

THE METABOLISM OF CHLOROQUINE
PHOSPHATE BY RAT LIVER CELL FRACTIONS



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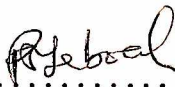
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DECLARATION

The experimental work described in this thesis was carried out by me at the Department of Biochemistry University of Ghana, and the Department of Chemical Pathology, University of Leeds. The work has been under the joint supervision of Professor G.S. Asante and Dr. Colin Toothill (external supervisor). Drs. Larway and Gyang are members of the supervisory committee.

I certify that this work has not previously been accepted for any degree, and it is not being concurrently submitted in candidature for any other degree.

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ABBREVIATIONS AND INTRODUCTORY NOTES

1. The abbreviations used in the text are as follows:-

DMSO	=	Dimethylsulphoxide
G6P	=	Glucose-6-Phosphate
G6PD	=	Glucose-6-Phosphate dehydrogenase
I. R.	=	Infrared
NAD ⁺	=	Oxidized form of Nicotinamide-adenine dinucleotide
NADP ⁺	=	Oxidized form of Nicotinamide-adenine dinucleotide phosphate
NADPH	=	Reduced form of Nicotinamide-adenine dinucleotide <i>phosphate</i>
P.P.O.	=	2-5-diphenyl-oxazol
P.O.P.O.P.	=	1,4-di-(2-(5-phenyl-oxazol)-benzene).
SKF 525-A	=	β -diethylaminoethyl diphenyl- <u>n</u> -propylacetate
TCA	=	Trichloroacetic acid
TLC	=	Thin layer chromatography
U.V.	=	Ultraviolet

2. All melting points (M.P.) are quoted in degrees centigrade and are uncorrected.
3. 'SN' stands for Survey number; and the number that follows it identifies the various drugs in the files of the survey of Antimalarial Drugs committee on Medical Research; U.S.

CONTENTS

<u>PART I</u>	<u>Page</u>
<u>INTRODUCTION AND LITERATURE REVIEW</u> ...	1
Enzymic Basis of Drug metabolism ...	4
Microsomal Drug Metabolism ...	4
Conjugation Reactions ...	6
Factors affecting hepatic microsomal enzyme activity...	7
(i) Species Variation in Drug metabolism ...	8
(ii) Environmental factors influencing Drug metabolism ...	11
(iii) The effect of sex, age, diet and nutritional status, diurnal variation and liver pathology on hepatic microsomal enzyme activity ...	19
Chloroquine ...	21
Metabolism of Chloroquine: N-dealkylation ...	22
<u>PART II</u>	
<u>MATERIALS AND METHODS</u> ...	28
Materials ...	29
General Methods ...	31
Preliminary studies: Standardization of isolation procedures ...	31
M1: Preparation of subcellular fractions of the rat liver ...	37
M2: Incubation of cell fraction with chloroquine and other 4-amino quinoline derivatives ...	41

	<u>Page</u>
M3: Extraction Procedures	... 42
M5: Chromatography	... 43
M6: Estimation of Acetaldehyde	... 43
<u>PART III:</u> RESULTS AND DISCUSSIONS	... 47
SECTION I: Metabolism of Chloroquine	... 48
Preliminary Investigations	... 48
(A) Liver incubation studies	... 48
(B) Analysis of Urine of chloroquine-treated humans	... 49
(C) Rats on chloroquine	... 64
SECTION II: Subcellular distribution of chloroquine metabolizing activity.	... 66
SECTION III: Metabolism of ¹⁴ C-labelled chloroquine phosphate by rat liver microsomes	... 71
SECTION IV: Synthesis of metabolites of chloroquine	... 75
I Introduction	... 75
I 1-hydroxy-4-pentanone oxime	... 78
2 4-amino-1-pentanol	... 84
3 4-(4'-hydroxy-1'-methylbutylamino)-7-chloroquinoline	... 84
4 Conversion of the 'Carbinol' to the 4'-aldehyde derivative	... 96
5 The chloride derivative and its conversion to metabolite E	... 97
(a) Preparation of the chloride derivative	... 97
(b) Conversion of the chloride derivative to metabolite E	... 100

SECTION V:	<u>Identification of U.V.-absorbing spots</u>	Page
	<u>and the in vitro metabolism of specific</u>	
	<u>quinoline derivatives</u>	... 108
(A)	Identification of U.V. -absorbing spots on chromatograms	... 108
(B)	<u>In vitro</u> studies on the metabolism of reference quinoline compounds used as standards in Section VA	... 111
SECTION VI:	Investigations into the mechanism of the <u>in vitro</u> degradation of chloroquine by hepatic microsomal fraction of rat liver homogenate	... 114
(A)	Distribution of products of hepatic microsomal incubation mixture of chloroquine between unchanged drug and metabolites thereof, in presence or absence of SKF 525-A	... 116
(B)	Acetaldehyde estimation in the hepatic microsomal incubation mixtures of chloroquine	... 117
(C)	Measurements of rate of oxygen consumption in hepatic microsomal incubation mixtures of chloroquine	... 119
PART IV:	CONCLUSIONS	... 120
	Suggestion for further studies	... 132
	References	... 134

LIST OF FIGURES

FIGURES	<u>Page</u>
1 METABOLISM OF CHLOROQUINE <u>IN VIVO</u> (McChesney <u>et al</u> , 1966)	... 24
2 Standard calibration curve for chloroquine phosphate in 0.1M acetic acid	... 34
3 Schematic representation for fractionation of rat liver cell components	... 38
4 Standard calibration curve for estimation of acetaldehyde (Modified method of Gupta and Robinson, 1971).	... 45
5 Ultraviolet spectrum of eluate (in 0.1M HCl in methanol) of spot I (Rf 0.04) on thin layer chromatogram of urine extract	... 53
6 Ultraviolet spectrum of eluate (in 0.1M HCl in methanol) of spot II (Rf 0.19) on thin layer chromatogram of urine extracts	... 55
7 Ultraviolet spectrum of eluate (in 0.1M HCl in methanol) of spot III (Rf 0.47) on thin layer chromatogram of urine extracts	... 57
8 Ultraviolet spectrum of eluate (in 0.1M HCl in methanol) of spot V (Rf 0.65) on thin layer chromatogram of urine extracts	... 59
9 Ultraviolet spectrum of eluate (in 0.1M HCl in methanol) of spot VIII (Rf 0.78) on thin layer chromatogram of urine extracts	... 61
10. Ultraviolet spectrum of eluate (in 0.1M HCl in methanol) of spot XII (Rf 0.89) on thin layer chromatogram of urine extracts	... 63

FIGURES		<u>Page</u>
11	Scheme for the synthesis of known metabolites of chloroquine	... 77
12	Infra red spectrum of 1-hydroxy-4-pentanone oxime in Nujol	... 80
13	Infra red spectrum of 4-amino-1-pentanol (KBr disc)	... 83
14	Infra red spectrum of the 4-hydroxy derivative: 4-(4'-hydroxy-1'-methylbutylamino)-7-chloroquinoline (in Nujol)	... 86
15	Ultraviolet spectrum of the 4'-hydroxy derivative of chloroquine in 0.1M HCl	... 88
16	Ultraviolet spectrum of the 4'-aldehyde derivative in CCl ₄	... 91
17	Infra red spectrum of the 4'-aldehyde derivative of chloroquine (metabolite E) in Nujol	... 93
18	Infra red spectrum of DMSO	... 95
19	Infra red spectrum of 4-(4'-chloro-1'-methylbutylamino)-7-chloroquinoline in Nujol	... 99
20	Infra red spectrum of 4-(4'-amino-1'-methylbutylamino)-7-chloroquinoline	... 102
21	Ultraviolet spectrum of the primary amine derivative (Fig. 19) in 0.1M HCl	... 104
22	Infra red spectrum of 4-amino-7-chloroquinoline	... 107

FIGURES

Page

- | | | | |
|----|---|-----|-----|
| 23 | Mass spectrum of eluate of spot VII on thin layer chromatogram of extract from incubation mixtures with chloroquine | ... | 109 |
| 24 | Proposed pathway for the metabolism of chloroquine phosphate | ... | 130 |

LIST OF TABLES

<u>TABLE</u>		<u>Page</u>
1	Subcellular distribution of chloroquine degradation .	
	(a) Thin layer chromatograms of ether-extract, pH 10.5, of incubation mixtures of various subcellular fraction .	67
	(b) Incubation mixtures of various subcellular fractions extracted with iso-amyl alcohol-dichloroethane (10:90) mixture, pH 10.5 .	68
2	Quantitative pattern of metabolism of (3- ¹⁴ C) chloroquine by rat liver microsomes using various extracting solvents; pH 10.5 .	73
3	(a) The identities of U.V. -absorbing areas on the chromatogram of the extract of the incubated mixture containing chloroquine and the microsome/cytoplasm system as shown by matching standards .	110
	(b) TLC analysis of the extracts of the incubated mixture containing the microsome/cytoplasm system, and a reference quinoline compound as substrate .	112

ABSTRACT

1. The metabolism of chloroquine phosphate by the various subcellular fractions of the rat liver homogenate has been studied.

(a) Both the mitochondrial and soluble fractions were found to contain enzyme systems that degraded chloroquine to varying degrees.

(b) The maximum activity of chloroquine degradation was however found in the microsomal fraction.

2. In vitro studies on the metabolism of (ring-3-¹⁴C) chloroquine led to the conclusion that the microsomal subcellular fraction was capable of breaking chloroquine down to a minimum of eight metabolites in which the 4-aminoquinoline nucleus remained intact.

3. A number of 4-aminoquinoline derivatives, including the 4'-hydroxy, the 4'-aldehyde, the primary amine (SN 13617) derivatives of chloroquine have been successfully synthesized and characterized.

4. Using the synthesized derivatives of chloroquine as chromatographic standards, four of the eight metabolites of chloroquine (see '2' above) were identified as the primary amine derivative (SN 13617), the 4'-hydroxy and the 4'-aldehyde derivatives and 7-chloro-4-aminoquinoline.

5. Based on in vitro studies on the metabolism of both chloroquine and the synthesized derivatives by the hepatic microsomal fraction of rat, it has been tentatively suggested that the metabolic degradation of chloroquine proceeds by the formation of the following metabolites, in the order:

- i) The hydroxychloroquine, HCQ (ie. the analogue of chloroquine in which a hydroxyl group has been substituted in the 2-position of N-ethyl groups).
- ii) The secondary amine derivative (SN 13616);
- iii) The primary amine derivative (SN 13617);
- iv) The 4'-aldehyde derivative, which is largely converted into the 4'-hydroxy derivative.

6. An alternative pathway for the secondary amine derivative (SN 13616) has also been proposed. Through this pathway, chloroquine may be converted through SN 13616 and an unidentified intermediate V, to a final product 4-amino-7-chloroquinoline. It has also been demonstrated that in presence of SKF 525A, the metabolism of chloroquine is apparently activated through the latter alternative pathway.

7. Evidence is also presented to show that the proposed pathway for chloroquine metabolism might be common to both the rat and man.

PART 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION AND LITERATURE REVIEW

Drug metabolism refers to the chemical alterations of a drug produced by the biological environment, and represents one aspect of the physiological disposition or fate of the agent.

One main natural function of drug metabolism is the formation of more polar and water soluble derivatives which leads to an accelerated termination of the pharmacological activity of the drug and its rapid excretion. Brodie (1964) points out that if there were no process such as drug metabolism, it would take the body about a hundred years to terminate the action of pentobarbital, which is lipid-soluble and cannot be excreted without being metabolised.

Although most drug metabolites are less biologically active than their parent compounds, in quite a few instances, a more toxic metabolite is produced. This has been shown to be true with sulphadiazine and sulphamerazine (Williams, 1959). The anti-malarial drug primaquine has also been shown to be metabolized in man to a compound of high oxidant properties, which, though an active antimalarial, is very toxic and induces hemolysis in individuals lacking G6PD (WHO, 1967).

Thus drug metabolism does not necessarily imply detoxication or loss of pharmacological activity. In numerous other instances, the administered drug is really only a "pro-drug"

which is biotransformed into a more pharmacologically active metabolite. The demethylation of imipramine to desmethylinipramine; the desulphuration of parathion to paraoxon; the oxidation of diethylene glycol to oxalate, and the conversion of α -methyldopa to α -methylnorepinephrine- are all ~~other~~ instances where drug metabolism has produced compounds of enhanced pharmacological potency.

Most drugs are metabolized in the liver, and drug metabolizing enzymes have been found to occur in the soluble, mitochondrial and microsomal fractions of the hepatic cell (La Du et al, 1971). The most common routes of drug metabolism involves oxidation, reduction, hydrolysis and conjugation (Williams, 1959). Very often a drug is subjected to several competing pathways simultaneously, and the extent of formation of the various metabolites depends on the relative rates of the various interactions. In addition, very commonly, metabolic reactions proceed sequentially, and oxidation, reduction or hydrolysis reactions are followed by conjugation of these derivatives. For instance, the alkyl side chain of a drug may be oxidized to an alcohol which then forms a conjugate with glucuronic acid; an ester may be hydrolysed to a carboxylic acid which is then coupled with glycine, or an aromatic azo compound is reduced to an amine derivative which is subsequently acetylated. Though it is not very easy to predict precisely the pathways of metabolism in a given species,

enough information is however available to the extent that by analogy, the major routes of metabolism for most drugs can be anticipated.

ENZYMIC BASIS OF DRUG METABOLISM

The general pattern of drug metabolism is common to all species. The basic pattern is usually biphasic. The initial phase of the process consists of reactions classified as oxidations, reductions and hydrolyses, and the second as syntheses (Williams, 1967). These reactions are controlled by tissue enzymes. Reactions which are classified under the initial phase are many and varied, and they are carried out by enzymes which appear to be of two kinds. The first consists of enzymes which are involved in the metabolism of the natural substrates of the body and thus have natural and foreign substrates (i.e. para-metabolic enzymes); and the second are the "drug-metabolizing" enzymes which appear to be concerned only with foreign compounds (xenometabolic enzymes) and are located mainly in the endoplasmic reticulum of the liver cell.

MICROSOMAL DRUG METABOLISM

The metabolism of a foreign compound by hepatic microsomes was first described by Mueller and Miller (1949, 1953). They showed that the microsomal fraction of a liver homogenate catalysed both the reductive splitting of the azo linkage and the oxidative N-demethylation of amino-azo dyes. Their reaction required either NADP or NAD; and molecular oxygen. Brodie et al.

(1955) later showed that a similar enzyme, localised in hepatic microsomes, was responsible for the metabolism of many other drugs; and required molecular oxygen, Mg^{++} and $NADP^+$ or NADPH generating systems viz:

G6PD/ $NADP^+$ /G6P and isocitrate dehydrogenase/isocitrate/ $NADP^+$ systems (Gillette et al, 1968).

This requirement for both a reducing agent and molecular oxygen places the microsomal drug metabolizing system within the external mixed function oxidase classification of Mason (1957, 1965) - whereby, NADPH reduces a component in the microsomes which reacts with molecular oxygen to form an 'active oxygen' intermediate. The 'active oxygen' intermediate is then transferred to the drug. Gillette (1963) formulated the overall reaction as follows:

- 1) $NADPH + A + H^+ \longrightarrow AH_2 + NADP^+$
- 2) $AH_2 + O_2 \longrightarrow \text{"Active } O_2\text{"}$
- 3) $\text{"Active } O_2\text{"} + \text{Drug} \longrightarrow \text{Oxidized Drug} + A + H_2O$

Overall Reaction



Major components of the microsomal drug metabolizing system include the following:

- 1) NADPH-cytochrome c reductase, the flavin enzyme involved in the oxidation of NADPH. This reductase is currently thought to reduce cytochrome P-450 directly or indirectly through a non-heme iron

protein or some other unidentified carrier.

- 2) Cytochrome P-450 - a hemoprotein, which, in its oxidized form, represents 'A' in equations 1 and 3 in the preceding paragraph. Evidence for the involvement of cytochrome P-450 in drug metabolism includes the fact that certain substances such as deoxycholate and steapsin (Gillette et al., 1957; Posner et al., 1961), which destroy the drug metabolizing activity of microsomes, have also been found to convert cytochrome P-450 to the inactive form of the hemoprotein, cytochrome P-420 (Omura & Sato 1964).
- 3) NADPH - cytochrome P-450 reductase which functions in the reduction of oxidized cytochrome P-450.

A wide variety of oxidation reactions that have been shown to occur in microsomes includes deamination, O-,N- and S-dealkylation, hydroxylations of alkyl and aryl hydrocarbons, epoxidation, N-hydroxylation etc. (Brodie et al., 1958; Gillette, 1963; 1966).

Conjugation Reactions: Conjugation reactions or what has been classified as syntheses may occur when the drug contains a group, usually OH, COOH, NH₂ or SH, which is suitable for combining with a natural compound provided by the body to form readily excreted water - soluble metabolites. If the drug does not contain these groups, it may acquire them through the initial phase I reactions, i.e. by oxidation, reduction or hydrolysis. Thus whereas phenol,

which contains a hydroxyl group, can undergo conjugation directly, benzene, which does not contain a group suitable for conjugation, acquires one by being oxidized to phenol in the body.

The compounds provided by the body for conjugation (i.e. conjugating agents) are derived from materials involved in normal carbohydrate, protein and fat metabolism, and include glucuronic acid, glycine, cysteine, methionine for methylation, sulphate, acetic acid and thiosulphate for sulphur. Apart from these, some species provide glucose (insects), glutamine (man), and ornithine (birds) for conjugation. The conjugating agents, however, do not, as a rule, react directly with the drug or its phase I metabolite, but do so either in an "activated form" or with an "activated form" of the drug. These activated forms are usually nucleotides, and the reaction between the nucleotide and the drug or conjugating agent as the case may be is catalysed by an enzyme. Thus for glucuronide synthesis, the glucuronic acid is used in the form of the nucleotide, uridine diphosphate glucuronic acid (UDPGA) which, under the influence of the enzyme glucuronyl transferase, transfers its glucuronyl residue to the drug.

Factors Affecting Hepatic Microsomal Enzyme Activity

Both physiological and environmental factors are known to influence the most likely metabolic pathways of an agent. These are listed below ~~and only two have been discussed in detail.~~ and only two have been discussed in detail.

- (1) Species and strain of animal used.
- (2) Interaction of other drugs and environmental contaminants.
- (3) Age, sex and weight of animal.
- (4) Dose and frequency of administration of the drug.
- (5) Diet and nutritional status.
- (6) Route of administration.
- (7) Time of administration (circadian ^{rhythm} ~~system~~, light, seasonal changes).
- (8) Subsequent interaction with other enzymes (~~p~~-glucuronidase, sulph^aase, deacetylase for which drug conjugates may be substrates).
- (9) Pregnancy and physiological abnormalities.

(1) Species Variation in Drug metabolism

Species variation in the metabolism of foreign compounds ^{is} ~~are~~ not unexpected, since such variations often occur in the metabolism of some natural metabolites of the body and are explicable in enzymic terms. For instance, the inability of man to synthesize L-ascorbic acid is due to the lack of the liver (or kidney in some other species) microsomal enzyme, L-gulonolactone oxidase, which converts L-gulonolactone to 2-Keto-L-gulonolactone which then isomerises to ascorbic acid (Chatterjee et al., 1961).

Species variation in metabolism of a drug may appear as:

(a) qualitative differences in the actual pathways of metabolism and/or; (b) quantitative differences in pathways of metabolism which are common to several species. The qualitative differences probably result from the presence in a particular species of an enzyme which is not found in others, or the absence in a particular species of an enzyme otherwise generally distributed in the animal world. Quantitative differences could be due to variations in the amount of an enzyme or its inhibitor, or to the occurrence of an enzyme possibly located in another tissue, reversing the reaction, or to an enzyme competing for the same substrate. Thus the in vitro demethylation of ethylmorphine by liver microsomal preparations of mouse, rat, guinea pig and rabbit shows a four fold variation in rate, the mouse preparation being the most active and the rabbit the least active (Davies et al, 1969). These workers also showed that at the enzymic level, species differences parallel differences in NADPH-cytochrome P-450 reductase and may be related to the rate of reduction of the cytochrome P-450 substrate complex in different species.

Although the main causes of species variation in drug metabolism seem to be associated with the tissue enzymes, it has been found that other factors such as the gastrointestinal flora may be involved in certain cases (Scheline, 1968). In spite of the fact that the gut bacteria are common to many

species, they have been found to vary in numbers and location in the intestine of different species and they could vary within the same species according to the nature of the diet. Thus, since many foreign compounds are taken orally, it is possible for species variation in the metabolism of some compounds to depend partly upon the gut flora. A striking example of species variation influenced by gut bacteria is to be found in the metabolism of homoprotocatechuic acid. This acid is dehydroxylated to m-hydroxyphenyl ~~acetic~~ ^{acetic} acid in the rat and rabbit, but the excretion of this metabolite is suppressed almost to zero if the animals are pretreated with antibiotics (Williams, 1971).

Aromatic dehydroxylation of this compound seems therefore to depend on the gut flora. Homoprotocatechuic acid, however, is also methylated in these animals to 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) but the output of this metabolite is unaffected by pretreatment with antibiotics. The rabbit dehydroxylates twice as much as the rat (Dacre et al., 1968) a clear indication that the rabbit differs from the rat in its gut flora (Scheline, 1968), and that species differences in the extent of dehydroxylation apparently depend on the gut flora.

