance in chloroquine medication. Niger.J.Pharm., 9 (2), 63-69.

FISCHER, V.W. and FITCH, C.D. (1975). Affinity of chloroquine for bone. J.Pharm.Pharmac., 27, 527-529.

FITCH, C.D. (1969). Chloroquine-resistance in malaria: deficiency of chloroquine binding. Proc.Natl.Acad.Sci. USA, 64, 1181-1187.

FITCH, C.D., CHEVLI, R. and GONZALEZ, Y. (1975). Chloroquine-resistance in Malaria: Variations of substrate-stimulated chloroquine accumulation. J.Pharm.Exptl.Ther., 195 (3), 389-396.

FITCH, C.D., GONZALEZ, Y. and CHEVLI, R. (1975). Amodiaquine Accumulation by Mouse Erythrocytes infected with Plasmodium berghei. J.Pharmacol.Exptl.Ther., 195 (3), 397-403.

FITCH, C.D., NG, R.C.R. and CHEVLI, R. (1978). Erythrocyte surface: Novel determinant of Drug Susceptibility in Rodent Malaria. Antimicrob. Agents Chemother., 14 (2), 185-193.

FITCH, C.D., YUNIS, N.C., CHEVLI, R. and GONZALEZ, Y. (1974). High-affinity Accumulation of Chloroquine by Mouse Erythrocutes infected with Plasmodium berghei. J.Clin.Invest., 54, 24-33.

FORSDYKE, D.R. (1975). Evidence for a relationship between chloroquine and complement from studies with lymphocyte mitogens: Possible implication for the mechanism of action of chloroquine in disease. Can.J.Microbiol., 21, 1581-1586.

FREEDMAN, A. and STERNBERG, V.L. (1960). Chloroquine in rheumatoid

GALVAO, A.L.A., GUSMAO, H.H., JUAREZ, E., SCHMID, A.W., RICCI, A. and MELLO, J.V. De (1961). Archos Fac.Hig.Saude. Publ.Univ. Sao Paulo, 15, 201, as quoted by Pinder (1973).

GARCIA-MARTIN (1963). Proc. 7th Int.Congr.Trop.Med.Malar., Rio de Janeiro, 5, 137, as quoted by Pinder (1973).

GARNHAM, P.C.C. (1966). "Malaria Parasites and other Haemosporidia." Blackwell, Oxford.

GEORGHIOU, G.P., ARIARATNAM, V. and BREELAND, S.G. (1972). Development of resistance to carbamates and organophosphorous compounds in Anopheles albimanus in nature. Bull.Wld.Hlth.Org., 46, 551-554.

GERSHANOVICH, V.N., BURD, G.I., BOL'SHAKOVA, T.N., ERLAGAEVA, R.S., UMYAROV, A.M. and GABRIELYAN, T.R. (1977). System of Glucose Transport and Expression in Escherichia coli. Mikrobiologiya, 46, (5), 912-919.

GIGLIOLI, G., RUTTEN, F.J. and RAMJATTAN, S. (1967). Interruption of malaria transmission by chloroquinized salt in Guayana, with observation of a chloroquine-resistant strain of Plasmodium falciparum. Bull.Wld.Hlth.Org., 36, 283-301.

GOLDMAN, L. and PRESTON, R.H. (1957). Am.J.Trop.Med.Hyg., 6, 654-657, as quoted by Thompson and Werbel, 1972.

GOOD, N.E. and IZAWA, S. (1972). Hydrogen Ion Buffers. Methods in Enzymology, 24, 53-68.

GOOD, N.E., WINGET, G.D., WINTER, W., CONNOLLY, T.M., IZAWA, S. and SINGH, R.M.M. (1966). Hydrogen Ion Buffers for Biological Research. Biochem. 5, 467-477.

GRAY, C.T., WIMPENNY, J.W.T., and MOSSMAN, M.R. (1966). Regulation of metabolism in Facultative Bacteria. II. Effects of Aerobiosis, Anaerobiosis and Nutrition on the formation of Krebs Cycle Enzymes in Escherichia coli. Biochim.Biophys.Acta., 117, 33.

GUTTERIDGE, W.E., TRIGG, P.I. and BAYLEY, P.M. (1972). Effects of chloroquine on Plasmodium knowlesi in vitro. Parasitology, 64, 37-45.