Attempts to explain species variation in drug metabolism are usually made through in vitro studies; though these studies serve as an important guide to what happens in vivo, it is desirable that both types of studies are made together so that such factors as absorption, tissue distribution, biliary and urinary excretion, enterohepatic circulation and the role of gut bacteria can be taken into account in the overall picture of species variation.

Species variation in the conjugation reactions depends on the occurrence of the conjugating agent or the ability to form the necessary nucleotide or the amount of the transferring enzyme. It is well known for example, that the cat has a defect in glucuronide synthesis, a defect not in the animal's inability to provide glucuronic acid, or to make UDPGA, but in a deficiency of the enzyme glucuronyl transferase (Williams, 1971). Man is peculiar in being able to convert phenylacetic acid into phenylacetylglutamine. The rat is unable to do this because although it can make the intermediate nucleotide derivative phenylacetylCoA, it does not possess the necessary transferring enzyme which, however, occurs in the human liver and kidney (Williams, 1971).

(2) Environmental factors influencing Drug metabolism

Studies have shown that the duration and intensity of action of many drugs in animals depend on the activity of

drug-metabolizing enzymes localized in the microsomal fraction of liver homogenate which is derived from the endoplasmic reticulum of the liver cell. Many drugs and environmental chemicals stimulate or inhibit microsomal enzyme function in animals, and this is reflected in vivo by an altered metabolism and action of drugs, carcinogens and various normal body constituents, such as steroid hormones, thyroxine and bilirubin (Conney, 1967; Conney and Kuntzman, 1971). Chemicals found in man's environment that alter the action of drugs by stimulating microsomal enzyme activity in animals include halogenated hydrocarbon insecticides (Hart and Fouts, 1965; Conney et al, 1967), urea herbicides (Kinoshita and DuBois, 1970), volatile oils (Jori et al, 1970), polycyclic aromatic hydrocarbons (Conney 1967; Arcos et al, 1961, Welch et al, 1969; Nerbert et al 1969), dyes used as colouring agents (Radomski, 1961; Levin and Conney, 1967), nicotine and other alkaloids (Wenzel and Brodie, 1966), food preservatives (Gilbert and Golberg, 1965); Creaven et al, 1966); Botham et al, 1970), Xanthines (Mitoma et al, 1969), flavones (Miller et al, 1954); that occur in food.

The stimulatory effect of halogenated hydrocarbon insecticides on drug metabolism was discovered accidentally by Hart and Fouts (1965), after their animal living quarters were treated with chlordane. Studies on the metabolism and action

held up
 of drugs were ~~disrupted~~, and the effects of chlordane persisted for several weeks, even though insecticide treatment had stopped. Examples of other insecticides that stimulate drug metabolism in rats include DDT, methoxychlor, aldrin, dieldrin, heptachlor and benzene hexachloride (Conney et al, 1967). People exposed to DDT and lindane in a pesticide factory metabolized antipyrine about twice as rapidly as control population (Kolmodin et al, 1969). The effect of intensive occupational exposure to only DDT on drug and steroid metabolism has also been studied. In a population of DDT - factory workers with a serum and fat concentration of DDT-related substances 20 to 30-fold higher than control population, the half life of phenylbutazone in the serum was 19 percent lower, and the urinary excretion of 6 β -hydroxycortisol was 57 percent higher than in the control population (Poland et al, 1970).

The stimulatory effect of polycyclic aromatic hydrocarbons on the metabolism and action of drugs in animals is evident from the observation that the smoking of cigarettes enhances the metabolism of nicotine in man (Beckett and Triggs, 1967), lowers the concentration of phenacetin in the plasma (Pantuck et al, 1972) and induces enzymes in the placenta that hydroxylate benzo(a)pyrene (Welch et al, 1971), (Welch et al, 1968). Benzo(a)pyrene and other polycyclic hydrocarbons are present in cigarette smoke.

Environmental chemicals that inhibit microsomal function in animals include organophosphorus insecticides (Welch et al, 1967; Welch et al, 1969), carbon tetrachloride (Dingell and Heimberg, 1968; Levin et al, 1970), Ozone (Palmer et al, 1971), and carbon monoxide (Cooper et al, 1965; Conney et al, 1968). Changes in atmospheric pressure and respiratory oxygen can also influence drug metabolism (Kitawaga, 1968; Merritt and Medina, 1968).

The persistent administration of one drug can also affect the pharmacologic activity of another drug by stimulating or inhibiting its metabolic inactivation. Patients are often given several drugs at the same time and sometimes the combination results in an undesirable effect because one drug may inhibit or stimulate the metabolism of the other. For example, phenylbutazone and chloramphenicol inhibit the metabolic inactivation of tolbutamide in man, and this effect can result in hypoglycemia. Phenobarbital, on the other hand, decreases the action of other drugs by increasing the rate at which they are metabolized to pharmacologically inactive substances.

The magnitude of the change in drug action that may occur after enzyme induction in animals is illustrated by data obtained from rats given the muscle-relaxant drug zoxazolamine, which is metabolized by the liver microsomal hydroxylase to the inactive compound, 6-hydroxyzoxazolamine. A high dose of

zoxazolamine paralyses rats for more than 11 hours, but if they are treated with phenobarbital for 4 days before being given zoxazolamine, they are paralysed for only 105 minutes; if they are treated with benzopyrene 24 hours before the test they are paralysed for only 17 minutes with the same dose of zoxazolamine (Conney et al, 1960).

Ethanol, when consumed habitually, stimulates its own metabolism and the metabolism of various drugs in man. Alcoholics have been shown to metabolize tolbutamide more rapidly than nonalcoholics (Kater et al, 1969), and Conney and Burns (1972) have suggested that this should be considered when alcoholics are given tolbutamide for the treatment of diabetes. Alcoholics also have an increased concentration of the hepatic enzyme that metabolizes pentobarbital and other barbiturates and sedatives (Rubin and Lieber, 1968), and this helps to explain the increased tolerance of alcoholics to barbiturates and other sedatives when sober. A single large dose of ethanol, however, inhibits drug metabolism and this effect - in addition to the central depressant action of ethanol - helps explain the enhanced sensitivity of inebriated individuals to barbiturates and other sedatives (Rubin and Lieber, 1971).

Not only does long-term administration of drugs stimulate the metabolism of other compounds, but often the drug stimulates its own metabolism. Examples of drugs that stimulate their own metabolism in dogs include phenylbutazone, tolbutamide, probenecid, chlorcyclizine and hexobarbital (Welch et al, 1967). Welch and his associates actually demonstrated the accumulation of a demethylated metabolite, norchlorcyclizine, after ^{persistent} ~~continuous~~ administration of the parent compound - chlorcyclizine, to dogs. The development of tolerance to glutethimide in man results from the ability of glutethimide to stimulate its own metabolism and this may be also true for ethanol and meprobamate (Douglas et al, 1963; Schmidt et al, 1964). Tolerance to narcotics such as morphine and meperidine, on the other hand, does not result from their metabolism being enhanced (Conney and Burns, 1972).

The same factors that affect the metabolism of drugs in man have been shown to influence the metabolism of steroids (Conney, 1967; Conney and Kuntzman, 1971), lipid-soluble vitamins (Dent, et al, 1970; Rowe and Richens, 1970a; Hunter et al, 1971; Bergstedt et al, 1972; Halm et al, 1972) and other normal body constituents (Maurer et al, 1968). Treatment of rats with phenobarbital for several days increased the activity of enzymes in liver microsomes that hydroxylate androgens, estrogens, progestational steroids and adrenocortical

steroids (Levin et al, 1968). Several structurally unrelated chemicals that stimulate the activity of drug metabolizing enzymes also stimulate steroid hydroxylase activity. Examples of such compounds include phenobarbital, diphenylhydantoin, chlorcyclizine, norchlorcyclizine, orphenadrine, phenylbutazone and several halogenated hydrocarbon insecticides (Conney and Klutch, 1963; Kuntzman et al, 1964; Conney et al, 1966; Levin et al, 1968; Welch et al, 1971b). Conney and Burns (1972) have even suggested that the effect of insecticides on fertility in animals may be explained, at least, in part, by the induction of enzymes that metabolize progesterone and other steroids. Compounds that inhibit drug-metabolizing enzymes in liver microsomes also inhibit steroid metabolism and augment the action of steroids in animals. SKF 525-A, which potentiates the action of many drugs by inhibiting their microsomal metabolism (Axelrod et al, 1954), also potentiates the action of estrone on the rat uterus and the induction by cortisol of hepatic tyrosine transaminase in the rat (Kupfer and Peets, 1967).

Symptoms of vitamins D and K deficiencies have been observed following therapy with anticonvulsant drugs (Rowe and Richens, 1970b). Long term anticonvulsant therapy was associated with osteomalacia and with reduced concentration of calcium and elevated concentrations of alkaline phosphatase

in the serum; suggesting that drug-mediated enzyme induction might be responsible for the osteomalacia by causing increased metabolic inactivation of vitamin D (Dent et al, 1970; Rowe and Richens, 1970a). Administration of vitamin D to patients receiving anticonvulsants resulted in a return towards normal concentrations of calcium and activity of alkaline phosphatase in the serum. Further evidence that enzyme induction can decrease the action of vitamin D came from the finding that rats treated with phenobarbital are protected from loss of weight, hypercalcemia and renal calcinosis produced by the administration of a single large dose of calciferol (Rowe and Richens, 1970b).

Enzyme induction has also been explored for the treatment of certain metabolic diseases such as hyperbilirubinemia. The administration of barbiturates to animals enhances the enzymic glucuronidation of bilirubin by liver microsomes, stimulates bile flow, and accelerates the metabolism of bilirubin in vivo (Catz and Yaffe, 1962, Roberts and Plaa, 1967). Studies in man indicate that long term administration of phenobarbital lowers the concentration of bilirubin in the serum of patients with chronic intrahepatic cholestasis (Gold and Crigler, 1969) and in the serum of jaundiced infants (Gold and Crigler, 1966; 1969; Yaffe et al, 1966; Stern et al, 1970; Thompson and Williams, 1967; Arias et al, 1969). The decrease in plasma concentration

of bilirubin in subjects treated with phenobarbital was associated with a decreased half-life of bilirubin (Gold and Crigler, 1969). The effectiveness of phenobarbital in lowering the concentration of serum bilirubin in infants with congenital non-hemolytic jaundice suggested that these infants did not have a complete genetic block in their ability to synthesize the enzymes involved in bilirubin metabolism. This observation further suggests that "genetic diseases" might be treated with suitable enzyme inducers.

(3) The effect of sex, age, diet and nutritional status, diurnal variation and liver pathology on the hepatic microsomal enzyme activity.

The activity of drug metabolizing enzymes have been demonstrated to be sex-dependent in rats. For example, the liver microsomal fraction of male rats has higher oxidizing activity than that from females towards a number of substrates such as hexobarbital (Quinn et al, 1958) pentobarbital (Kuntzman et al, 1966), ethylmorphine (Davies et al, 1968) and meperidine (Axelrod, 1956). Such sex-dependent differences were shown to disappear during starvation (Kato and Gillette, 1965). Further differences in metabolism between sexually mature and immature animals have also been demonstrated.

The diet and nutritional status also influence the activity of the hepatic microsomal drug metabolizing enzymes; McLean and McLean (1966) found a loss of hydroxylating enzyme activity from the liver microsomes when rats were placed on a protein-free diet for 4 days. Rubin et al (1968), however found that, when rats were kept on a diet deficient in choline, casein, methionine and cysteine for 15 days there was an increase in microsomal aniline hydroxylase and nitro-reductase.

New born animals have little or no ability to metabolize drugs (Jondorf et al, 1959; Fouts and Adamson, 1959). However, treatment of new born rabbits with pentobarbital has been shown to enhance the ability of their liver enzymes to metabolize drugs like aminopyrine (Fouts and Hart, 1963),

Davies (1962) found a diurnal variation in the response to pentobarbital by mice. Vessel (1968) using hexobarbital confirmed this finding and demonstrated that sleeping time was longer and that hexobarbital oxidase activity was lower during the light than the dark periods.

The activities of hepatic microsomal drug metabolizing enzymes also depend on the liver pathology; and the activities of such enzymes as aminopyrine demethylase have been shown to decrease by as much as 60% in tumour-bearing male and female rats (Kato et al, 1968).

CHLOROQUINE

Chloroquine is a derivative of 4-amino-quinoline and has the chemical formula $C_{18}H_{20}ClN_3$. It is the drug of choice for the treatment of acute malaria. Chloroquine is also frequently employed in the treatment of collagen diseases especially rheumatoid arthritis and discoid lupus erythematosus (Goldman and Preston, 1957); in addition, it has been successfully used in the treatment of extra-intestinal amoebiasis (Conan, 1948; Lane, 1951), clonorchis infection and several other parasitic diseases. Its use in the treatment of bronchial asthma (Juul-Moller, 1961; Khoryavster, 1966) and in the control of epilepsy (Burns, 1966) has also been reported.

For treatment of an acute malaria, an initial dose of 1.0g is administered immediately. This is followed by 4 doses of 0.5g at intervals of 6-8 hours to make a total of 3.0g within three days (British Pharmaceutical Codex, 1968). As an anti-malarial prophylactic, about 5mg of base/kg body weight is taken once weekly for many years. In the treatment of collagen diseases which usually extends over many years, the daily dosages are 3.5 - 14 mg/kg body weight (Chinyanga et al, 1971). unmetabolized drug.

Metabolism of Chloroquine: N-dealkylation

Many drugs which are alkylamines undergo dealkylation in the body. Such compounds as ephedrine (Axelrod J. 1953), codeine, aminopyrine (Brodie and Axelrod, 1950), morphine (Elliot et al, 1954), meperidine (Burns et al, 1963), methylamphetamine (Axelrod, 1954) and mephobarbital (Butler, 1952), are all demethylated in vivo; the removal of ethyl groups has also been demonstrated with quinacrine (Williams, 1959). The transformation of the alkylamine side chain of chloroquine is therefore not unexpected.

Titus et al (1948) investigated the metabolism of chloroquine and several of its analogues in man, partly in an attempt to identify metabolites with higher levels of anti-malarial activity. They identified two processes of detoxification, the first involving conjugation with a uronic acid, and, the second being the successive removal of side chain groupings down to the quinoline nucleus which remained intact (Fig. 1).

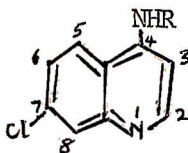
Kuroda (1962) and McChesney et al (1967) have provided experimental evidence from the study of urine extracts from human volunteers receiving chloroquine that the metabolic transformation of chloroquine in vivo results in the formation of:

1. The secondary (2°) amine (SN 13616)
2. The primary (1°) amine (SN 13617)

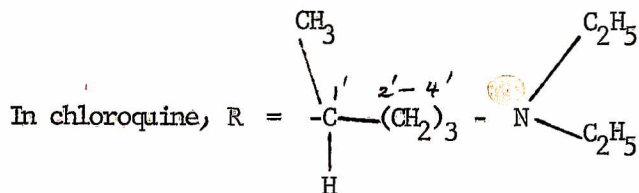
3. The 4'-aldehyde which, to a minor extent, is apparently reduced to the alcohol, in the monkey, this is principally converted to the 4'-carboxy compound.
4. The 4-amino-7-chloroquinoline which appears as a minor degradation product of chloroquine.

The structural formulae of these compounds are represented below according to the nature of the side chain in 4-amino-7-chloroquinoline.

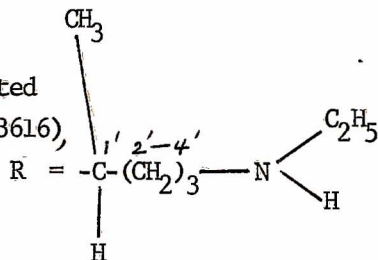
FIGURE I: Metabolites of Chloroquine in vivo
(McChesney et al., 1966)



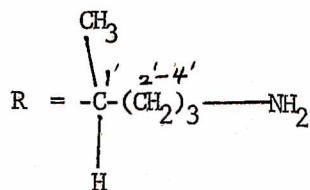
In 4-amino-7-chloroquinoline, R = H



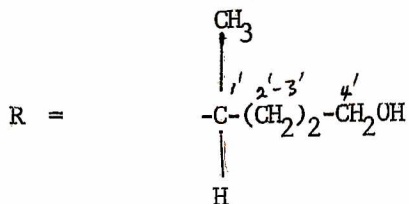
In mono-deethylated
derivative (SN 13616),



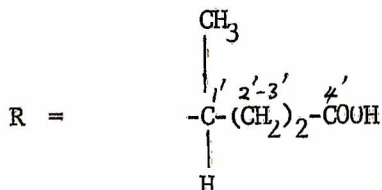
In di-deethylated
derivative,
(SN 13617)



In the 4'-Hydroxy derivative



In the 4'-carboxy derivative



The mono-ethyl derivative (SN 13616) has been shown to be the major metabolite (McChesney et al, 1954).

Chloroquine is slowly degraded in man, and plasma levels up to 52% of that measured at 3 hours after a normal therapeutic dose were found 5 days later (Alving et al, 1948); Berliner et al, 1948).

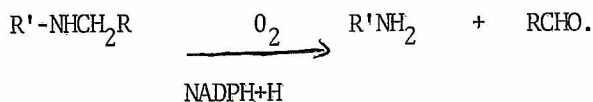
Muting (1962) compared the metabolism of the sulphate and the diphosphate of 4'-hydroxychloroquine in patients with chronic polyarthritis, and found that serum concentrations of the sulphate at various times up to 48 hours after administering a single dose of 400 mg were about 3 times higher than those of the diphosphate following the same dose. In each case, peak ~~1 sample was recorded after~~ 2 hours. The parent compound showed

levels of only 2-3 mg, whereas metabolites accounted for some 10-20%. These findings differ from those of Kuroda (1962), whose analyses revealed that, at least in solid tissues, the main compound present was the parent substance - chloroquine. Williams (1959) pointed ~~out~~^{not} that [^]only chloroquine but also some of its metabolites are potent antimalarials and probably the latter contribute to its total antimalarial activity.

The urinary excretion of chloroquine is slow and discontinuous (Zanca and Benatti, 1959). Three to five years after the last known injection of chloroquine, patients were found to be excreting the drug in the urine. The proportions of drugs and metabolites excreted in the urine following daily administration to man of chloroquine or hydroxychloroquine have been estimated as: parent compound 60%; 2^o amine (SN 13616) 37%, and 1^o amine (SN 13617) 3% (McChesney et al, 1966).

The biochemical mechanism responsible for the removal of alkyl groups have been studied in greater detail. Liver enzyme systems for N-dealkylation have been demonstrated by several authors (Gram and Fouts 1966; In the rabbit, rat and the mouse, the enzyme activity is known to be localized in the microsomal fraction of the cell with requirements for NADPH and oxygen. These

enzyme systems were shown by Gaudette and Coatney (1961) to be non-specific, as they are able to oxidatively dealkylate a whole spectrum of compounds with the concomitant production of aldehydes (Williams, 1959). This finding has been confirmed by later workers. If R' and R are aromatic and alkyl groups respectively, a typical dealkylation process is summarised as:



Little work has been done on the in vitro enzymic degradation of chloroquine to elucidate the formation of its metabolites. The only evidence that chloroquine is degraded by a microsomal enzyme system has come from the work of Gaudette and Coatney (1961) who reported a decrease in chloroquine levels following incubation with the 9,000 x g supernate of rabbit liver homogenates. They determined chloroquine levels by fluorimetry using the Aminco-Bowman spectrophotofluorimeter; and showed that various compounds including SKF 525A (Beta-diethylaminoethyl diphenylpropylacetate-HCl) and a number of other antimalarial drugs such as amodiaquine, constitute effective in vitro inhibitors to the metabolic transformation of chloroquine. They did not, however, report on the formation of any metabolites in their reaction system.

The present investigation is the first ~~very~~ detailed study⁹ on the in vitro degradation of chloroquine by preparations

PART II

MATERIALS AND GENERAL METHODS

MATERIALS AND METHODS

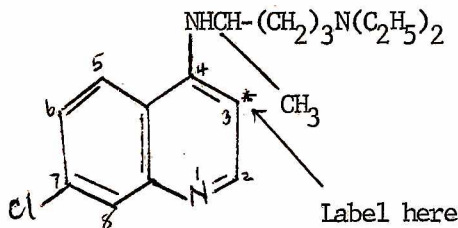
MATERIALS

Animals: Adult male Wistar albino rats (150-300g body weight) were used throughout.

Reagents: Unless otherwise stated, all chemicals were of A.R. grade and purchased from B.D.H.

Iodoplatinate reagent (Warhurst, 1973): One millilitre of 1% (w/v) platinum chloride and 25ml of 40% (w/v) KI were mixed and made up to 50 ml with distilled water.

(ring-3-¹⁴C) chloroquine (as phosphate) was purchased from New England Nuclear Corporation, Frankfurt, West Germany.



(ring-3-¹⁴C) chloroquine.

Instruments: U.V. and infrared measurements were made on a Unicam SP 8000 and SP 200 spectrophotometers respectively. Centrifugations were done in an M.S.E. 50 Ultracentrifuge.

All homogenisations were carried out with a Potter-homogeniser, using a teflon pestle. The type of homogeniser used has been shown to influence the total yield of microsomal drug metabolizing enzyme activity, and attention has been drawn to this fact (Von Jagow et al, 1965).

Thin layer silica gel plates with fluorescing indicator (Kieselgel 60 F₂₅₄) were used unless otherwise stated.

Radioactivity was counted in a liquid scintillation counter (Packard model 3380) which was operating at 60% efficiency.

GENERAL METHODS

Preliminary Studies: Standardization of Isolation Procedures

One problem facing investigators of drug metabolism lies in isolation and identification procedures for the drug and its metabolites from tissues and fluid systems. Attention was therefore directed towards standardizing the following procedures:

- I Solvent for extracted samples
- II Solvent system for extracting chloroquine from body tissues
- III Solvent systems for developing chromatograms.

Preliminary experiments involving the use of paper chromatography, to isolate and identify chloroquine, were based on the methods described by Kuroda (1962) and Clarke (1969).

I. Solvent System for Extracted Samples

1% Solutions of chloroquine phosphate in ethanol, 2M-acetic acid, 2M-HCl, 2M-NaOH and methanol were prepared. Five μ l portions of each solution were applied on Whatman No.1 filter paper. The papers were dried and chloroquine was detected by its fluorescence (light purple) under U.V. light, and also from the brown complex formed when spots of chloroquine are sprayed with iodoplatinate.

It was observed that chloroquine was sparingly soluble in ethanol and 2M NaOH and that spots corresponding to chloroquine in these solvents did not fluoresce under U.V. light. This was probably because the solubility of chloroquine in these solvents was too low to fall within the limit of detection.

Chloroquine was however soluble in methanol, 2M-HCl, and 2M acetic acid, and spots corresponding to chloroquine in these solvents fluoresced strongly under a U.V. lamp. These solvents or mixtures of them were therefore used to dissolve the chloroquine and/or metabolites in the extracts.

II. Choice of Solvent System for Extracting Chloroquine from Body Tissues and Fluids

Warhurst (1973) recommended extraction of the basic derivatives of chloroquine at pH 10 to 11 with n-hexane; whilst McChesney et al (1966, 1967) have successfully extracted chloroquine and its metabolites at pH 11, using an ethylene dichloride: iso-amyl alcohol (90:10) mixture. The latter authors also used ether for extracting chloroquine and its metabolites at pH 10 to 11 from body tissues.

The reliability of these extraction procedures was assessed by determining the percentage recovery of chloroquine from 5 ml of a solution containing 10 micromoles of chloroquine using each of the three possible extraction solvents.

FIGURE 2

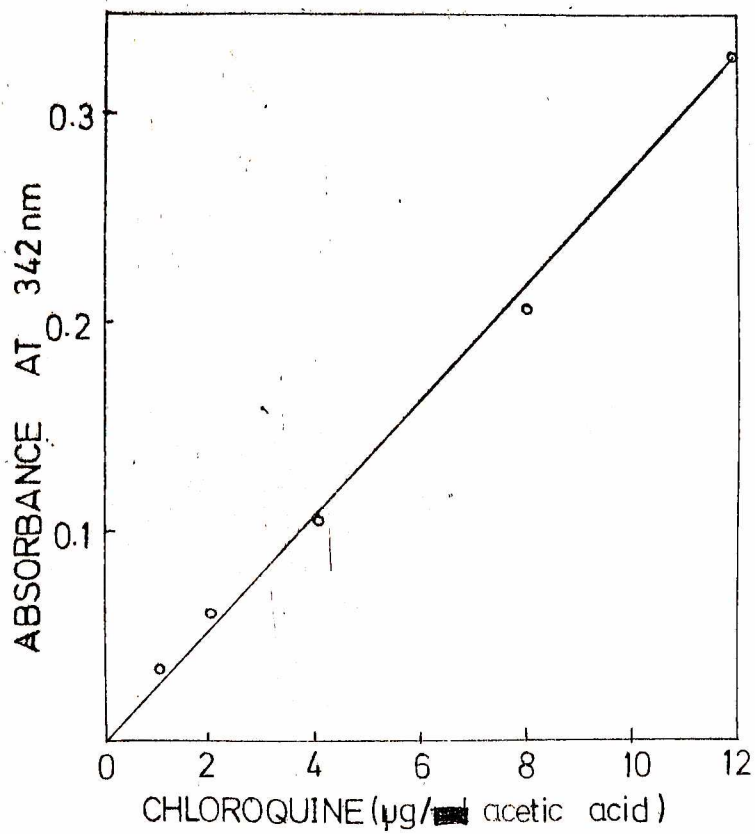


FIGURE 2

STANDARD CALIBRATION CURVE FOR THE ESTIMATION OF CHLOROQUINE
PHOSPHATE IN 0.1M ACETIC ACID

Three extractions with 25 ml of either ether or ethylene dichloride: iso-amyl alcohol system were made on 5^a ml portion of chloroquine solution. The solvent phase was evaporated to dryness; and the residue was taken up in either 10 ml of 0.1M-acetic acid or 0.1M-HCl in methanol. Appropriate dilutions were made, and the absorbance was read at 342 nm, which is the absorption maximum for chloroquine in 0.1M-HCl (Clarke, 1969).

A calibration curve for chloroquine phosphate in 0.1M-acetic acid (Fig. 2) was prepared using chloroquine phosphate concentrations of 0.5-20 µg/ml (Prouty and Kuroda, 1958).

Interpolation of the corresponding absorbance of the various extracts from the calibration curve gave recoveries of 87.2 ± 0.4 , 90.2 ± 0.3 , $94.1 \pm 0.3\%$ for the n-hexane, ether and the ethylene dichloride/iso-amyl alcohol systems respectively. (Data represents the mean and standard errors of recoveries made for three separate extractions for each solvent).

Both ether and ethylene dichloride: iso-amyl alcohol systems were used in subsequent studies.

III. Developing Solvents for Chromatograms

(a) Paper Chromatography: The choice of suitable developing solvents was studied by developing chromatograms of spots of 1% chloroquine solution in solvent using Whatman No.1 filter paper in the following developing solvents

- i) n-butanol saturated with 1 in 10 of 0.88 NH_3 solution
(Warhurst, 1973)
- ii) methanol: ammonia solution sp.gr. 0.88 (100:1.5 v/v)
- iii) n-butanol: glacial acetic acid: water (10:1:1.5 v/v/v)

In solvent systems (i) and (ii) the U.V. absorbing spot corresponding to chloroquine migrated with solvent front.

These solvents were therefore abandoned.

In system (iii) however, the U.V. absorbing spot corresponding to chloroquine phosphate was identified at an R_f value of 0.60. However a longer developing time of 16 hours was required. Attempts were therefore made to find a rapid and sensitive method of identifying chloroquine and possibly some of its metabolites.

(b) Thin Layer Chromatography (TLC): Warhurst (1973), McChesney et al (1966, 1967) and Clarke (1969) have used TLC to resolve and identify chloroquine and its metabolites. The above experiment (IIIa) was therefore repeated with thin layer plates without fluorescing indicator. Chromatograms were developed in the following systems:

- (i) methanol: conc. ammonia solution sp.gr.0.88(100:1.5)

- (ii) Ethylacetate: iso-propanol: 1 in 5 of 0.88 NH_3
(79:15:6)
- (iii) n-butanol saturated with 1 in 10 of 0.88 NH_3

System (i) gave the most rapid development - running time 40 mins. However, this system was found unsuitable because U.V. - absorbing spots corresponding to chloroquine appeared at the origin; and there was the obvious difficulty of not being able to identify metabolites with R_f 's lower than that of chloroquine.

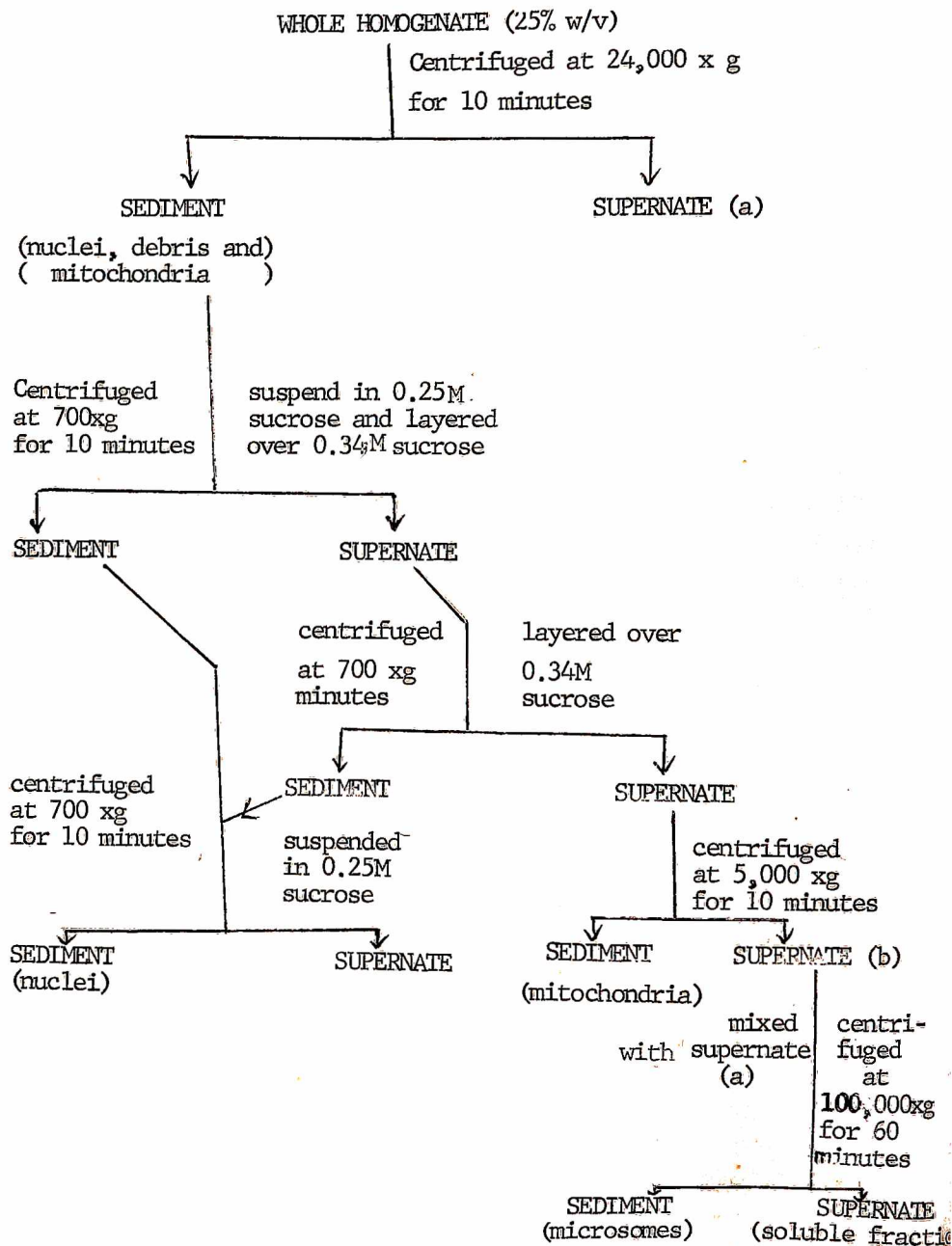
In system (iii) which required a longer running time of 6 hours, chloroquine had an R_f value of 0.63. System (ii) required 2 hours for development and chloroquine yielded R_f value of 0.47. System (ii) and, in certain cases, system (iii) were therefore used as the developing solvents in subsequent studies.

M I. A. Preparation of Subcellular Fractions of the Rat Liver

The method described by Gaudette and Coatney (1961) and La Du ~~et~~ al. (1971) was followed with slight modifications (as shown below).

The animals (fasted overnight) were sacrificed by a blow on the head, and their livers immediately excised. All subsequent operations were carried out in the cold at 0-4°C. The livers were freed of excess blood by immersing in a beaker of ice-cold 0.25M sucrose. The wet livers were rinsed, and a 25% (w/v) whole liver homogenate was prepared (3 ml of ice-cold sucrose solution for each gram of liver) by homogenizing the minced tissue for 1 min.

FIGURE 3: Schematic representation for fractionation of liver cell components (La Du et al., 1971)



The homogenate was centrifuged at 24,000 x g for 10 min. to sediment nuclei, unbroken liver cells, red blood cells, and mitochondria. Other liver cell components were fractionated according to the scheme in Figure 3.

(a) Isolation of nuclear-debris fraction: The sediment obtained after centrifugation at 24,000 x g was made to the original volume of the initial homogenate in cold isotonic sucrose. Half of this solution was layered over an equal volume of cold 0.34M sucrose. The mixture was centrifuged at 700 x g (in a bench centrifuge) for 10 min. The supernatant fluid was relayered over an equal volume of 0.34M sucrose and centrifugation at 700 x g repeatedly.

The loosely sedimented pellets in these two consecutive centrifugations were resuspended in 0.25M sucrose and recentrifuged at 700 x g for 10 mins. The supernatant fluid was removed and the sedimented nuclei-debris were washed with two portions of 0.25M sucrose solution. The washed pellets were then suspended in isotonic buffer pH 7.4 (1.15% KCl - 0.01M Na^+/K^+ phosphate).

(b) Isolation of Mitochondria: The supernatant fluid from the separation of nuclei-debris was centrifuged for 10 mins at 5,000 x g. The supernate was carefully drawn off and the brownish red mitochondria were washed twice by resuspending in 0.25M sucrose solution followed by centrifugation at 5,000 x g. The mitochondrial pellet was then resuspended in isotonic-K Cl to the original volume of the homogenate.

(c) Isolation of Microsomes: The supernate obtained in the centrifugation at 24,000 x g (a) and the post-mitochondria fraction in stage (b) were pooled and centrifuged at 100,000 x g for 60 mins. The fatty material at the top was removed. The supernatant fluid (known in the text as soluble fraction) was also removed and diluted with isotonic KCl to the original volume of the homogenate. The microsomal pellet was washed once by resuspending it in isotonic KCl and recentrifuging at 100,000 x g for 60 mins. The washed microsomes were resuspended in isotonic KCl to the same volume as the original homogenate.

B. Preparation of Microsomes from 9,000 x g Supernate

The whole homogenate (containing 250mg of liver/ml) prepared in MIA above was centrifuged at 9,000 x g for 10 mins. The 9,000 x g supernate was centrifuged at 105,000 x g for 60 mins. The fatty material at the top was gently removed and discarded. The supernatant fraction was removed and stored as the soluble fraction. The microsomal pellet was resuspended in 10 ml of 0.05M Na^+/K^+ phosphate buffer pH 7.4; and made up to the original volume of the crude homogenate.

M2 Incubation of Cell Fractions with Chloroquine and other
quinoline derivatives

The incubation system that was used to study the metabolism of chloroquine by various cell fractions prepared in method 1A was made up of the following:

Phosphate buffer 0.2M	pH 7.4
Chloroquine phosphate	10 μ mole
NADP	2 μ mole
Glucose-6-Phosphate	25 μ mole
Magnesium chloride	75 μ mole
Nicotinamide	100 μ mole

In a total volume of 2.0 ml

To this was added a 3.0 ml aliquot of each cell fraction.

Incubation was carried out in a shaking water bath, in an atmosphere of oxygen in 25 ml conical flasks. The flasks were incubated for 1 hour at 37°C, after which the reaction was stopped ~~and~~ by addition of 2.0 ml 10% trichloroacetic acid (TCA).

M3. Extraction Procedures

(a) Incubation Systems (McChesney et al, 1967)

Ten minutes after the addition of the TCA solution to the incubation system, the solution was centrifuged (3,000 x g: 10 min.) and the supernate collected and adjusted to pH 10.5 with NaOH (25% w/v). The resulting alkaline solution was then extracted with either three volumes of iso-amyl alcohol/ethylene dichloride (10:90, v/v) or ether.

(b) Urine of chloroquine treated humans

Human urine samples were adjusted to pH 11.0 with 1 in 5 of 0.88 NH_3 solution. Extractions of 150 ml portions were made with 3 x 200 ml aliquots of iso-amyl alcohol: ethylene dichloride (10:90, v/v) mixture by vigorous shaking. After each extraction, the phases were separated by centrifugation (3,000 x g: 10 min.).

(c) Liver Homogenate from Rats on Chloroquine

(i) Direct method (McChesney et al, 1967)

An aqueous homogenate of the livers was subjected to direct ether extraction, initially at pH 5.5 ('acidic fraction') and then after adjusting the homogenate to pH 11 ('basic fraction').

(ii) Acid Digest Method (Clarke, 1969)

To 20 ml of the aqueous liver homogenate was added 20 ml concentrated HCl. The resulting slurry was diluted with 40 ml distilled water and heated in a boiling water bath for 1 hour.

This was filtered and the residue washed with water. The filtrate was first extracted with ether ('acid fraction'), adjusted to pH 11.0 with NaOH and then re-extracted with three volumes of either ether or iso-amyl alcohol: ethylene dichloride mixture.

M4. Chromatography

All extracts were evaporated to dryness and the residue taken up in 1.0 ml methanol. Exactly 30 microlitres of the methanol extract was applied to a thin layer plate of silica gel, and the chromatogram was developed in the appropriate solvent system.

M5. Estimation of Acetaldehyde

Acetaldehyde was determined by a modification of the diffusion method of Gupta and Robinson (1966). Warburg flasks with quick-fit ground glass stoppers were used in place of the specially designed flasks described by Gupta and Robinson (1966). In the main part of the flask were placed 2.0 ml of 10% TCA and 3.0 ml distilled water. A 0.2 ml sample containing 0.1 - 0.54 μ mole of paracetaldehyde was added to the main part of the flask which ^{was} ~~was~~ stoppered.

The centre well contained 0.6 ml 0.015M semicarbazide HCl in 0.16M phosphate buffer pH 7.0. Precooled containers were used throughout to minimise the loss of acetaldehyde. The flasks were incubated at 37°C for 2 hours. A 0.4 ml aliquot of the acetaldehyde semicarbazide solution in the centre well was diluted with water

FIGURE 4

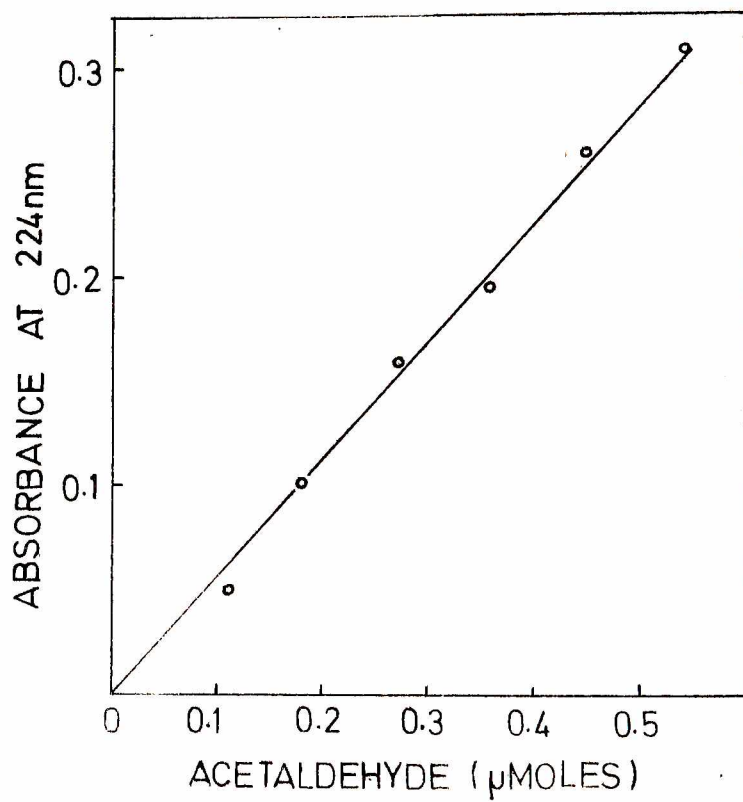


FIGURE 4

STANDARD CALIBRATION CURVE FOR THE ESTIMATION OF ACETALDEHYDE -
A MODIFIED METHOD OF GUPTA & ROBINSON (1966)

to 3.0 ml and the absorbance read at 224 nm against a blank containing an equivalent of semicarbazide solution. A standard curve was obtained (Fig. 4).

In the experimental estimation of acetaldehyde from incubation systems, the main part of the flask contained the incubation mixture. Two millilitres of 10% TCA was placed in the side-arm. After thirty minutes incubation, the TCA was added to the main part of the flask by carefully tilting the flask. The mixture was then incubated for an additional 2 hours at 37°C, after which 0.4 ml aliquot of the acetaldehyde semicarbazide solution in the centre well was taken and diluted to 3.0 ml and the absorbance read at 224 nm. The acetaldehyde concentration in the incubation system was obtained from the calibration curve (Fig. 4).

PART III

RESULTS AND DISCUSSIONS

PART IIISECTION 1METABOLISM OF CHLOROQUINE PHOSPHATEPreliminary Investigations

The ability to separate drugs and their metabolites from biological samples is an essential requirement of almost all investigations of drug disposition. Qualitative studies designed to determine whether a drug is metabolised, and if so, the identity of the metabolites have usually posed serious problems to investigators. The following preliminary investigations were therefore conducted to evolve a rapid and sensitive means of separating and identifying chloroquine and its metabolites from biological tissues.