HAHN, F.E. (1975). Chloroquine, in "Antibiotics III, Mechanism of Action of Antimicrobial and Antitumour Agents", ed. Corcoran, J.W. and Hahn, F.E., Springer-Verlag, Berlin-Heidelberg - New York.

HAHN, F.E. O'BRIEN, R.L., CIAK, J., ALLISON, J.L. and OLENICK, J.G. (1966). Studies on Mode of Action of Chloroquine, Quinacrine, and Quinine, and on Chloroquine Resistance. Milit.Med., 131 (Suppl.), 1071-1089.

HARINASUTA, T., MIGASEN, S. and BOONANG, D. (1962). UNESCO First Regional Symposium on Scientific Knowledge of Tropical Parasites, p.148, as quoted by Pinder, 1973.

HARINASUTA, T., VIRAVAN, C. and REID, H.A. (1967). Sulphormethoxine in chloroquine-resistant falciparum Malaria in Thailand. Lancet, 1, 1117-1119.

HONG, D.D. (1976). Chloroquine Phosphate, in "Analytical Profiles Drug Subst.", 5, 61-85, ed. Florey, K., Academic Press, New York-San Francisco - London.

HOPKINS and WILLIAMS, Biological Buffers, 1978.

HOUBA, V. and ADAM, M. (1964). Prispevek k mechanismu pusobeni resochinu. Casopis Lekaru Ceskych, 103, 540.

HOWELLS, R.E., PETERS, W. and FULLARD, J. (1970). Chemotherapy of rodent malaria. XIII. Fine structural changes observed in erythrocytic stages of Plasmodium berghei following exposure to primaquine and menaclone. Ann.Trop.Med. Parasit. 64 (2), 203-207.

IRVIN, J.L. and IRVIN, E.M. (1947). Spectrophotometric and Potentiometric Evaluation of Apparent Acid Dissociation Exponents of Various 4-Aminoquinolines. J.Am.Chem.Soc., 69, 1091-99.

KASHKET, E.R. and WONG, P.T.S. (1969). The intracellular pH of Escherichia coli. Biochim.Biophys.Acta, 193, 212-214.

KINNAMON, K.E. and ROTHE, W.E. (1975). Biological Screening in the U.S. Antimalarial Drug Development Programme. Am.J.Trop.Med. Hyg., 24 (2), 174-178.

KNOX, J.M. and OWENS, D.W. (1966). The chloroquine mystery. Arch. Dermatol., 94, 205-214.

KRADOLFER, F. and NEIPP, L. (1958). Experimental studies on amebicidal, antibacterial, and antiparasitic phenanthroline compounds. Antibiot.Chemother., 8, 297-308.

KURNICK, M.B. and RADCLIFFE, I.E. (1962). Reaction between DNA and quinacrine and other antimalarials. J.Lab.Clin.Med., 60, 669-687.

KURODA, K. (1962). Detection and Distribution of Chloroquine Metabolites in Human Tissues. J.Pharmacol.Exptl.Ther., 137, 156-161.

LAING, A.B.G. (1968). Hospital and Field trials of sulphormethoxine

with pyrimethamine against Malaysian strains of Plasmodium falciparum and P.vivax. Med.J.Malaya, 23, 5-19.

LAING, A.B.G. (1970). Studies on the chemotherapy of malaria. I. The treatment of overt falciparum malaria with potentiating combinations of pyrimethamine and sulphormethoxine or Dapsone in the Gambia. Trans.R.Soc.Trop.Med.Hyg., 64, 562-568.

LA MONTAGNE, M.P., MARKOVAC, A. and MENKE, J.R. (1977). Antimalarials 10. Synthesis of 4-substituted Primaquine Analogues of Candidate Antimalariais. J.Med.Chem., 20 (9), 1122-1127.

LANSBURY, J. and MENDUKE, H. (1963). A double-blind comparison of the anti-rheumatic properties of amopyroquine-HCl (Propoquin) and chloroquine diphosphate. Arthritis Rheum., 6, 349-355.

LANTZ, C.H. and VAN DYKE, K. (1971). Studies concerning the Mechanism of Action of Antimalarial Drugs - II. Inhibition of the Incorporation of Adenosine-5-monophosphate-^{3}H Into Nucleic Acids of Erythrocyte-free Malarial Parasites. Biochem.Pharmac. 20, 1157-1166.