1-A Liver Incubation Studies: Crude liver homogenate and its 9.000 x g supernate prepared according to general method MTB were incubated as previously described under general methods M2. After the reaction had been stopped, the mixture was extracted by the methods of McChesney et al (1967) and also by that of Warhurst (1973) M3. The extracts were examined using TLC plates without

fluorescence indicator; the chromatograms were examined under U.V. light and also with the iodoplatinate spray.

Results and Discussion : In eight such experiments, only one U.V. - absorbing spot corresponding to chloroquine phosphate was observed with R_f 0.47; and this produced a bluish-brown complex with the iodoplatinate spray. No spots corresponding to any metabolites of chloroquine ^{were} ~~was~~ found.

These findings suggest that either (i) the amounts of chloroquine metabolites formed in the incubation systems were too small to be detected; in which case, a highly sensitive method of identification was required, or (ii) no metabolites of chloroquine were formed in the various incubation systems.

I-B Analysis of urine of chloroquine treated humans

McChesney et al (1967) successfully isolated and identified chloroquine and its metabolites in urine of volunteers on chloroquine. It was decided to use this method and obtain some metabolites of chloroquine for studying in detail; and particularly, for developing more sensitive methods of identification.

Two human volunteers were given chloroquine at a dose of 250 mg daily. Urine was collected at 12 hour intervals after the first dose - three such 12 hour samples of urine were collected from each volunteer. The urine samples were pooled, diluted and extracted according to general method M3b. The extracts were then analysed

chromatographically using method M4. Thin layer plates with and without fluorescing precoating were used in this experiment.

The U.V. absorbing areas on various chromatograms were removed from the silica gel plates and the compounds contained in them eluted with 0.1 M-HCl in methanol. The U.V. spectra of the eluates corresponding to the various fluorescing spots were obtained.

Results, Discussion and Conclusions: Using TLC plates without fluorescing precoating, four fluorescent spots were found under U.V. light. They had Rf values of 0.04, 0.47, 0.65 and 0.78 respectively in the ethyl acetate: isopropanol: 1 in 5, 0.88 NH_3 solvent system.

Authentic chloroquine which was run as standard on the same plate was observed at Rf 0.47. The spots of Rf values of 0.65 and 0.78 showed a very weak fluorescence. All the spots, however, reacted with iodoplatinate reagent to form a very unstable bluish-brown complex.

On the silica gel plates with fluorescing precoating, however, 12 spots were seen under the U.V. light using the same solvent system. These showed up as bright purple spots on the fluorescent background of the silica gel plates. All these U.V.-absorbing areas were numbered 1 to XII in order ^{of} ~~the~~ increasing Rf values. 11

Only spots I (Rf=0.04), II (Rf 0.19), III (Rf 0.47), V (Rf 0.69), VII (Rf 0.78) and XII (Rf: 0.89) formed the bluish-brown complex with iodoplatinate spray.

The U.V. spectra of eluates (0.1M HCl in methanol) corresponding to spots I, II, III and V (figures 5, 6, 7, 8) were all similar with maxima at 346, 332, 263, 238 and 224 nm. Spot III (Rf 0.47) was identified as chloroquine since authentic chloroquine run as standard on the same plate had the same Rf. Clarke (1969) reported the absorption maxima of chloroquine in 0.1M-HCl as 257, 329, 343 nm; and in 0.1M-H₂SO₄ as 221.5, 236, 257.5, 329, and 343 nm.

There was a close similarity in the behaviour of compounds eluted from spot I (Rf 0.04); II (Rf 0.19); and V (Rf 0.69) with respect to their reaction with iodoplatinate reagent and U.V. spectra. They could therefore be metabolites of chloroquine, Lending weight to this argument is the fact that McChesney et al (1967) using the same solvent systems, found metabolites of chloroquine at similar Rf values. Compounds eluted from spots I, II, and V were considered to be metabolites of chloroquine and are referred to in the text as UI, UII, and UV respectively.

FIGURE 5

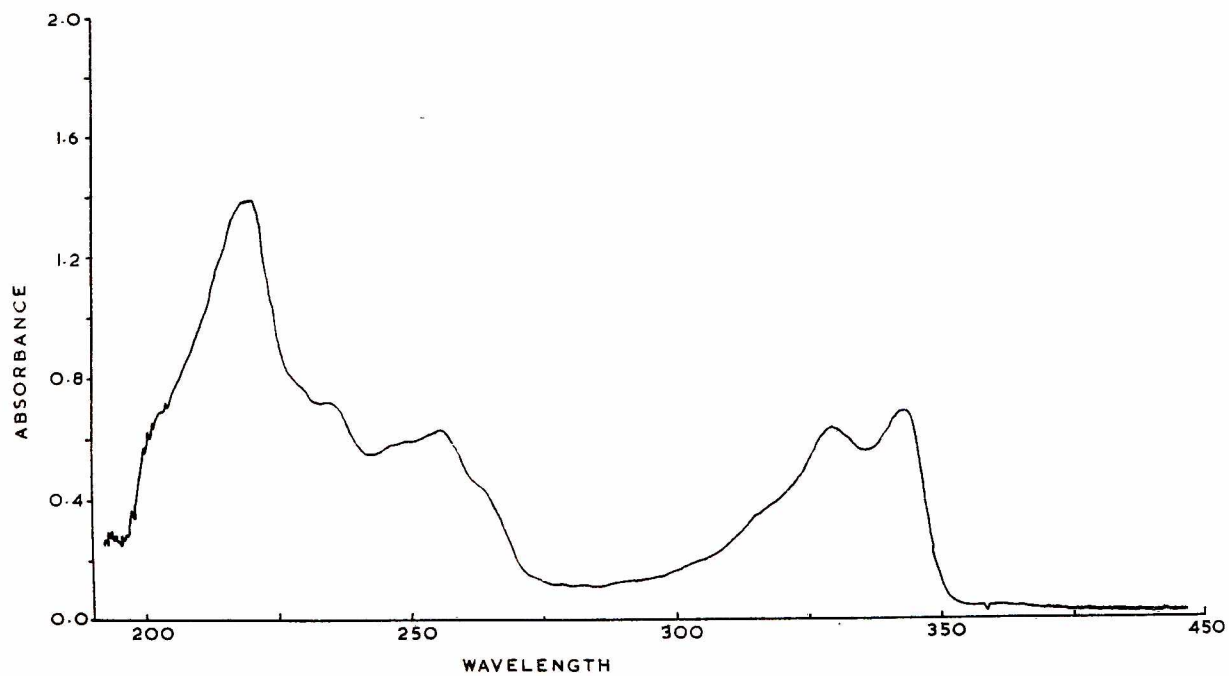


FIGURE 5*

ULTRA VIOLET SPECTRUM OF ELUATE (IN 0.1M-HCL IN METHANOL)
OF SPOT 1 (Rf 0.04) ON THIN LAYER CHROMATOGRAM OF URINE

EXTRACT

λ_{max} at 346, 332, 263, 238

and 224 nm

*Spot was also positive to iodoplatinate reagent.

FIGURE 6

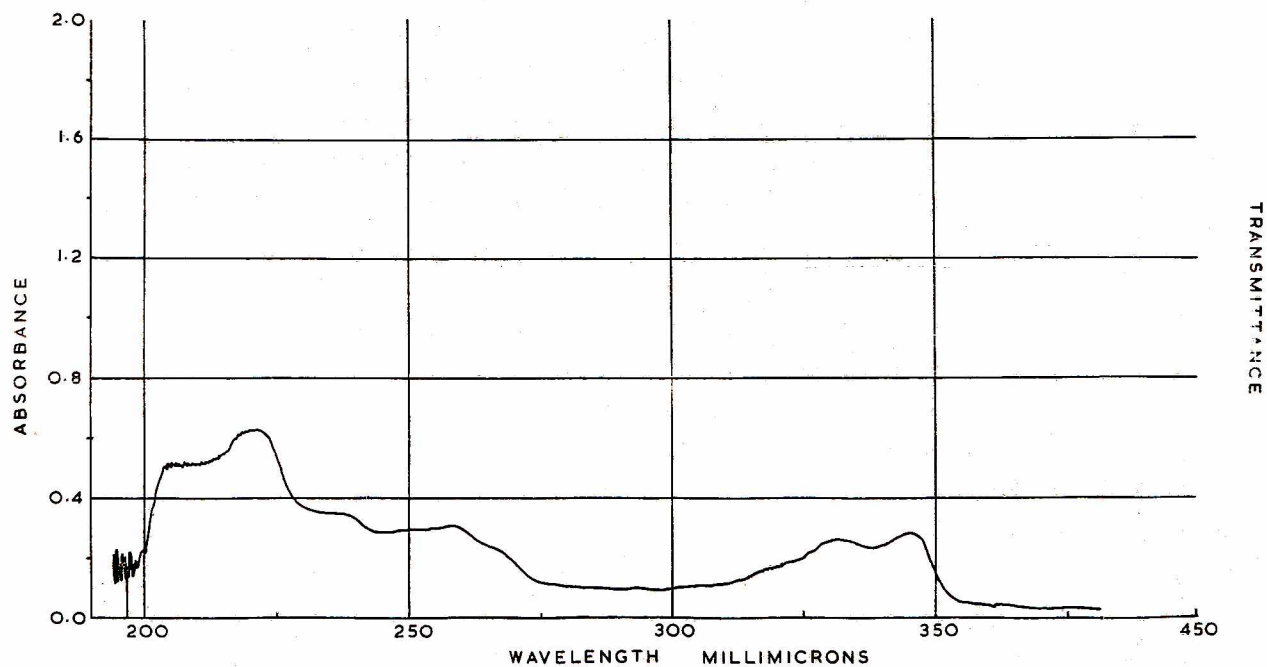


FIGURE 6*

ULTRAVIOLET SPECTRUM OF ELUATE (IN 0.1M
HCl IN METHANOL) OF SPOT II (Rf 0.19) ON
THIN LAYER CHROMATOGRAM OF URINE
EXTRACT

λ max at 346, 332, 263, 237
and 224 nm

* Spot was also positive to iodoplatinate reagent.

FIGURE 7

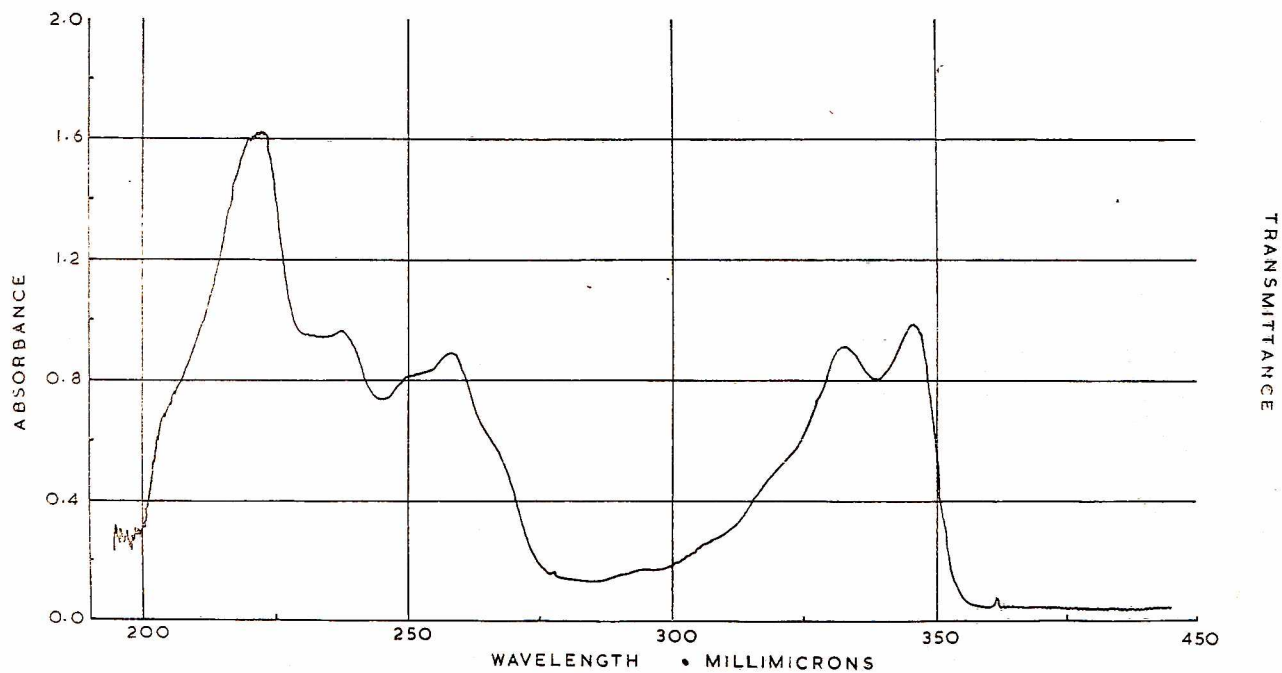


FIGURE 7*

ULTRAVIOLET SPECTRUM OF ELUATE (IN 0.1M HCL
IN METHANOL) OF SPOT III (Rf 0.47) ON THIN
LAYER CHROMATOGRAM OF URINE

EXTRACT

λ max at 346, 332, 263, 237
and 224 nm

* Spot was positive to iodoplatinate spray.

FIGURE 8

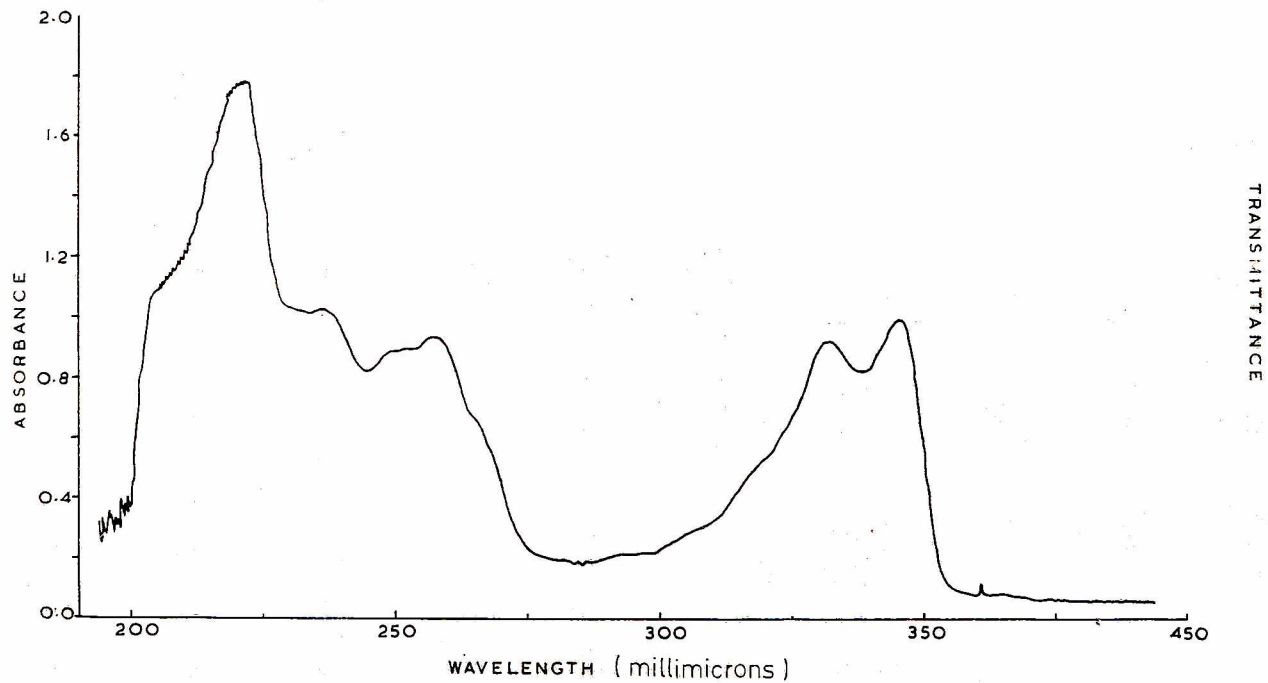


FIGURE 8*

ULTRA VIOLET SPECTRUM OF ELUATE (IN
0.1M HCl IN METHANOL) OF SPOT V (Rf 0.69)
ON THIN LAYER CHROMATOGRAM OF URINE
EXTRACT .

λ_{max} at 346, 332, 263, 237
and 224 nm

* Spot was positive to iodoplatinate reagent.

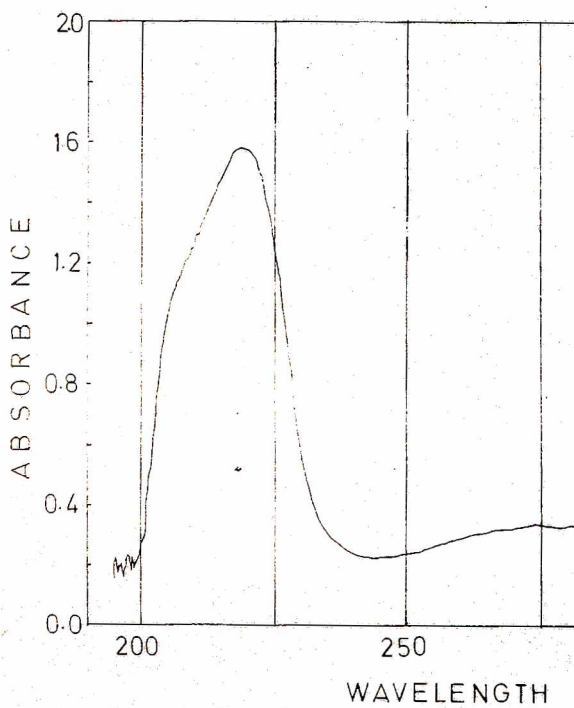


FIGURE 9

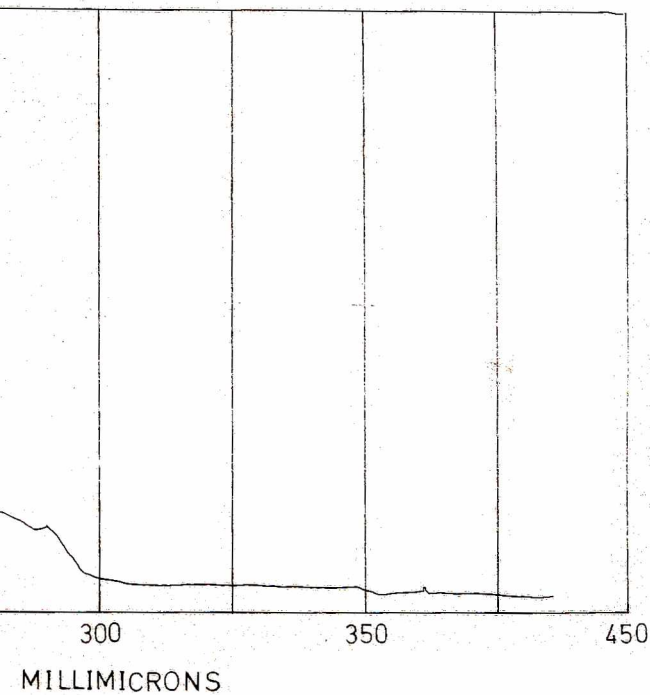


FIGURE 9*

ULTRA VIOLET SPECTRUM OF ELUATE (IN
0.1M HCl IN METHANOL) OF SPOT VII (Rf 0.78)
ON THIN LAYER CHROMATOGRAM OF URINE

EXTRACT.

λ_{max} 289, 280, 224 nm

;

* Spot was also positive to iodoplatinate reagent.

FIGURE 10

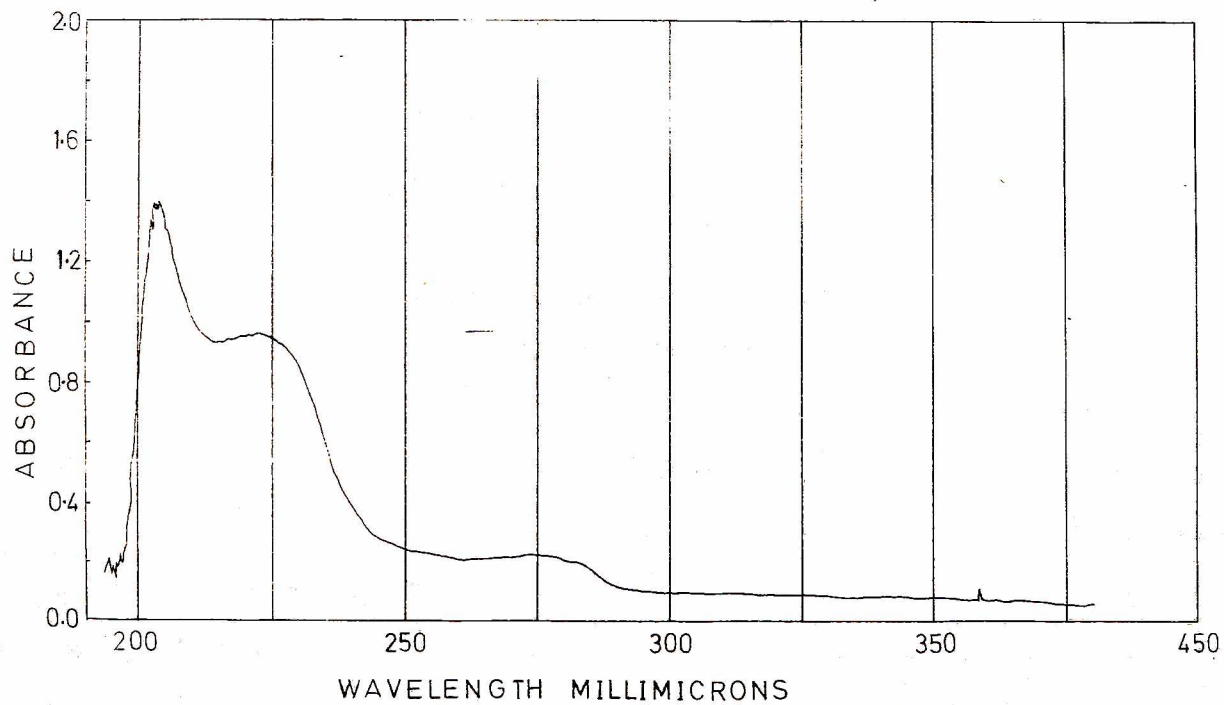


FIGURE 10*

ULTRA VIOLET SPECTRUM OF ELUATE (IN 0.1M
HCl IN METHANOL) OF SPOT XII (Rf 0.89) ON
THIN LAYER CHROMATOGRAM OF URINE.