MABERTI, S. (1960). Archos.Venez.Med.Trop.Parasit.med. 3, 239, as quoted by Pinder (1973).

MACKERRAS, M.J. and ERCOLE, Q.N. (1949). Trans.R.Soc.Trop.Med.Hyg. 42. 443-454, as quoted by Peters (1970).

MACOMBER, P.B., O'BRIEN, R.L. and HAHN, F.E. (1966). Chloroquine: Physiological Basis of Drug Resistance in Plasmodium berghei. Sci., 152, 1374-1375.

MACOMBER, P.B., PRINZ, H. and TOUSIMIS, A.J. (1967). Morphological Effects of Chloroquine on Plasmodium berghei in Mice. Nature, Lond., 214, 937-939.

MARCHIAFAVA, E. and BIGNAMI, A. (1894). Translation by Thompson, J.H. from 1st edn., The New Sydenham Society, London 1-428, as quoted by Peters, W. (1970).

MARTINDALE,(1977). Extra Pharmacopoeia, 27th edn., British Pharmaceutical Press, London.

McCHESNEY, E.W., BANKS Jnr., W.F. and SULLIVAN, D.J. (1965). Metabolism of chloroquine and hydroxychlorcquine in albino and pigmented rats. Toxicol.Appl.Pharmacol. 7 (4), 627-636.

McCHESNEY, E.W., CONWAY, W.D., BANKS Jnr., W.F., ROGERS, J.E. and SHEKOSKY, J.M. (1966). Studies of the metabolism of some compounds of the 4-amino-7-chloroquinoline series. J.Pharmacol.Exptl. Ther. 151, 482-493.

McCHESNEY, E.W., FASCO, M.J. and BANKS, W.F. (1967). The metabolism of chloroquine in man during and after repeated oral dosage. J.Pharmacol.Exptl.Ther. 158, 323-331.

McDONALD, W.C. (1967). Inhibition of spore outgrowth in Bacillus subtilis by chloroquine. Can.J.Microbiol. 13, 611-613.

McGREW, S.B. and MALLETTE, M.F. (1962). Energy of maintenance in Escherichia coli. J.Bacteriol. 83, 844-850.

McKELVEY, T.P.H., LUNDIE, A.R.T., WILLIAMS, E.D.H., MOORE, H.S., and WORSELEY, D.E. (1968). Chloroquine-resistant Malaria in West Malaysia. Br.Med.J., 4, 703-704.

MEGO, J.L. and CHUNG, C.H. (1979). Effects of some antimalarials and related substances on intralysosomal proteolysis. Biochem. pharmacol., 28, 465-470.

MICHEL, R. (1968). Comparative Study of Sulphalene-pyrimethamine combination and Sulphalene alone in the chemoprophylaxis for malaria for a large group of people. Méd.trop., 28 (4), 488-494.

MIDDLETON, K.R. (1976). "Studies on the Mode of Antibacterial Action of Chloroquine". Ph.D. Thesis, University of Bradford, Bradford, England.

MIDDLETON, K.R. and WISEMAN, D. (1974). Effect of Chloroquine on the growth and viability of Escherichia coli. J.Pharm.Pharmac. 26, Suppl. 99p - 100p.

MOORE, D.V. and LANIER, J.E. (1961). Am.J.Trop.Med.Hyg., 10, 5, as quoted by Pinder (1970).

MORRIS, C.R., ANDREW, L.W., WHICHARD, L.P. and HOLBROOK, D.J. (1970). The Binding of Antimalarial Aminoquinolines to Nucleic Acids and Polynucleotides. Molec.Pharmac., 6, 240-250.

MOST, H., LONDON, I.M., KANE, C.A., LAVIETES, P.H., SCHROEDER, E.F. and HAYMAN, J.M. (1946). J.Am.Med.Assoc., 131, 963-967, as quoted by Middleton (1976).

NAGARAJAN, K. (1968). Metabolism of Plasmodium berghei. III. Carbon Dioxide Fixation and Role of Pyruvate and Dicarboxylic Acids. Exptl. Parasit., 22, 33-42.

NAKAYAMA, H. and HANAWAIT, P. (1974). Sedimentation Analysis of Deoxyribonucleic Acid from Thymine-starved Escherichia coli. J.Bacteriol., 121 (2). 537-547.

NEIDHARDT, F.C., BLOCH, P.L. and SMITH, D.F. (1974). Culture Medium for Enterobacteria. J.Bacteriol. 119 (3), 736-747.

NG, W.C., FUNG, W.P., COLBOURNE, M.J. and GILLES, H.M. (1969). Chloroquine-resistant malaria in Singapore. Ann.Trop.Med. Parasit., 63, 313-316.

NORMAN, C. and COMBE, S. (1975). Stimulatory Effect of the Lysosomal Stabilizer, Chloroquine, on the Respiration and Motility of Fresh and Aged Bovine Spermatozoa. J.Reprod.Fert., 44, 481-486.

NUSSENZWEIG, R.S., VANDERBERG, J. and MOST, H. (1969). Protective immunity produced by the injection of x-irradiated sporozoites of Plasmodium berghei. IV. Dose response, specificity and humoral immunity. Milit.Med., 134, 1176-1182.

O'BRIEN, R.L., OLENICK, J.G. and HAHN, F.E. (1966). Reactions of quinine, chloroquine and quinacrine with DNA and their effects on DNA and RNA polymerase reactions. Proc.U.S.A. Natl.Acad. Sci., 55 (6), 1511-1517.

OHKAWA, T. (1975). Studies of Intracellular Thymidine Nucleotides, Thymineless Death and the Recovery after Re-addition of Thymine in Escherichia coli K-12. Eur.J.Biochem., 60, 57-66.

OLURINOLA, P.F. (1967-1972). From experience and personal observation during hospital Pharmacy Practice.

PARKER, F.S. and IRVIN, J.L. (1952). The interaction of chloroquine with nucleic acids and nucleoproteins. J.Biol.Chem., 199, 897-909.

PETERS, W. (1970). "Chemotherapy and Drug Resistance in Malaria." Academic Press, London and New York.

PINDER, R.M. (1973). "Malaria". Scientechnica (Publishers) Ltd., Bristol.

PIRAME, Y., PATAZQ-CROUTZET, J., DEJEU, G. and SAWADOGO, R. (1968). Résultats observés après une injection unique de Sulphormethoxine dans le traitment de L'accès palustre à Plasmodium falciparum à l'hôpital de Ouagadougou (Haute-Volta). Bull.Soc.Path. exot., 61, 389-392.

POLET, H. and BARR, C.F. (1969). Uptake of Chloroquine-3-H^3 by Plasmodium knowlesi in Vitro. J.Pharmacol.Exptl.Ther., 168, 187-192.

POLET, H. and CONRAD, M.E. (1969). The Influence of Three Analogs of Isoleucine on in Vitro Growth and Protein Synthesis of Erythrocytic Forms of Plasmodium knowlesi. Proc.Soc.Exp.Biol.Med., 130, 581-586.

POLET, H., CONRAD, M.E. and BARR, C.F. (1969). In vitro studies on the amino acid metabolism of Plasmodium knowlesi and the antiplasmodial effect of the isoleucine antagonists. Milit.Med., 134, 939-944.

POPERT, A.J., MEIJERS, K.A.A., SHARP, J. and BIER, F. (1961). Chloroquine diphosphate in rheumatoid arthritis. A controlled trial. Ann.Rheum.Dis., 20, 18-35.

POWELL, R.D. and TRIGGERT, W.D. (1968). Drug resistance of parasites causing human malaria. Ann.Rev.Med. 19, 81-102.

PROUTY, R.W., and KURODA, K.J. (1958). Spectrophotometric determination and distribution of chloroquine in human tissue. J.Lab.Clin.Med., 52, 477-480.

RACHMELER, M., GERHART, J. and ROSNER, J. (1961). Limited thymidine uptake in Escherichia coli due to an inducible thymidine phosphorylase. Biochim.Biophys.Acta, 49, 222-225.

RAZDAN, R.K., BRUNI, R.J., MEHTA, A.C., WEINHARDT, K.K. and PAPANASTASSIOU, Z.B. (1978). A New Class of Antimalarial Drugs: Derivatives of Benzothiopyrans. J.Med.Chem. 21 (7), 643-649.