EXTRACT.

λ_{max} at 280, 225, 203 nm

* Spot was also positive to iodoplatinate reagent.

The U.V. spectra of eluates of spots VII and XII were however different (Figures 9, 10) and it is possible that they were not metabolites of chloroquine even though they formed the bluish-brown complex with the iodoplatinate reagent.

These experiments demonstrated that the urine samples used contained at least three metabolites of chloroquine. It also showed that silica gel plates with fluorescing precoating can be used to give a rapid and sensitive means of resolving and identifying chloroquine, its metabolites and other fluorescing compounds without spraying with reagents that might otherwise contaminate samples.

1-C Rats on Chloroquine

Having developed a sensitive method for identifying chloroquine and its metabolites, it was now necessary to demonstrate that rats do in fact metabolise chloroquine.

Four adult male rats were put on water containing chloroquine phosphate (500 mg/litre). The animals were then sacrificed after one week, and their livers immediately removed and homogenised in distilled water. The liver homogenate was extracted using general method M3 and the extract was analysed chromatographically as in method M4.

Results and Discussion

A thin layer chromatographic analysis of the extract using the ethyl acetate: iso-propanol: 1 in 5, 0.88 NH₃ solvent system, revealed two U.V. absorbing spots of Rf 0.04 and 0.47 - the latter spot (Rf 0.47) being chloroquine and the other spot (Rf 0.04) a metabolite. This was a clear suggestion that rats really do metabolize chloroquine and that at least one of the metabolites was retained in the liver.

SECTION II

Subcellular Distribution of Chloroquine metabolizing activity

Most drugs are metabolized in the liver, and metabolizing enzymes have been found to occur in the various subcellular fractions. This section describes experiments which were conducted to study the pattern of metabolism of chloroquine in the various subcellular fractions of the rat liver homogenate.

The various subcellular fractions were prepared according to the general method M1.A. The microsomal fraction was more often prepared by method M1B because it was faster to prepare microsomes by this method.

Chloroquine phosphate (10 μ moles) was incubated with various subcellular fractions using the system described under general methods M2. The incubation ^{mixture}s were extracted with both iso-amyl alcohol: dichloroethane (10:90, v/v) mixture or ether. Each extract was analysed by TLC. Extracts of the urine of humans on chloroquine were also run on the same silica gel plate along with extracts from the various incubation systems.

Results and Discussion: Tables 1(a) and 1(b) show the pattern of chloroquine metabolism by the various subcellular fractions for instances in which incubation mixtures had been extracted with ether or iso-amyl alcohol: dichloro-ethane mixtures.

Table 1: SUBCELLULAR DISTRIBUTION OF CHLOROQUINE DEGRADATION

Ten micromoles of chloroquine phosphate were incubated for 30 minutes with the various subcellular fractions of rat liver homogenate using the incubation system in M2. Extraction at pH 10.5 with (a) ether and (b) iso-amyl alcohol - dichloro ethane (10:90) mixture respectively; and the extracts were analysed by T.L.C.

U.V. - absorbing areas on chromatograms of various extracts are referred to in the table as I, II, III, etc. - in order of increasing Rf values.

^{Results of}
(a) Thin layer chromatograms of ether-extract of incubation mixtures of various subcellular fractions.

Subcellular Fractions	U.V. absorbing areas on chromatograms and their respective Rf							
	Rf	I(0.04)	II(0.19)	III(0.47)	IV(0.65)	V(0.69)	VI(0.72)	VII(0.78)
Crude homogenate			+	+	+			
9,000 x g supernate			+	+	+			
Mitochondria		+	+	+	+	+	+	
Microsomes		+	+	+	+	+	+	+
Microsomes soluble fraction		+	+	+	+	+	+	+
Soluble fraction		+	+	+	+	+		
Urine		+	+	+	+	+	+	+

+ implies spots absorbed strongly under U.V. light.

+ indicates the spots are not always observed on chromatograms.

- (b) Incubation mixtures of various subcellular fractions extracted with iso-amyl alcohol - dichloroethane (10:90) mixture at pH 10.5.

In brackets are the corresponding Rf values of ultraviolet absorbing compounds isolated from urine of human volunteers using the same extraction system (McChesney et al, 1966).

Subcellular Fraction	Ultraviolet - absorbing areas on various chromatograms and their corresponding Rf's.								
	I 0.04	II 0.19 (0.17)	IIa 0.22 (0.19)	IIb 0.33 (0.31)	III 0.47	IV 0.65 (0.63)	V 0.69	VI 0.72	VII 0.78
Crude homogenate		+			+	+			
9,000 xg supernate		+			+	+			
Mitochondria	+	+	±	±	+	±	±		
Microsomes+									
Soluble Fraction	+	+	+	+	+	+	±	±	±
Soluble Fraction	+	+	±	±	+	+			
Urine	+	+			+	+	+	+	+

+ implies spots absorbed strongly under U.V. light.

± indicates that spots are not always observed on chromatograms.

Tables 1(a) and (b) show that the pattern of metabolites isolated from an incubation mixture in this experiment depends on the extracting solvent system: compounds contained in the U.V. absorbing areas corresponding to spots IIa and IIb (table 1b) are apparently insoluble in ether at pH 10.5. Elution of spots IIa and IIb, however demonstrated that the substances present in them had the same ultraviolet absorbing spectra as that of the quinoline derivatives (maxima at 325 and 343 nm in 0.1M-HCl in methanol).

Another observation from tables 1(a) and (b) was that chloroquine was degraded to many more metabolites by the microsomal or microsome + soluble fractions.

Thin layer chromatographic analyses of extracts of "Blank" incubation systems (containing - subcellular fraction, NADP, G6P but no chloroquine) showed no U.V. - absorbing spots. This was an indication that chloroquine was the precursor of all the U.V. - absorbing spots. It was also observed that chloroquine was metabolized in the microsomal + soluble fraction incubation system to at least seven metabolites.

Tables 1(a) and (b) show that all the ultra violet - absorbing areas on chromatograms of incubation systems containing microsomes/ soluble fraction, matched seven of the twelve ultra-violet-absorbing spots found on chromatograms of urine extracts. In either case, and, particularly, the extracts from the various incubation

systems, the ultimate precursor of UV - absorbing compounds was chloroquine, Hence the observation that the metabolic pattern of chloroquine degradation by rat liver fractions in vitro is similar to that of humans in vivo suggests that chloroquine metabolism may follow a pathway common to both man and the rat.

SECTION IIIMetabolism of (ring-3-¹⁴C) chloroquine phosphate

Results obtained from section II indicated that microsomes or a solution of microsomes + soluble fraction contained enzymes that metabolize chloroquine to at least seven ultraviolet - absorbing compounds. It was therefore necessary to confirm that all the U.V. - absorbing areas were really derived from chloroquine. This was done by using (ring-3-¹⁴C) chloroquine in the incubation system involving microsomes plus soluble fraction.

A 3.0 ml solution of microsomes plus soluble fraction (1.5 ml of each) was incubated aerobically for 30 minutes with 10 μ mol (16 μ Ci) of (ring-3-¹⁴C) chloroquine at 37°C, in the presence of the NADPH- generating system previously described in general methods M2. A similar incubation system serving as the 'control' contained microsome plus soluble fraction (3.0ml), NADP, G6P and unlabelled chloroquine. The reaction was stopped at the end of 30 minutes by the addition of 2.0 ml 10% TCA. The pH of the incubation mixture was adjusted to 10.5 using 1 in 5, 0.88 NH₃ solution in water, and the duplicate incubations were extracted with three 25 ml portions of ether, and with iso-amyl alcohol/dichloroethane mixture. The extracts for each solvent ~~were~~ were pooled; and 25 ml portion of the organic phase was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 1.0 ml methanol.

One hundred microlitre portion of the methanol extract was applied to a thin layer plate of silica gel; and the chromatogram was developed using the ethylacetate/iso-propanol/ 1 in 5, 0.88 NH_3 in water (79: 15: 6 v/v/v/) solvent system. Three chromatograms were run for each extract.

For the quantitative determination of labelled chloroquine and its metabolites, fluorescent spots were scraped from the developed TLC plate. The silica gel was carefully transferred to counting vials and mixed with 10 ml scintillation fluid containing Dioxane 1 litre, Naphthalene 120 g, PPO 4g, POPOP, 75 mg, (Butler, 1961). The radioactivity was measured in a liquid scintillation counter and the results were expressed as % of total radioactivity extracted (Table 2).

The inhibitory effect of SKF 525-A on the metabolism of labelled chloroquine was also studied using methods described above. The incubation system contained 2.0 μmole SKF 525-A, labelled chloroquine and the NADPH-generating system described in general methods.

TABLE 2

QUANTITATIVE PATTERN OF METABOLISM OF (3-¹⁴C) CHLOROQUINE BY
 RAT LIVER MICROSOMES USING VARIOUS EXTRACTING SOLVENTS

EXTRACTING SOLVENT Rf**	QUANTITATIVE PATTERN OF METABOLITES AS % OF TOTAL RADIOACTIVITY EXTRACTED								
	I (0.04)	II (0.19)	IIa (0.22)	IIb (0.33)	III (0.47)	IV (0.65)	V (0.69)	VI (0.72)	VII (0.78)
Ether	2.55 ±0.50	1.62 ±0.20	-	-	86.34 ±0.60	4.70 ±0.51	2.70 ±0.22	2.09 ±0.15	-
Iso-amyl alcohol: dichloro- ethane (10:90w/v)	2.77 ±0.02	3.14 ±0.55	1.42 ±0.02	5.09 ±0.05	66.21 ±0.08	4.78 ±0.06	13.32 ±0.40	3.22 ±0.06	0.05
Ether extracts* of incuba- tion mixture of micro- somes + soluble fraction + ¹⁴ C-chlo- roquine +2 μmole SKF 525-A	3.94 ±0.14	4.04 ±0.14	-	-	64.93 ±0.06	3.34 ±0.08	14.32 ±0.08	0.60 ±0.05	4.00 ±0.06

The data in Table 2 represents the mean and standard error of three determinations of three experiments, where each experiment was conducted with the pooled livers from three rats.

*About 5% of the quinoline bases did not move from the origin and could not be characterized.

**In brackets are the corresponding Rf values.

Results and Discussion

The quantitative distribution of the metabolites of chloroquine extracts using two different extraction media is shown in Table (2). All spots (I-VII) fluoresced under the U.V. light. Their Rf values were identical to those in Tables 1(a) and (b). It can also be seen in Table 2 that SKF 525-A is apparently activating the metabolism of chloroquine through the formation of metabolite V.

Table 2 shows that the compounds contained in the U.V. absorbing areas are all radioactive. This suggests that compounds contained in the eluates of the various U.V. - absorbing spots are metabolites of chloroquine. These derivatives of chloroquine have variable solubilities in the different extracting solvents used - metabolites II(a) and II(b) are not extracted into ether whereas appreciable amounts of these metabolites - 1.42% and 5.09% respectively, are extracted into the iso-amyl alcohol; dichloroethane solvent system. The results of the ether extraction ~~are~~^{are} however shown in Table 2, as a more efficient solvent for extracting chloroquine from such incubation systems than the iso-amyl alcohol/dichloroethane mixture. The latter solvent on the other hand, extracted comparatively substantial amount of metabolite V which contained 13.32% of the total radioactivity.

SECTION IV

SYNTHESIS OF METABOLITES OF CHLOROQUINE

INTRODUCTION:

Unsuccessful attempts were made to isolate the metabolites of chloroquine ^{on a large scale} from urine of humans on chloroquine ~~on a large scale~~ by means of preparative TLC of their urine extracts, and also by running such urine extracts through silica gel columns. It had been presumed that if large quantities of the metabolites could be successfully obtained by this method, their identity could have been worked out by such analytical methods as IR, mass spectra, NMR, qualitative organic analysis.

As an alternative to this unsuccessful attempt, some of the known metabolites were synthesized. Carmack et al. (1947) and McChesney et al. (1967) prepared some of the metabolites of chloroquine through the scheme of synthesis provided in Figure 11. Their methods were followed but with certain modifications. For example, the conversion of 4-(4'-chloro-1'-methylbutylamino)-7-chloroquinoline to the primary amine derivative, 4-(4'-amino-1'-methylbutylamino)-chloroquinoline was effected through Gabriel's condensation reaction between potassium phthalimide and the corresponding chloride.

FIGURE 11.

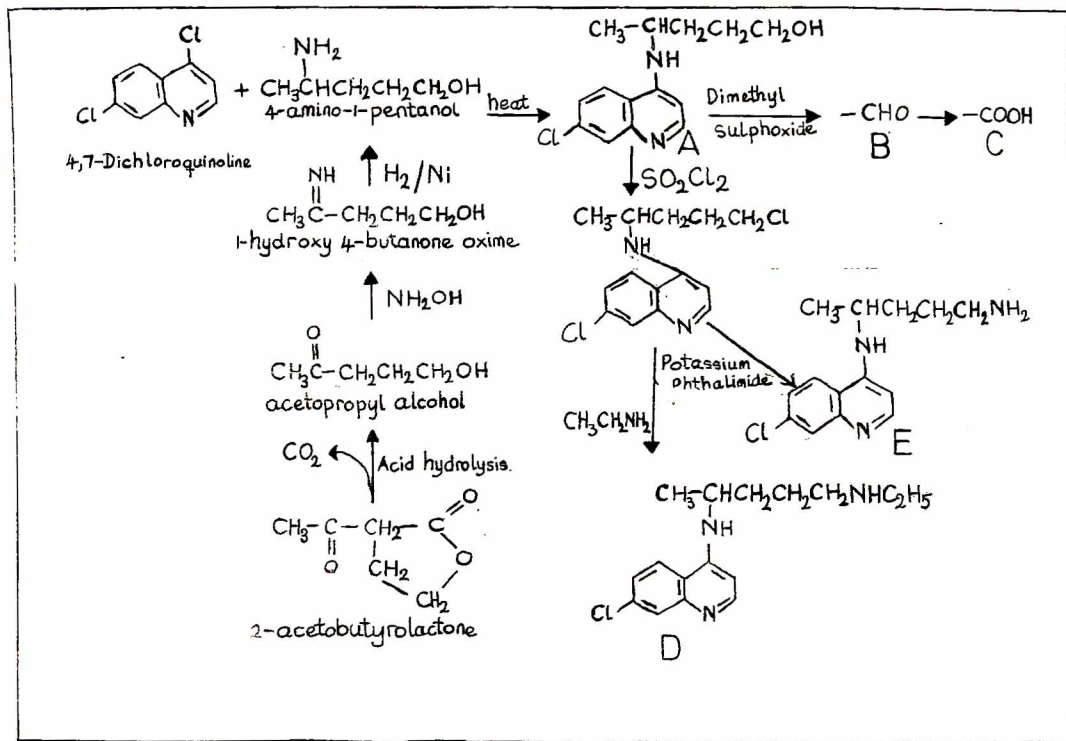


FIGURE 11SCHEME FOR THE SYNTHESIS OF SOME KNOWN METABOLITES
OF CHLOROQUINEKEY to figure:

- A: 4-(4'-hydroxy-1'-methylbutyl amino)-7-chloroquinoline
or the 4'-hydroxy derivative
- B: The 4-(-7'-Chloro-4'-quinonylamino)-4-methyl-1-butanol
- D: 4-(4'-ethylamino-1'-methylbutylamino)-7-chloroquinoline
or the Secondary amine derivative (*SN 13616).
- E: The Primary amine derivative (*SN 13617).

*SN(for Survey Number) identifies the drug in the files of the
Survey of Antimalarial Drugs; Committee on Medical Research,
U.S.A.

This was preferred to the direct reaction with liquid ammonia (Carmack et al, 1947), because the available laboratory facilities were not adequate to supply the high pressure conditions. Moreover, the route via the phthalimido derivative is known to be reliably efficient for all conversions of organic halides into amino derivatives (Sheeman and Bothofer, 1950).

EXPERIMENT:

Acetobutyrolactone (99%) and 4, 7-dichloroquinoline were purchased from Aldrich Chemical Company Limited.

(1) 1-Hydroxy-4-pentanone oxime (Fig. 11)

A solution of 200g of acetobutyrolactone in 600ml of 5% hydrochloric acid was stirred and heated on a mantle under reflux in a round bottom flask for three hours. The colourless mixture turned yellow during the course of the refluxing and then dark brown later in the experiment.

The solution was then cooled and treated with 150.6g of hydroxylamine hydrochloride and 144g. sodium carbonate monohydrate. To this mixture, 80g solid sodium hydroxide was added with stirring and cooling to maintain the temperature below 60°C. The final mixture was left to stand at room temperature for 24 hours.

FIGURE 12

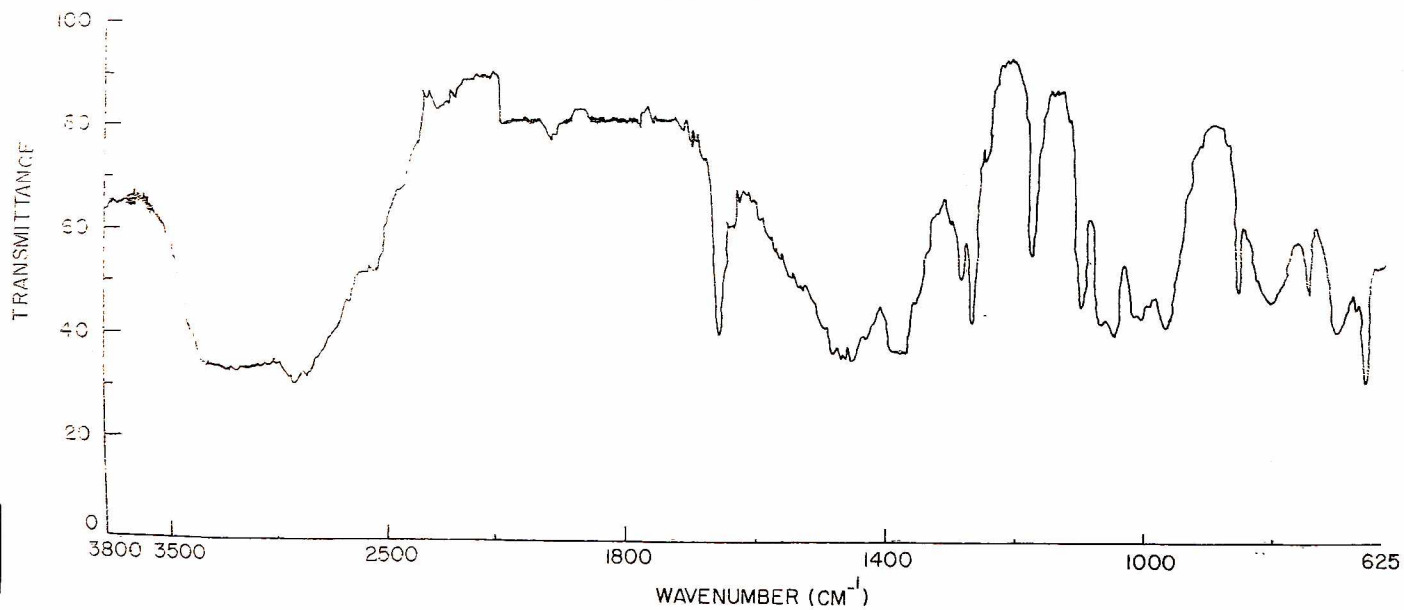


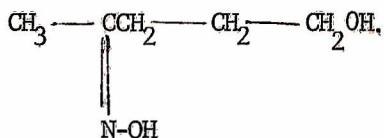
FIGURE 12Infra red spectrum of 1-hydroxy - 4 - pentanone oxime in Nujol

Position of absorption

CM -1

3200-3350	Strong intermolecular H-bonded O-H (str.)
2950	Nujol peak probably obscuring saturated C-H and C-C (Str.)
1660	C = N (Str.)
1450-1480	C-H (deformations)
1270-1280	O-H (bending) probably due to -O-H of Oxime
1010-1280	C-O (Str.)

The information given above together with the recorded m.p.
(which agrees with literature) is consistent with the following
structure.