REITER, H. and RAMAREDDY, G. (1970). Loss of DNA behind the Growing Point of Thymine-starved Bacillus subtilis 168. J.Mol.Biol. 150, 533-548.

RICHARD, W.H.G. (1966). Active Immunization of Chicks against Plasmodium gallinaceum by Inactivated Homologous Sporozoites and Erythrocytic Parasites. Nature, Lond., 212, 1492-1494.

RIECKMANN, K.H. (1971). Determination of the Drug Sensitivity of Plasmodium falciparum. J.Amer.Med.Assoc., 217 (5), 573-578.

RIECKMANN, K.H. and LOPEZ-ANTUNANO, F.J. (1971). Chloroquine resistance of Plasmodium falciparum in Brazil detected by a simple in vitro method. Bull.Wld.Hlth.Org., 45, 157-167.

RIECKMANN, K.H., MRENA, J.E. and CAMPBELL, G.H. (1978). Malarial Immunity Induced by Infection with Cultured Parasites of Plasmodium falciparum. J.Parasitol. 64, 750-752.

RIECKMANN, K.H., TRENHOLME, G.M., WILLIAMS, R.L., CARSON, P.E., FISCHER, H. and DESJARDINS, R.E. (1974). Prophylactic activity of Mefloquine hydrochloride (WR 142490) in drug-resistant malaria. Bull.Wld.Hlth.Org., 51, 375-377.

ROSKOSKI, R. and JASKUNAS, S.R. (1972). Chloroquine and Primaquine Inhibition of Rat liver Cell-free Polynucleotide-dependent Polypeptide Synthesis. Biochem.Pharmac., 21, 391-399.

RUSSELL, A.D. (1974). Factors influencing the activity of antimicrobial agents: an appraisal. Microbios, 10, 151-174.

RYE, R.M. (1969). "Some Factors Affecting Division and Growth in Escherichia coli", Ph.D. Thesis, University of Bradford, England.

SALT, W.G. (1970). "The Uptake of CTAB by Bacteria in Relation to its Antibacterial Action." Ph.D. Thesis, University of Bradford, England.

SCHELLENBERG, K.A. and COATNEY, G.R. (1961). The influence of antimalarial drugs on nucleic acid synthesis in Plasmodium gallinaceum and P. berghei. Biochem. Pharmac., 6, 143-152.

SCHIEBEL, L.W. and MILLER, J. (1969a), Milit.Med., 134, 1074, as quoted by Pinder (1973).

SCHIEBEL, L.W. and MILLER, J. (1969b). Cytochrome oxidase activity in Platelet-free Preparations of Plasmodium knowlesi. J.Parasit., 55, 825-829.

SEDLACZEK, L., CZERNIAWSKI, E. and ZABLOCKI, B. (1965). The effect of glucose concentration on the energy balance in Escherichia coli cultures. Bull.Acad.Polon.Sci.Ser.Sci.Biol., 13 (5), 295-299.

SERVICE, M.W. (1977). Control of Malaria, in "Ecological Effects of Pesticides", Linnean Society Symposium Series, No. 5, ed. Perring, F.H. and Mellanby, K., Academic Press, London.

SHAMBLEE, D.A. and GILLESPIE Jr., J.S. (1979). Antimalarials 4. Trichloronaphthalene Amino Alcohols. J.Med.Chem. 22 (1), 86-89.

SHANNON, J.A., EARLE Jr., D.P., BERLINER, R.W. and TAGGART, J.V. (1948). The chemotherapy of human malarias. I. Method for the quantitative assay of suppressive antimalarial action in vivax malaria. J.Clin.Invest. 27 (3), 66-74.

SHERMAN, I.W. and MUDD, J.B. (1966). Malaria Infection (Plasmodium lophurae): Changes in Free Amino Acids. Sci., N.Y., 154, 287-288.

SHERMAN, I.W. and TING, I.P. (1966). Carbon Dioxide Fixation in Malaria (Plasmodium lophurae). Nature, Lond., 212, 1387-1388.

SHERMAN, I.W. and TING, I.P. (1968). Carbon Dioxide Fixation in Malaria. II. Plasmodium knowlesi (Monkey Malaria). Comp.Biochem. Physiol., 24, 639-642.