The report of Carmack et al. (1947) gives the impression that the pH of the mixture after standing for 24 hours should be on the alkaline side, but contrary to their observation, the pH of the mixture was 6.3. The pH was therefore adjusted to 7.4 (as recommended by Carmack et al. ⁽¹⁹⁴⁷⁾ with 1.8g NaOH pellets, and the crude 1-hydroxy-4-pentanone oxime was extracted with ether for another 24 hours in a liquid - liquid extraction apparatus. Ether was then removed in a vacuum evaporator. The crude oxime recovered from the ether was distilled as a pale yellow viscous liquid, b.p. 106-108°C (1.00mm). The yield from acetobutyrolactone was 80%. The oxime crystallized after 14 days standing at room temperature and a sample recrystallised from ethyl acetate formed colourless crystals. Carmack et al. (1947) reported that the oxime crystallised spontaneously upon standing. The crystals were chromatographically pure, giving only one spot, R_f 0.42, when developed with iodine vapour. These data together with the infra-red spectrum (Fig. 12) indicated that a hydroxyoxime had been formed.

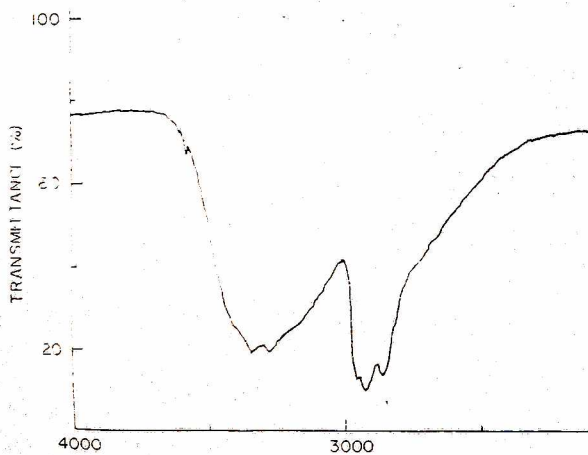


FIGURE 13

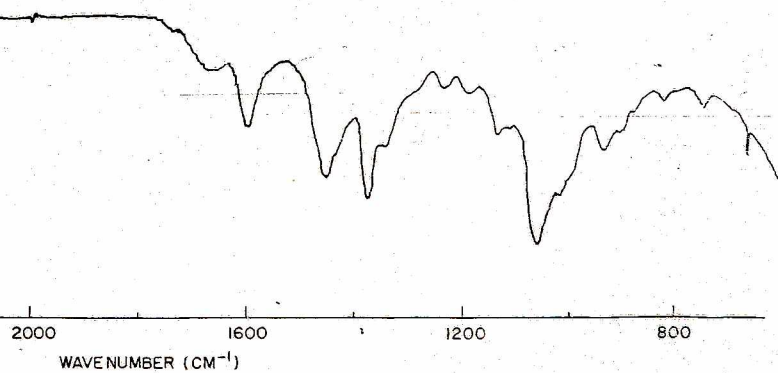


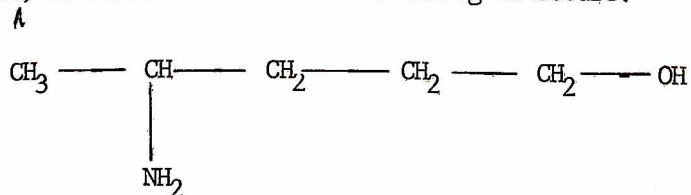
FIGURE 13Infra red spectrum of 4-amino-1-pentanol (K Br disc)

Position of absorption

CM-1

3280-3350	Strong intermolecular hydrogen-bonded -O-H (Str.); or N-H (unsymmetrical and symmetrical Str.) of primary amines.
2960-2860(s)	Saturated C-H and C-C (Str.) probably associated with CH ₂ and - CH ₃
1670 (weak)	C-N- Stretch of unreacted oxime
1600 (s)	N-H (Bending)
1380	O-H (Bending)
1060	This peak may be given by C-O (Str.)

The information above together with the recorded m.p. of the compound synthesized (which compares favourably with the ^{value} literature) is consistent with the following structure:



(2) 4-amino-1-pentanol (Fig. 11)

Distilled but not recrystallised 1-hydroxy-4-pentanone oxime (70 g) was hydrogenated over about 3,0g Raney nickel catalyst at an initial pressure of 125 atmospheres. No solvent was used. The temperature was carefully raised during 30 minutes to 60°C, and eventually to 75°C. Carmack et al. (1947) stopped the consumption of hydrogen after one mole equivalent of it had been consumed in their reaction. In the absence of a device to measure the rate of uptake of hydrogen, hydrogenation was carried out for 24 hours. The decision to carry out hydrogenation for 24 hours was based on evidence from TLC and infra red studies on several trial hydrogenations that 24 hours was the minimum time required for an almost complete hydrogenation of the oxime under the experimental conditions. The product of hydrogenation was distilled under reduced pressure from water pump at 118-120°C. The yield was 72%. The product was confirmed by infra red spectrum (Figure 13).

(3) 4-(4'-hydroxy-1'-methylbutylamino)-7-chloroquinoline-metabolite A (Refer to Fig. 11)

A mixture of 9.8 g (0.1 mole) of 4-amino-1-pentanol and 10 g (0.05 mole) of 4, 7-dichloroquinoline was carefully heated in a 250 ml round bottomed flask fitted with an absorbent cotton wool plug - through which passed a thermometer.

FIGURE 14

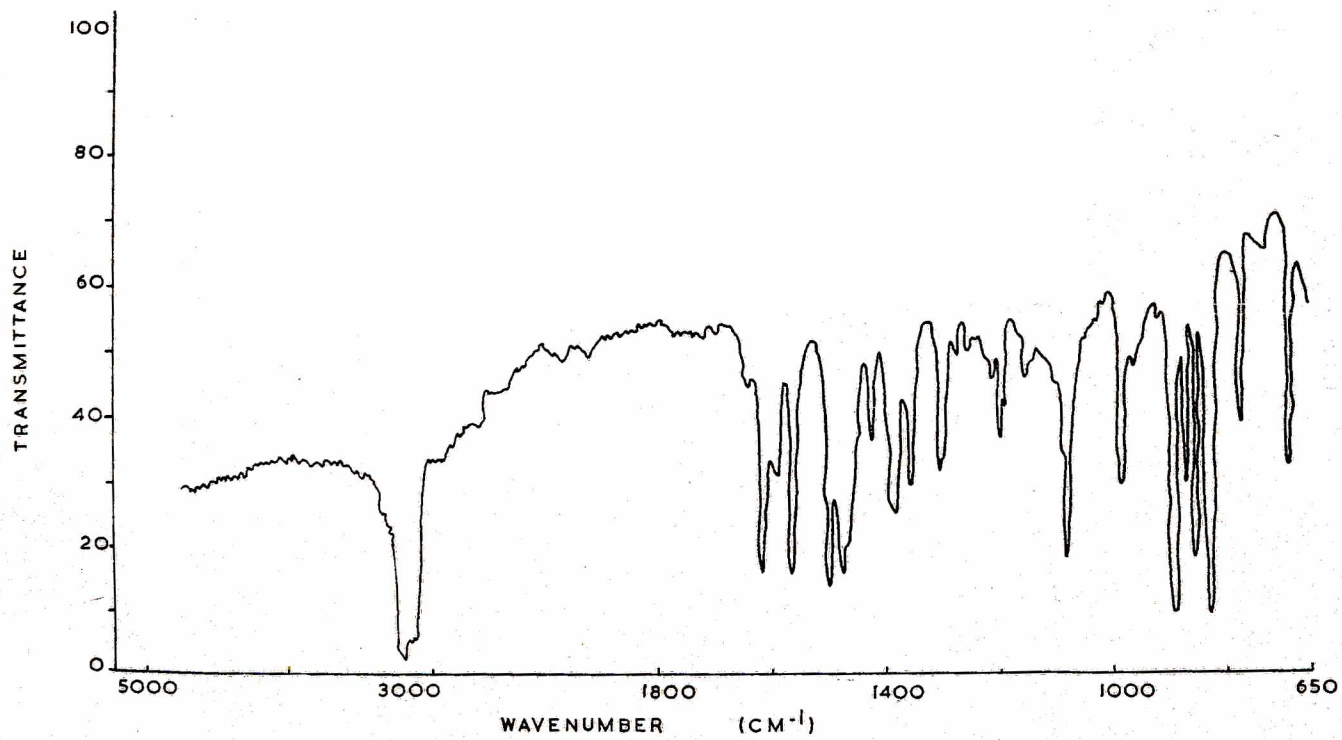


FIGURE 14

Infra red spectrum of the 4'-hydroxy derivative:

4-(4'-hydroxy-1'-methylbutylamino)-7 chloroquinoline

(in Nujol)

Position of absorption CM^{-1}

3,400 - 3,500	Hydrogen bonded - O-H (Str.)
2990 - 3,000	Nujol peaks
1600	N-H (Str.) for 2° amines
800	C - Cl (Str.)

FIGURE 15

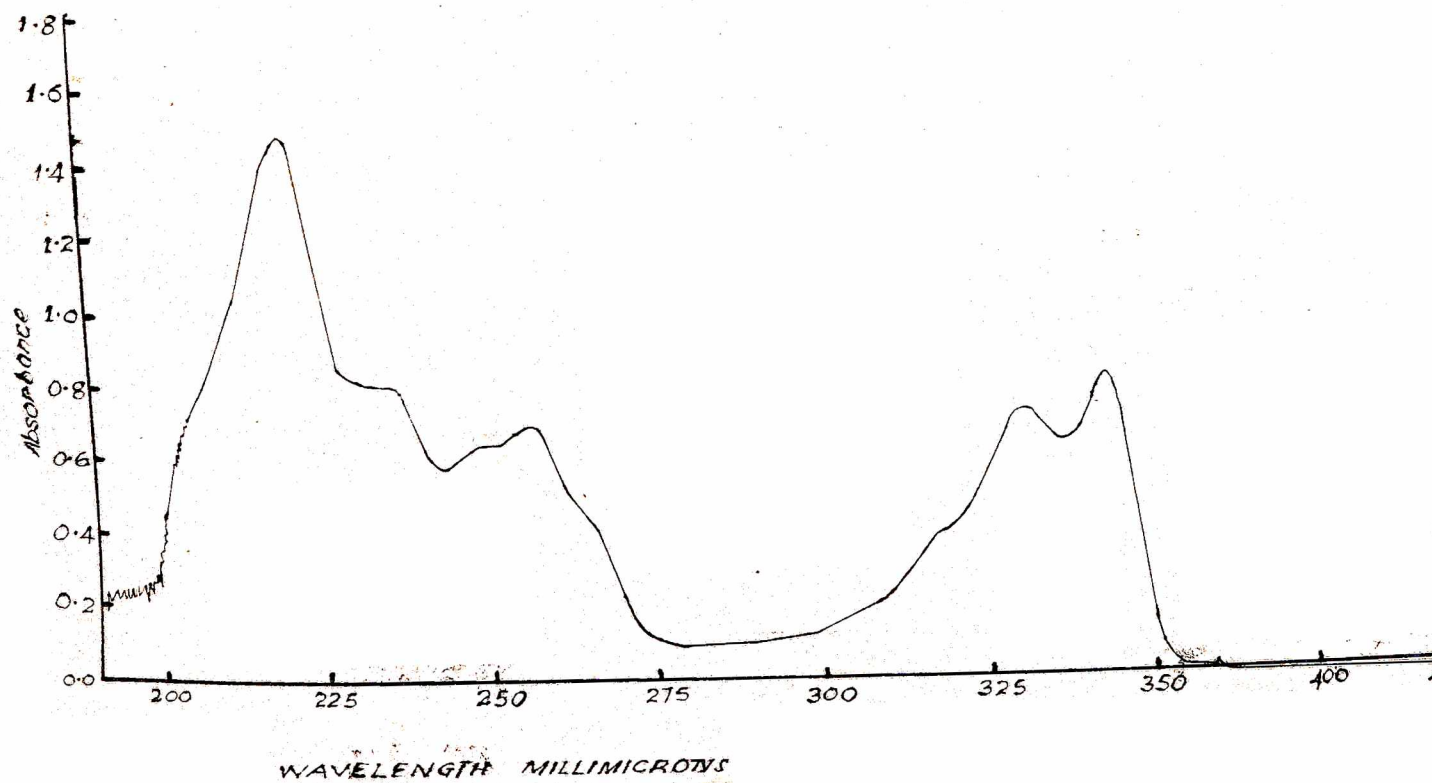
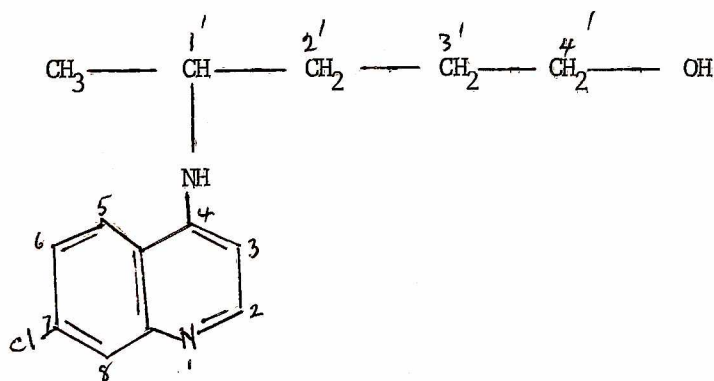


FIGURE 15

Ultra violet spectrum of the 4'-hydroxy derivative
of chloroquine in 0.1M-HCl

λ_{max} .. at 220, 330 and 343 nm; are characteristic of
 the spectrum of the quinoline nucleus of chloroquine.

This together with information in Figure 14 suggest
 the formula of the compound synthesized to be,



By means of an oil bath, the mixture was gradually brought to $152^{\circ} \pm 4^{\circ}\text{C}$, and held at this temperature for four hours. The flask was occasionally agitated to ensure thorough mixing. No sign of uncontrollable violence, reported by Carmack et al, (1947) was observed in this reaction.

After 4 hours of heating, the mixture was cooled to 100°C and poured into water. The liquid product immediately solidified into a sticky mass. The solid product was then left in cold water overnight to induce crystallisation. The solid product was washed with water, filtered and recrystallised twice from 95% ethanol, m.p. $178-181^{\circ}\text{C}$. The m.p. in literature is $175-178^{\circ}$ (McChesney et al, 1966) and $179-181^{\circ}\text{C}$ (Carmack et al, 1947) with yield of 90%. TLC of the recrystallised product using ethyl acetate: iso-propanol: 1 in 5, 0.88 NH_3 (79: 16: 6 v/v/v/) mixture yielded only one U.V. absorbing spot, Rf: 0.65. The product was confirmed by infra red (Figure 14) and ultra violet (Figure 15) spectra. The U.V. spectrum was identical to that of chloroquine having maxima at 343, 330 nm, characteristic of the quinoline nucleus of chloroquine.

90

FIGURE 16

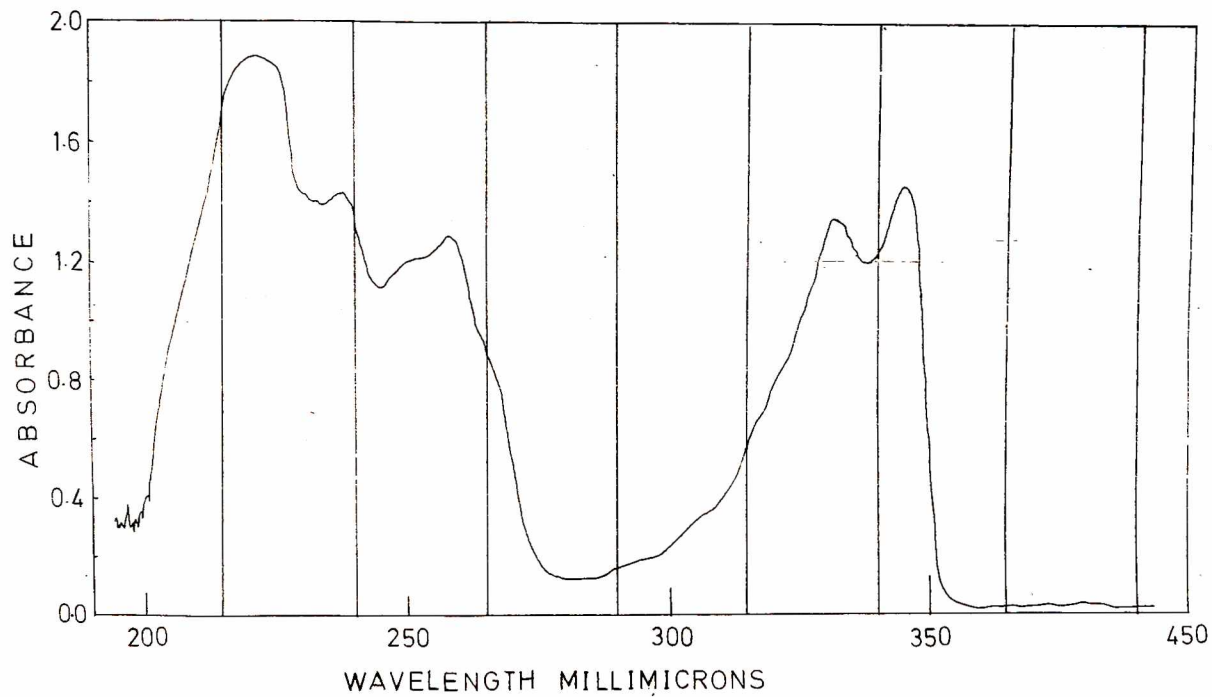


FIGURE 16

ULTRA VIOLET SPECTRUM OF THE 4'-ALDEHYDE

DERIVATIVE IN CCl_4

FIGURE 17

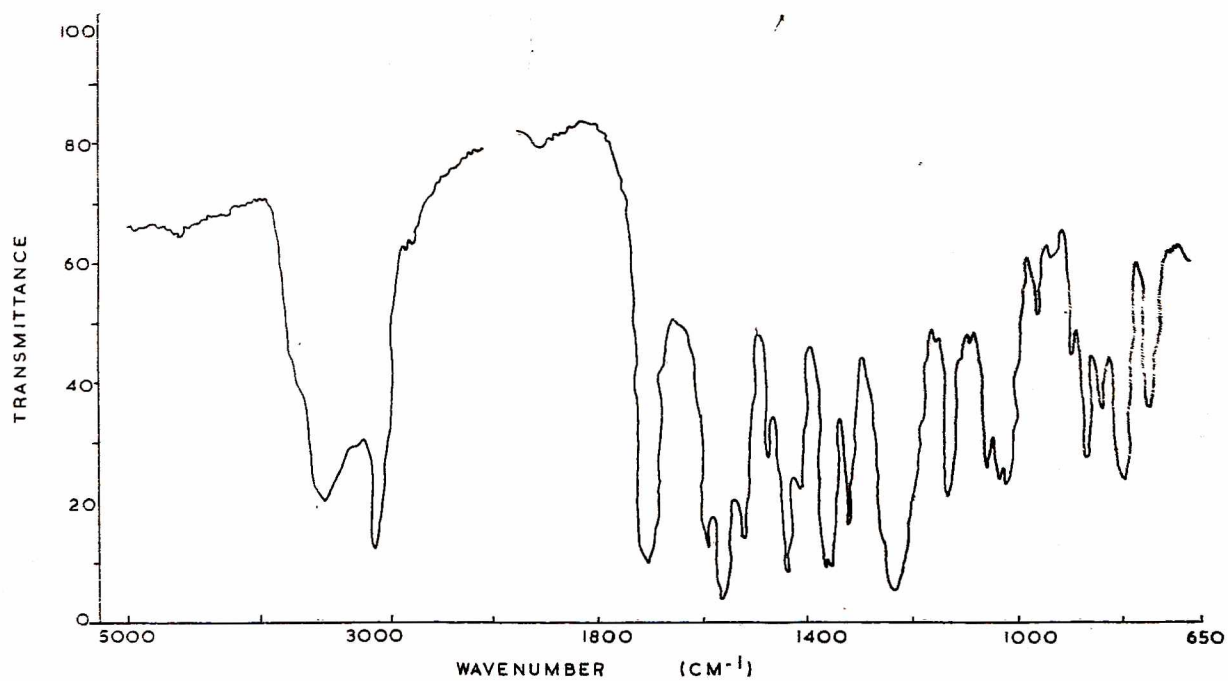


FIGURE 17

Infra red spectrum of the 4'- aldehyde derivative
of chloroquine in Nujol

Position of absorption

CM-1

3,300 Probably N-H (Str,) of quinoline ring

2900 Nujol peak

1705 C = O (Str,) of carbonyl group

1525-1610 Probably N-H (Str,) of 2° amines

1520-1600' Aromatic C-H vibration frequencies,

800 Peak of DMSO (Figure 18) absent;
indicating no contamination by DMSO,

Structure

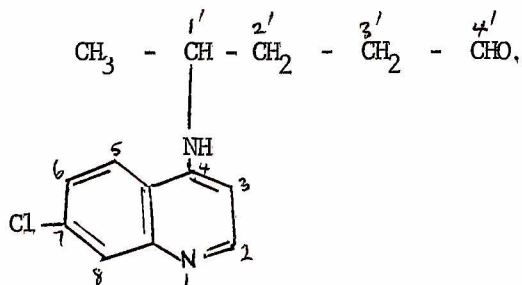


FIGURE 18

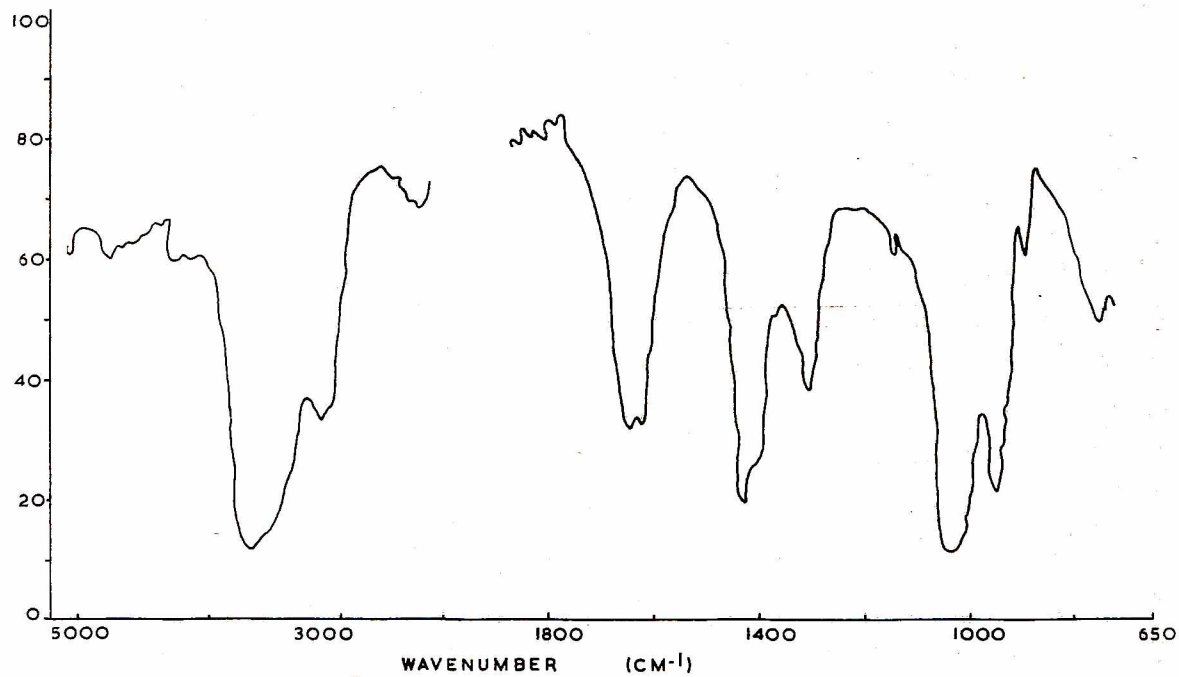




FIGURE 18

INFRARED SPECTRUM OF DMSO

4. Conversion of metabolite A (the carbinol) to metabolite B
(the 4'-aldehyde derivative, Figure 11)

A mixture of 0.5g of the carbinol, 3.2 ml of DMSO and 2.0 ml of acetic anhydride were left to stand at room temperature for three days. The product was dissolved in 15.0 ml chloroform. The chloroform solution was washed twice with 20.0 ml water. The solution was dried over anhydrous sodium sulphate and evaporated to dryness leaving a yellow solid. Thin layer chromatography, of the product yielded two spots - one corresponding to the carbinol Rf. 0.65; and the other to the 4'-aldehyde Rf. 0.72 (see Fig. 11). The product was confirmed by comparing I.R. spectra of the solid product (Figure 17) and that of DMSO (Figure 18). U.V. spectra of the compound was also obtained (Figure 16).

5. The chloride derivative, (4'-chloro-1'-methylbutylamino)-7-chloroquinoline and its conversion to metabolite (E) (Fig. 11, page 89)

(a) Preparation of the chloride derivative

Eleven grams of the carbinol (recrystallised) in 30 ml of chloroform was treated with 6.0 g SOCl_2 in 5 ml chloroform, and the temperature was allowed to rise to the boiling point. As soon as the solid had dissolved, the reagent was removed by distillation.

Excess solvent was removed using a rotary evaporator. The product at this stage was a dark brown viscous oil: 95% alcohol was added and the mixture was shaken to dissolve the oil. Excess 4M NaOH was added. Much shaking was required to solidify the oil. The dirty brown solid was recrystallised by first boiling in alcoholic solution containing activated charcoal, filtering the hot solution, and adding excess 4M-NaOH to induce crystallisation. Colourless crystals were obtained herein referred to as the chloride derivative, m.p. $130-132^\circ\text{C}$ compared with m.p. in literature $129-130^\circ\text{C}$ (Carmack et al, 1947). TLC of an alcoholic solution of this chloride derivative with ethylacetate: iso-propanol: 1 in 5, 0.88 NH_3 (79: 16: 5) as running solvent showed a spot under U.V. lamp (254 nm) with Rf of 0.80. On the same plate, the carbinol was located with Rf 0.65. The product was confirmed with infra red spectrum. The disappearance of -OH stretch at 3400 cm^{-1} (Figure 19) suggests that conversion from carbinol to chloride was complete.

FIGURE 19

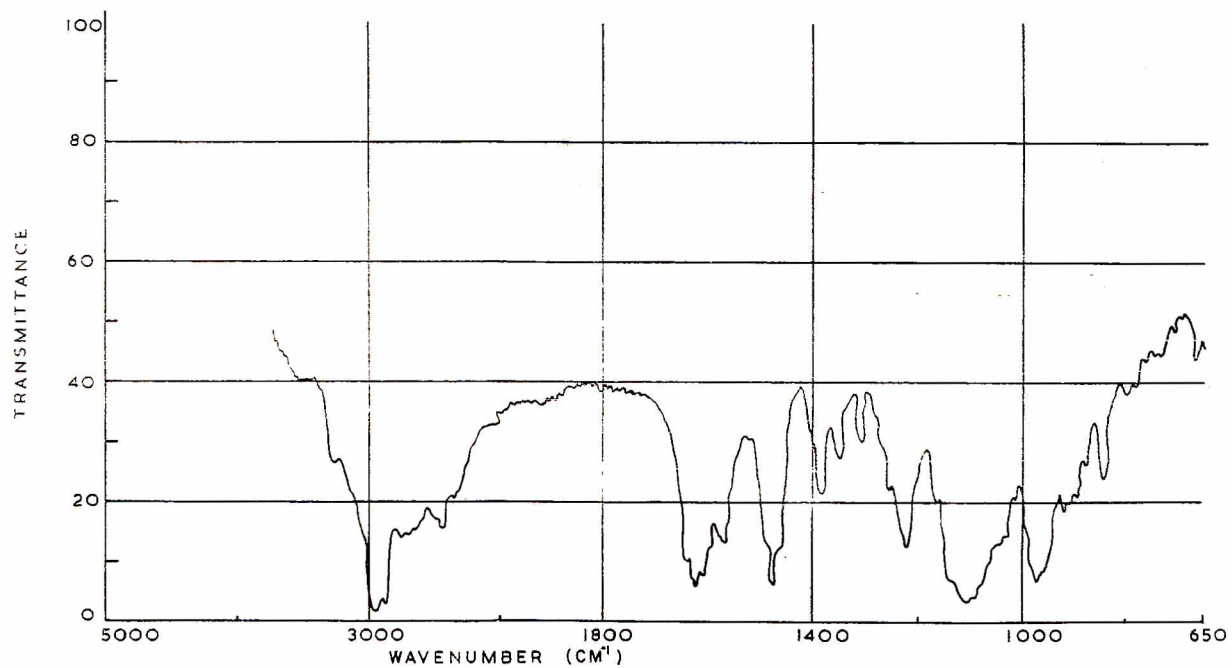


FIGURE 19INFRA RED SPECTRUM OF 4-(4'-CHLORO-1'-METHYLBUTYL
AMINO)-7-CHLOROQUINOLINE IN NUJOL

Position of absorption,

(cm^{-1})

1620 - 1560

N - H - bending for 2° amine,
or quinoline H - H

2950 - 2890

Nujol peaks

805

C - Cl (Str.) for quinoline nucleus.

(b). Conversion of chloride derivative to metabolite E (Fig. 11)

Potassium phthalimide (0.7 g; 0.027 mole) was added to a solution of 1.0g (0.025 mole) of the chloride in 2.5 ml dimethylformamide. The mixture was refluxed on a water bath for 3 hours. Twenty millilitres of chloroform were added, and the mixture was poured into 70 ml water. The aqueous phase was separated and extracted with 10 ml portions of chloroform. The combined chloroform layer was washed with 0.2M NaOH (to remove unreacted phthalimide) and 20 ml of water. After drying overnight over anhydrous sodium sulphate, the chloroform was removed in a rotary vacuum evaporator (at 70°C). The oily product was treated with ether and a small amount of insoluble material was removed by filtration. The resultant yellowish green oil solidified on standing overnight. Seventeen millilitres of methanol and 0.4 ml of 85% aqueous hydrazine hydrate solution were added to the solid and the mixture refluxed for one hour. After cooling, 8.0 ml of water was added and the methanol was removed in a rotary vacuum evaporator. Concentrated HCl (0.8 ml) was added to the residual aqueous suspension and the mixture was heated under reflux for another one hour. After cooling to 0°C, crystalline phthalhydrazine was removed by filtration.

FIGURE 20

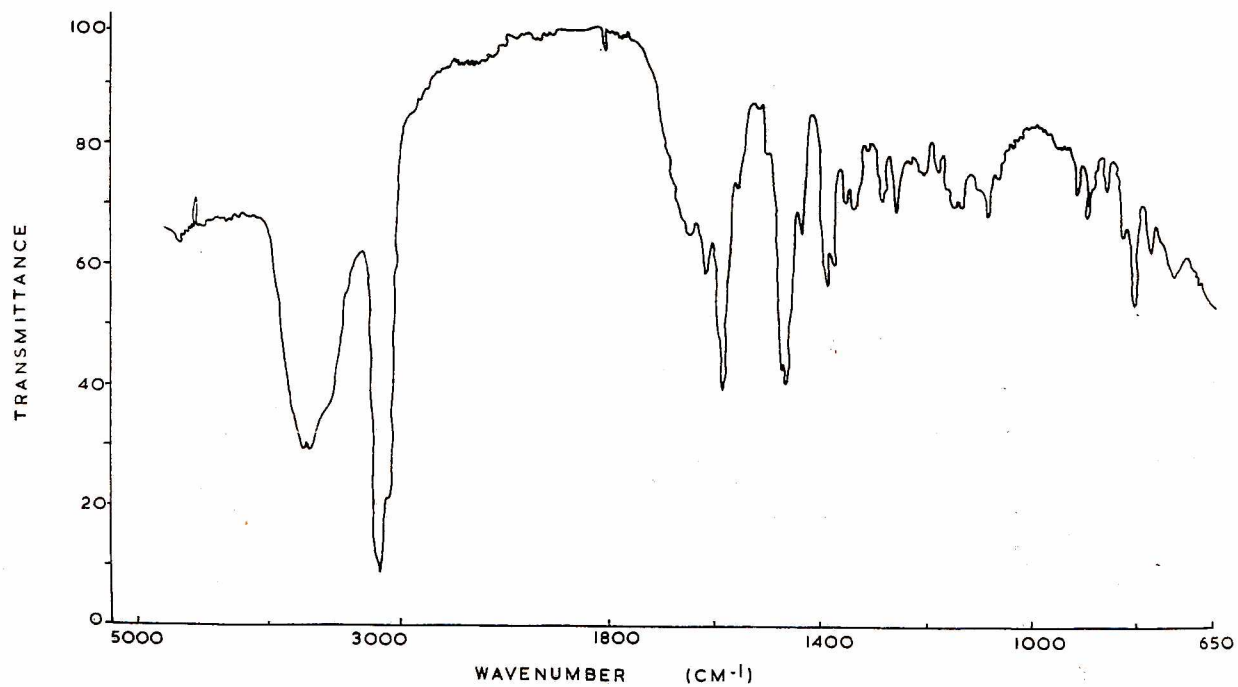


FIGURE 20

Infra red spectrum of the primary amine derivative:

4-(4'-amino-1'methylbutylamino)-7-chloroquinoline in Nujol

Position of absorption

CM-1

2990	Nujol peak possibly obscuring C-H (Str.)
3500-3505	N-H (Str.) for 1 ^o amines
1610	-H-H (Str.) for 2 ^o amines
1590	Probably quinoline - NH (Str.)
800	C - Cl,

FIGURE 21

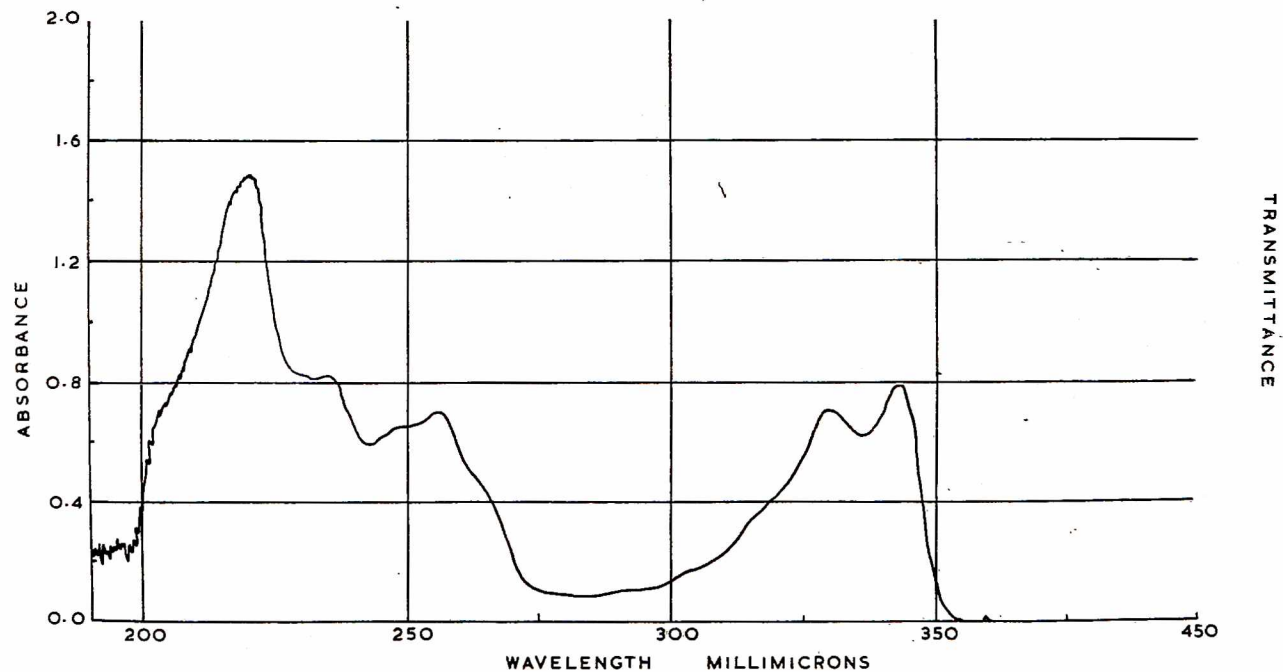
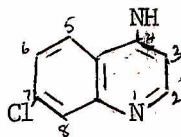


FIGURE 21ULTRA VIOLET SPECTRUM OF THE PRIMARY (1⁰) AMINE DERIVATIVEIN 0.1M H CL

The maxima at 220, 330 and 343 nm are similar to those of the quinoline nucleus of chloroquine.

This together with information from the infra red spectrum (Figure 20) suggests the formula of the compound

prepared to be $\text{CH}_3 - \overset{1'}{\text{CH}} - \overset{2'}{\text{CH}_2} - \overset{3'}{\text{CH}_2} - \overset{4'}{\text{CH}_2} - \text{NH}_2$



The filtrate was then concentrated under reduced pressure to remove most of the HCl, and the moist residue was dissolved in 17 ml water. A small amount of insoluble material was removed by filtration, and 2M NaOH was added to precipitate the product. Thin layer chromatography of the product with the ethyl acetate system showed two spots with Rf of 0.04 and 0.80 respectively. The chloride and the carbinol derivative run on the same plate were located and gave spots with Rf s of 0.9 and 0.65 respectively. This indicated that the spot with Rf. 0.80 was probably a side-product of the hydrolysis reaction, but not any of the precursors of the 4(4'-amino-1'methylbutylamino)-7-chloroquinoline (metabolite E). The product was purified by running two-way preparative TLC on 2 mm thick plates, using ethylacetate-iso-propanol; 1 in 5, 0.88 NH₃ and n-butanol saturated with 10% NH₃ in water, as running solvents respectively.

The U.V. absorbing spot (Rf: 0.04) was scraped from the plate and was eluted with 0.1M HCl in ethanol. Two molar NaOH was added to precipitate the product as a pale yellow solid, m.p. 98-100°C and yield 52%. The identity of the product was determined by U.V. and I.R. spectra (Figures 20 and 21).

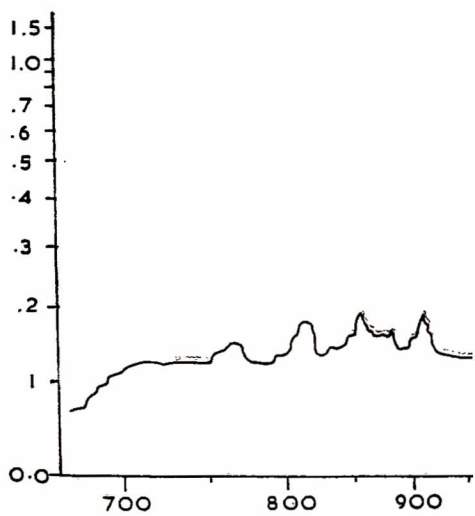


FIGURE 22 —

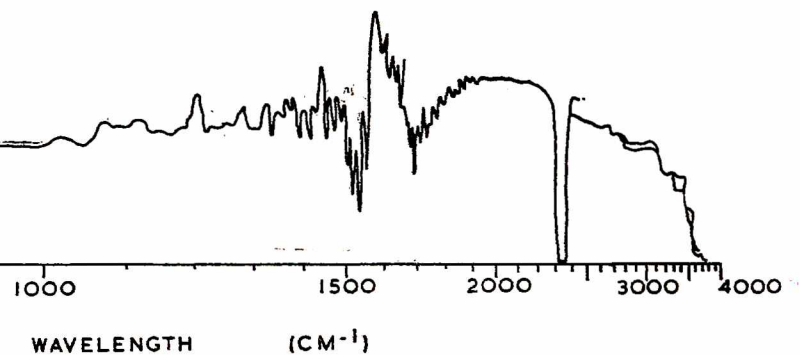


FIGURE 22INFRA RED SPECTRUM OF 4-AMINO-7-CHLOROQUINOLINE (KBr Disc)*

Position of absorption

(cm^{-1})

1540	N-H bending for 2 ^O amine (probably of quinoline N - H.)
1340-1280	N-H (str.) Primary aromatic amines.
805	C - Cl (Str.)

*Purchased from Rhone Chemicals, Polence - Paris.

SECTION VIDENTIFICATION OF U.V. . . . ABSORBING SPOTS AND THE IN VITROMETABOLISM OF SPECIFIC QUINOLINE DERIVATIVESA. IDENTIFICATION OF U.V. . . . ABSORBING SPOTS ON CHROMATOGRAMS

The identity of the substances located at the ultraviolet-absorbing areas on the various chromatograms was established by chromatographing samples of known reference compounds simultaneously.

With exception of 7- chloro - 4 - aminoquinoline, all the reference compounds used as standards were synthesized, purified and characterized as in section IV. These include, the 4'-aldehyde derivative, and 4'-hydroxy derivative and the primary amine derivative, SN 13617 (see page 77). 7-chloro-4-aminoquinoline was purchased from Rhone-Poulence-Paris, and had the following physical properties. It was a white crystalline solid m.p. 149-150°C, Cl, 19.85%; N, 15.68% (calc), and C, 64.47% (calc). It was insoluble in water but soluble in CCl₄, dimethyl formamide, ethanol and ethylene glycol.

Thin layer chromatographic analysis of extracts of the incubated mixture containing chloroquine and the microsome/soluble fraction system was carried out as in section II (page 66) with the reference compounds listed above as standards. Alcoholic solutions of the reference compounds were used.