SHETTY, R.V. and BLANTON, C.D. (1978). Synthesis of 2-substituted Primaquine analogs as potential antimalarials. J.Med.Chem., 21, (9), 995-998.

SHOEMAKER, J.P. (1978). Fifty-five Percent Complete Remission of Mammary Carcinoma in Mice with 5-Fluorouracil and Chloroquine. Cancer Res., 38 (9), 2700-2702.

SIDDIQUI, W.A., KRAMER, K. and RICHARD-CRUM, S.M. (1978). In Vitro Cultivation and Partial Purification of Plasmodium falciparum Antigen Suitable for Vaccination Studies in Aotus Monkeys. J.Parasitol., 64 (1), 168-169.

SILVA, T.A. Da (1966)., Am.J.Publ.Hlth., 56, 72. as quoted by Pinder (1973).

SIU, P.M.L. (1967). Carbon Dioxide Fixation in Plasmodia and the Effect of Some Antimalarial Drugs on the Enzyme. Comp.Biochem.Physiol. 23, 785-795.

SKELTON, F.S., PARDINI, R.S., HEIDKER, J.C. and FOLKERS, K. (1968). Inhibition of coenzyme systems by Chloroquine and other Antimalarials. J.Am.Chem.Soc., 90, 5334-5336.

SKELTON, F.S., REITZ, P.J. and FOLKERS, K. (1970). Coenzyme Q. CXXII. Identification of Ubiquinone-8 Biosynthesized by Plasmodium knowlesi, P. cynomolgi and P. berghei. J.Med.Chem.Chem., 13, 602-606.

SORSBY, B-D. (1970). "Some Aspects of Chloroquine Metabolism", Ph.D. Thesis, University of Bradford, England.

STASTERS, K.C. and WINKLER, K.C. (1963). Carbohydrate Metabolism of Staphylococcus aureus. J.Gen.Microbiol. 33, 213-229.

STOLLAR, D. and LEVINE, L. (1963). Antibodies to Denatured Deoxyribonucleic Acid in Lupus Erythematosus Serum. V. Mechanism of DNA-Anti-DNA Inhibition by Chloroquine. Arch.Biochem.Biophys. 101, 335-341.

TANABE, K., CHEN, E.H., VERMA, B.L., SAGGIOMO, A.J., and NODIFF, E.A. (1978). Modification of Primaquine as Antimalarials. 2. 5-Phenylthio and 5-anilino derivatives of Primaquine. J.Med.Chem. 21 (1) 133-136.

TARAYRE, J.P. and LAURESSERGUES, H. (1978). Action of Chloroquine on pleurisy due to Bordetella Pertusis hypersensitivity. J.Pharm. Pharmac., 30, 599-600.

THEAKSTON, R.D.G., ALI, S.N. and MOORE, G.A. (1972). Electron microscope autoradiographic studies on the effect of chloroquine on the uptake of tritiated nucleosides and methionine by Plasmodium berghei. Ann.Trop.Med.Parasitol., 66, 295-302.

THEAKSTON, R.D.G., HOWELLS, R.E., FLETCHER, K.A., PETERS, W., FULLARD, J. and MOORE, G.A. (1969). The Ultrastructural Distribution of Cytochrome Oxidase Activity in Plasmodium berghei and P. gallinaceum.

Life Sci. 8 (2), 521-529.

THOMPSON, P.E. and WERBEL, L.M. (1972). "Antimalarial Agents: Chemistry and Pharmacology", Medicinal Chemistry, 12, Academic Press, New York and London.

THURSTON, J.P. (1953). The Morphology of Plasmodium berghei before and after treatment with drugs. Trans.Roy.Soc.Trop.Med.Hyg., 47, 248-256.

TURNBULL, J.D., BIAGI, G.L., MEROLA, A.J. and CORNWELL, D.G. (1971). Structure-Activity Relationship for N,N'-Bis(Dichloroacetyl) Diamines and Substituted Naphthoquinones in the Inhibition of Mitochondria Electron Transport. Biochem.Pharmac., 20, 1383-1391.

VAN DYKE, K., SZUSTKIEWICZ, C. LANTZ, C.H. and SAXE, L.H. (1969). Studies concerning the Mechanism of Action of Antimalarial Drugs - Inhibition of the Incorporation of Adenosine-8-^{3}H into Nucleic Acids of Plasmodium berghei. Biochem.Pharmac., 18, 1417-1425.

VERDRAGER, J., RICHE, A. and CHHEANG, C.M. (1969). Action de L'association diaphenylsulphone-pyrimethamine sur le paludisme a Plasmodium falciparum au Cambodge. Bull.Wld.Hlth.Org., 40, 319-324.

VERDRAGER, J., RICHE, A., and CHHEANG, C.M. (1967). Traitment du paludisme a "P. falciparum" par les sulfamides-retard., Pr.Méd. 75, 2839-40.

VICTOR, T.A., HAHN, F.E. and HANSEN, E.A. (1971). A Preliminary Nuclear Magnetic Resonance Study of the Effect of pH on the Structure of Chloroquine Diphosphate. Progr.Mol.Subcell.Biol., 2, 90-102.

WARHURST, D.C. and HOCKLEY, D.J. (1967). Mode of Action of Chloroquine on Plasmodium berghei and P. cynomolgi. Nature, Lond., 214, 935-936.

WARHURST, D.C. and WILLIAMSON, J. (1970). Ribonucleic Acid from Plasmodium knowlesi before and after chloroquine treatment. Chem.Biol.Interact., 2, 89-106.

WHITEHOUSE, M.W. (1962). Structure-action Relationships among Drugs Acting on Connective Tissues (Anti-rheumatic Agents). Nature, Lond., 194, 984-985.

WHITEHOUSE, M.W. and BOSTRÖM, H. (1965). Biochemical Properties of Anti-inflammatory Drug - VI. The Effects of Chloroquine (Resochin), Mepacrine (Quinacrine) and some other potential metabolites on Cartilage metabolism and oxidative Phosphorylation. Biochem. Pharmacol., 14, 1173-1184.

WHITEHOUSE, M.W. and BOSTRÖM, H. (1962). The Effect of Some Anti-Inflammatory (Anti-rheumatic) Drugs on the metabolism of Connective tissues. Biochem.Pharmacol., 11, 1175-1201.

WILMOT, A.J., POWELL, S.J. and ADAMS, E.B. (1959). Chloroquine compared with chloroquine and emetine combined in amebic liver abscess. Am.J.Trop.Med.Hyg., 8, 623-624.

WISELOGLE, F.Y. (1946). "A Survey of Antimalarial Drugs, 1941-1945". J. Edwards. Ann Arbor, Michigan.

WISEMAN, D. (1969). "The Mode of Antibacterial Action of Chlorhexidine." Ph.D. Thesis, University of Bradford, England.

WISEMAN, D. (1972a). The effect of pH on the inhibitory activity of chloroquine against Escherichia coli. J.Pharm.Pharmac., 24, Suppl. 162 P.

WISEMAN, D. (1972b). The Uptake of Chloroquine by Escherichia coli. J.Pharm.Pharmac., 24, Suppl., 161 P.

WOODRUFF, A.W. (1978). "Protozcal Infection and Infestation: Malaria". in "Prices Textbook of the Practice of Medicine." (ed.Scott, R.B.), 12th Ed. pp. 178-188, Oxford University Press, London.

WORLD HEALTH ORGANISATION (1971). Techn. Rep.Ser. No. 467, as quoted by Pinder (1973).

WORLD HEALTH ORGANISATION (1975). *Ecology and Control of Vectors in* Public Health. 1st Report of the WHO Expert Committee on Insecticides. Tehn.Rep.Ser. No. 585, 88pp.

YOSHINAGA, T., TSUTSUMI, Y., TSUNODA, K. and YAMAMURA, Y. (1970). Antimalarial property of 4-methoxy-6-sulphanilamido-pyrimidine monohydrate (Sulphamonomethoxine: DJ 1550) and its effect on the chloroquine-resistant malaria. Arzneim-forsch, 20, 1206-1210.

ZUCKERMAN, A., HAMBURGER, J. and SPIRA, D. (1967). Active Immunization of Rats with a Cell-free Extract of the Erythrocytic Parasites of Plasmodium berghei. Exptl. Parasitol., 21, 84-97.

ZVAIFLER, M.J. and RUBIN, M. (1962). Arthritis Rheum. 5, 330, as reported by Pinder (1973).