MASS SPECTRUM OF ELUATE OF SPOT VII ON THIN LAYER
CHROMATOGRAPH OF EXTRACTS OF INCUBATION MIXTURES

RBH 337-75/1

BASE PEAK 100% TSD

M/E CORRECTION = 321

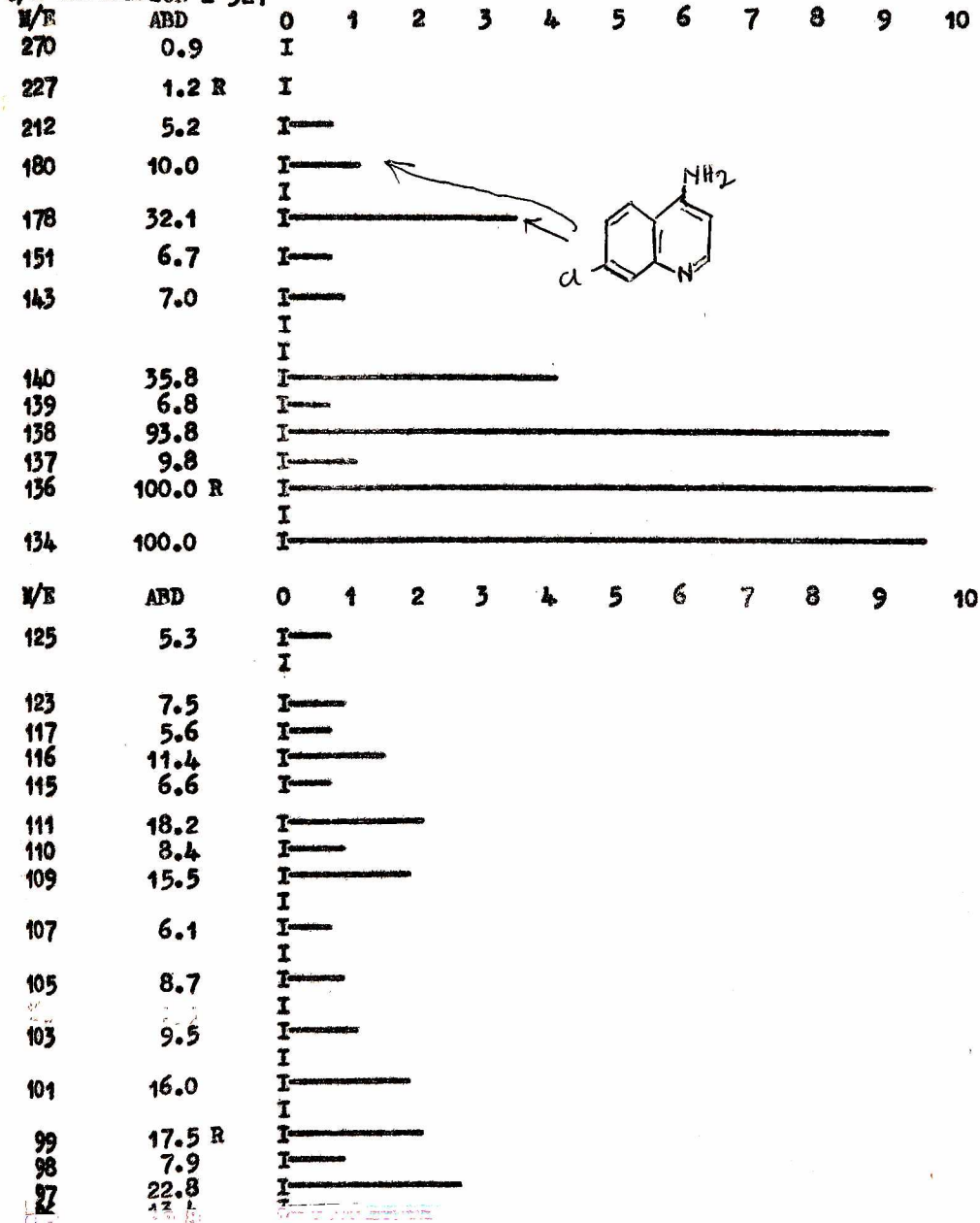


TABLE 3a

The identities of compounds on the chromatogram of the extract of the incubated mixture containing chloroquine and the microsomal/cytoplasm system, as shown by matching standards

U.V. -absorbing spot*	Spots from reaction mixture Rf	Corresponding matching standard
I	0.04	The primary amine derivative (SN 13617)
III **	0.47	Unchanged chloroquine
IV	0.65	4-(4'-hydroxyl-1'-methylbutylamino)-7-chloro-quinoline or the 4'-hydroxy derivative
VI	0.72	The 4' -aldehyde derivative
VII	0.78	7-chloro-4-aminoquinoline

* U.V. - absorbing spots are numbered I to VII in order of increasing Rf values as in Table 1a (See page 66)

** This had already been identified in Section I (page 51) as

RESULTS

The identities of the compounds on the chromatogram of extract of the incubated mixture containing chloroquine and microsome/cytoplasm system as shown by matching standards is presented in Table 3a. The identity of spot VII was further confirmed by running a mass spectrum on the eluate of spot VII. The resulting spectra in Figure 23 show peaks at m/e ratio of 180 and 178 in the proportion of 1:3.2, thus corresponding to 7-chloro-4-aminoquinoline in which Cl is ^{37}Cl and ^{35}Cl isotopes respectively. Incidentally, this is the normal isotopic composition of Cl and all its derivatives, ie. ^{37}Cl : ^{35}Cl (Williams and Fleming, 1973).

Four of the U.V. absorbing spots were not identified in this experiment. These are spots II (Rf 0.19), IIa (Rf 0.22), IIb (Rf 0.33) and V, Rf 0.69 (See page 68).

B. IN VITRO STUDIES ON THE METABOLISM OF REFERENCE

QUINOLINE COMPOUNDS USED AS STANDARDS IN SECTION VA

In another experiment to study the sequence of metabolism of chloroquine and its derivatives, a 0.1% (w/v) solution of each of the reference compounds (in ethanol) used in Section VA was incubated with the microsome/cytoplasm system using the incubation system M2 given in the general methods (page 44). Incubation was carried out for one hour at 37°C ; and each incubation mixture was

TABLE 3b

TLC analysis of the extracts of the incubated mixture containing the microsome/cytoplasm system with a reference quinoline compound as substrate

Reference quinoline substrate	Observation on thin layer chromatogram of extract	Discussion Comments
7-chloro-4-aminoquinoline	Only one U.V.-absorbing spot Rf 0.78, and corresponding to unchanged 7-chloro-4-aminoquinoline was observed.	7-Chloro-4-aminoquinoline was probably not metabolized by rat liver microsomes to any U.V.-absorbing derivative.
The 4'-aldehyde derivative.	Two U.V.-absorbing spots were observed - a faint one at Rf 0.72, corresponding to the unchanged 4'-aldehyde; and another strong U.V.-absorbing spot at Rf 0.65, corresponding to the 4'-hydroxy derivative.	Comparing ^{ing} the intensities of the U.V.-absorbing spots, it appears that most of the 4'-aldehyde derivative is converted <u>in vitro</u> by rat liver microsomes into the 4'-hydroxy derivative
The 4'-hydroxy derivative	Only one U.V.-absorbing spot was observed at Rf 0.65, corresponding to the unchanged 4'-hydroxy derivative.	The 4'-hydroxy derivative was probably not further metabolized <u>in vitro</u> by the rat liver microsome/cytoplasm system.
The primary amine derivative (SN 13617)	Two U.V.-absorbing spots corresponding to the 4'-hydroxy derivative, Rf 0.65, the unmetabolized primary amine derivative, Rf 0.04 and a weak U.V.-absorbing spot of the 4'-aldehyde derivative Rf 0.72 were observed. Other ultraviolet-absorbing spots were detected on the plates, but the ultraviolet spectra of their eluates did not correspond to that of any known compound of the 4-aminoquinoline series.	The primary amine derivative SN 13617 (page 66) is metabolized <u>in vitro</u> by the rat liver microsome/cytoplasm system into at least two other metabolites, namely: the 4'-hydroxy and the 4'-aldehyde derivatives. In the absence of a labelled atom in the nucleus of the primary amine derivative, it would be difficult to establish the precursor of the compounds contained in the eluates of the other U.V.-absorbing spots.

extracted with ether and also with dichloroethane: iso-amyl alcohol mixture. Each extract was subjected to thin layer chromatographic analysis as in section VA and the results obtained are summerised in table 3b.

SECTION VI

Investigations into the mechanism of the in vitro
degradation of chloroquine by hepatic microsomal
fraction of rat liver homogenate

The in vitro oxidation of tertiary amine drugs and other foreign compounds has been extensively studied over the past three decades; and, of the different pathways by which tertiary amines can be oxidized, oxidative N-demethylation has been thoroughly studied (Gillette, 1969).

The work of McMahon and Sullivan (1964) suggests a mechanism requiring a direct oxidative attack on the methyl group of an N-methyl tertiary amine to form the N-hydroxy methyl derivative which would readily decompose to the secondary amine and formaldehyde. On the other hand, the experiments of Fish et al (1955) and the subsequent kinetic studies of Ziegler and Pettit (1964) suggest that, the initial oxidative attack occurs on the nitrogen atom to yield an intermediate N-oxide. This oxygenated intermediate is then dealkylated by a second enzyme system. Upon subsequent studies by the latter investigators on N, N-dimethyl-aniline for example, Ziegler and Pettit suggested further that the microsomal system catalysing the oxidative N-dealkylation of lipid soluble dialkylaryl amines could be separated into two

partial reactions (i.e. the N-oxide synthesizing and the N-oxide dealkylating systems). Based on the subsequent demonstration that the enzymic dealkylation of the N-oxides of simple methylarylamines is inhibited by SKF 525-A (Machinist et al, 1966), Zeigler and Pettit (1966), demonstrated the accumulation of the N-oxide of dimethylaniline in presence of SKF 525-A.

In part, the extensive investigations on the pathway of metabolism of simple methyl arylamines via N-oxides can be attributed to the use of a sensitive spectrophotometric assay for the estimation of formaldehyde developed by Nash (1953). Although the N-oxidation of tertiary amines has been demonstrated in many vertebrates (Uehleke and Stahn, 1966) including man (Kuntzman et al, 1967), studies on the in vitro N-oxidation of certain other alkylamines, such as ethyl arylamines have been hampered by lack of sensitive micro methods for the estimation of acetaldehyde - a side product of N-dealkylation of most ethylamines and for the detection of a wide variety of amine oxides.

This chapter discusses attempts made to demonstrate the probable formation of the N-oxide of chloroquine and the concomitant production of acetaldehyde when chloroquine is incubated with the microsomal fraction of rat liver homogenates.

(a) Distribution of the products of the incubation of [^{14}C]-
Chloroquine with hepatic microsomes between the unchanged
drug and metabolites thereof, in presence of SKF 525-A

Ten micromoles of (ring-3- ^{14}C) chloroquine (16 μCi) was incubated for 30 minutes at 37°C with the hepatic microsomal fraction in presence of SKF 525-A (2 μmole). An ethereal extract of the incubated mixture was then analysed by thin layer chromatography and quantitative determination of the U.V. absorbing areas was made by the method in section III (page 71). The motive behind this experiment was that if the oxidative dealkylation of chloroquine really proceeded through the formation of the N-oxide intermediate, and if the subsequent oxidation of the N-oxide formed was inhibited by SKF 525-A, then, the presence of SKF 525-A in the incubation system should lead to the accumulation of the U.V. - absorbing spot corresponding to the N-oxide.

Results:

Table 2 (page 73) shows that the ether extract of incubation systems containing SKF 525-A yielded a mean of 64.93% of the 4-aminoquinoline bases as the unchanged drug. About 5% of the quinoline bases did not move from the origin and could not be characterised. In addition, however, the same extract contained 14.32% of 4-aminoquinoline bases in the form of the compound contained in spot V.

In a similar extract of incubation systems containing no SKF 525A, the compound in the U.V. - absorbing spot V accounted for only 2.70% of the total 4-aminoquinoline bases. There were also slight increases in the percentage of compounds contained in spots II and III and corresponding decreases in those in spots IV and VI in extracts of incubation mixtures containing SKF 525A, compared to the levels of these compounds from extracts of incubation systems without the SKF 525A. Metabolite VII accounted for 4% of the total quinoline bases in the extract of the former incubation system whilst no trace of it was found in extracts of the latter incubation systems.

(b) Acetaldehyde estimation in hepatic microsomal incubation mixtures of chloroquine

Acetaldehyde is one of the products of dealkylation reactions involving ethylamines (Williams, 1959). Attempts were therefore made to estimate the rate of acetaldehyde production in the microsomal incubation systems using a modified method of Gupta and Robinson (1966) as outlined under method (M5).

Acetaldehyde concentrations measured using this method ^{were} ~~not~~ not sensitive enough to measure low rate of acetaldehyde production and allow any subsequent kinetic studies on chloroquine degradation, based on acetaldehyde measurements, to be made.

Two other unsuccessful attempts were made in a search for sensitive methods for the estimation of acetaldehyde. The first was a modification of the method of Nash (1953) for the estimation of formaldehyde. This is based on the formation of a corresponding pyridine ring, diethyl 1, 4-dihydrocollidine, from a reaction between acetaldehyde, acetylacetone and NH_3 . Nash (1953) reported a yield of 51% for the pyridine compound formed from this reaction, and that the compound had an absorption maximum at 353 nm. Estimation of acetaldehyde by this method might have had an advantage over the diffusion method of Gupta and Robinson (1966) in that acetaldehyde was trapped as it was formed in the solution to form the pyridine compound. However, the rate of acetaldehyde production measured by this method was still too low for it to be used in studies of the microsomal degradation of chloroquine.

The other unsuccessful attempt on the estimation of acetaldehyde was based on the micro diffusion method of E. Conway (1960). This method was however found unsuitable since thin layer chromatographic analysis of extracts of incubation mixtures on which acetaldehyde estimation using this method was made did not reveal any U.V. - absorbing spot. The method was therefore abandoned on the grounds that the use of vigorous conditions such as 0.66N H_2SO_4 and tungstic acid probably destroyed the quinoline nucleus of chloroquine - the substrate.

(c) Measurements of rate of Oxygen consumption in hepatic microsomal incubation mixtures of chloroquine

In a further search for analytical methods for conducting kinetic studies on chloroquine oxidation, attempts were made to measure the rate of oxygen-consumption in the hepatic microsomal incubation system. The dealkylation enzymes are presumed to catalyse the consumption of one molecule of oxygen per molecule of substrate consumed (Mason, 1957, 1965).

Oxygen measurements were made using an oxygen electrode (Rank model). The sensitivity of measurements made by this method was so negligible that no subsequent kinetic studies on the metabolism of chloroquine based on the rate of oxygen consumption could be made.

PART IVC O N C L U S I O N S

The data on the subcellular distribution of chloroquine degradation by the hepatic cellular fractions (tables 1a and 1b) is very interesting especially in view of the fact that a lot of the efforts of most biochemical pharmacologists has been directed towards the solubilization of lipid-soluble microsomal enzyme systems. Thin layer chromatographic analysis of both the ether and the dichloroethane - iso-amyl alcohol extracts of incubation mixtures of chloroquine shows that, the soluble fraction (105,000 x g supernate) of rat liver homogenates contains enzymes responsible for the in vitro degradation of chloroquine. The soluble fraction could therefore be used for metabolic studies involving chloroquine. Also important in the results presented in tables 1(a) and 1(b) is the further observation that, the mitochondrial fraction of the rat liver homogenate degraded chloroquine to a some extent. This possibility has been mentioned by La Di et al (1971). However, consistent with current thinking, the maximum activity for chloroquine degradation has been found (tables 1a and 1b) to be localised in the microsomal fraction.

The data on the metabolism of (ring-3-¹⁴C) chloroquine (table 2) indicates that chloroquine was the precursor of all the

nine ultraviolet absorbing spots which appeared on chromatograms of the dichloroethane - iso-amyl alcohol extracts. This implies that the microsomal fraction of the rat liver homogenate contains enzyme systems that metabolize chloroquine into a minimum of eight metabolites in which the 4-aminoquinoline base is intact - one of the nine ultraviolet absorbing spots (Rf 0.47) was identified as unchanged chloroquine. Four other U.V. - absorbing spots were also identified using reference 4-aminoquinoline derivatives as standards. These are the primary amine derivative (Rf 0.04), the 4'-hydroxy derivative (Rf 0.65), the 4'-aldehyde derivative (Rf 0.72) and 7-chloro-4-aminoquinoline (Rf 0.78) (see table 3a page III 0). These results are also consistent with the work of McChesney et al. (1966), in which they observed eight ultraviolet absorbing spots on thin layer chromatograms of urine extracts from monkeys on chloroquine. Using the same solvent system, McChesney and his associates located all eight U.V.-absorbing spots at the position (Rfs) as those of the eight U.V. spots observed in these experiments (table 2). It is therefore not surprising that the identity of compounds contained in some of the U.V. - absorbing areas in these experiments (section III) parallels that made by McChesney and his associates (1966) - spot I (Rf 0.04), being the primary amine; spot III (Rf 0.47), the unchanged chloroquine; spot IV (Rf 0.65), the 4'-hydroxy derivative and spot VII (Rf 0.78), the 7-chloro-4-aminoquinoline.

In their proposed metabolic pathway for the in vivo degradation of chloroquine, McChesney and his associates alluded to the 4'-aldehyde derivative as a very important intermediate which, to a minor extent, is apparently reduced to the 4'-hydroxy derivative. For the first time, the 4'-aldehyde has not only been identified as a metabolite of chloroquine (table 3a, page 110) but also, its subsequent metabolism to the 4'-hydroxy derivative has actually been demonstrated (table 3b) (page 112). In fact, it has been further demonstrated through in vitro studies in this thesis that, the 4'-aldehyde is largely converted to the 4'-hydroxy derivative by the microsomal fraction of the rat liver (refer to Table 3b page 112). This latter finding appears contrary to the proposal of McChesney and his associates, that, the 4'-aldehyde is a major metabolite in vivo which, to a minor extent, is reduced to the 4'-hydroxy derivative.

The disagreement in the results in this thesis and that proposed by McChesney and his associated (1966) is however not unexpected. It has already been stated elsewhere in the literature review (page 11) that such factors as absorption, tissue distribution, the role of gut bacteria etc. dictate the overall picture of species variation in drug metabolism; and that investigations based on either in vivo or in vitro studies alone are not sufficient to account for species variations in drug metabolism. Perhaps the differences in the results recorded in this thesis and that proposed by McChesney et al. (1966) might be a clear example of this fact.

It is however evident from both results that the 4'-aldehyde is not only an intermediate in chloroquine metabolism, but that it is to some extent converted to the 4'-hydroxy derivative.

No attempt was however made to detect the 4'-carboxy derivative, which has been shown to be the principal metabolite in the monkey (McChesney et al, 1966); its probable formation therefore cannot be ruled out. The observation that the 4'-hydroxy derivative is not further metabolized by hepatic microsomal enzyme systems is contrary to the conjecture by McChesney et al, (1966) that the 7-chloro-4-aminoquinoline is formed by the successive degradation of the butylamino side chain through the 4'-hydroxy derivative was not metabolized further by the microsomal enzyme system, it appears the 7-chloro-4-aminoquinoline observed on thin layer chromatograms of chloroquine might have been formed directly from chloroquine by an alternative pathway. The 7-chloro-4-aminoquinoline was also not metabolized further by the microsomal fraction; and it could probably be the end product of the alternative pathway being proposed.

The secondary amine (SN 13616) could not however be synthesized in this laboratory for lack of suitable glassware to withstand the high pressure conditions required for the reaction between ethylamine and 4-(4'-chloro-1'-methylbutylamino)-7-chloroquinoline (Fig. 11). It is however interesting to note that the Rfs of the compounds so far identified in these experiments are

identical in most cases to the Rf's of the same compounds identified by McChesney and his associates who used the same solvent system. For example, in both these experiments, and that of McChesney and his associates, the unchanged drug has been identified at Rf 0.47, the 4-amino-7-chloroquinoline at Rf 0.78. The 4'-hydroxy derivative which was located by McChesney and his associates at Rf 0.63, was however observed in these experiments at Rf 0.65. McChesney et al. (1966) also located the secondary amine (SN 13616) derivative at Rf 0.17. Spot II in these experiments (tables 1a, 1b and 2) with Rf 0.19 is close to the location for SN 13616; and, this, together with the fact that the U.V.-spectrum of spot II (Fig. 6) has identical characteristics (max at 330 and 343 nm) as the 4-aminoquinoline bases, suggest\$that spot II in tables 1a, 1b and 2 is probably the secondary amine derivative (SN 13616).

By a similar argument, one could also speculate that spot IIa, Rf 0.33 (tables 1a and 2) is probably hydroxychloroquine (i.e, the analogue of chloroquine in which a hydroxyl group has been substituted in the 2- position of one N-ethyl group). McChesney and his associates using the same solvent system, identified authentic hydroxy-chloroquine at Rf 0.31.

The implication of hydroxychloroquine as a metabolite of chloroquine would not contradict modern theories of drug metabolism.

It would only support the mechanism proposed by McMahon and Sullivan (1964) that the initial process in N-dealkylation of tertiary amines involves a direct attack on the alkyl group of N-alkyl tertiary amines to form the N-hydroxy alkyl derivative; which in turn readily decomposes to give a secondary amine and the corresponding aldehyde. The possibility of hydroxychloroquine being an intermediate of chloroquine metabolism is further supported by the further observation of McChesney et al (1966) that thin layer chromatograms of urine extracts of humans on hydroxychloroquine indicated the following composition: hydroxychloroquine, 49%; SN 13616 - the secondary amine, 21%; SN 13617 - the primary amine, 6%.

McChesney and his associates also demonstrated through in vivo experiments that SN 13616 is the precursor of SN 13617, the 4-hydroxy-derivative and the 4-amino-7-chloroquinoline, together with small amounts of other unidentified compounds. It has also been shown in these experiments that the primary amine, SN 13617 is metabolized in vitro, to the 4'-aldehyde and the 4'-hydroxy derivatives. In the light of these findings, it is being proposed that the metabolic degradation of chloroquine in vitro by the hepatic microsomal enzyme systems of rat proceeds by the formation of the following metabolites in order of:

- (i) the hydroxychloroquine,
- (ii) the secondary amine (SN 13616)
- (iii) the 4' - aldehyde which is largely converted to the 4' - hydroxy derivative.

But for the addition of hydroxychloroquine, the present proposed mechanism is in accord with the concept proposed by McChesney et al (1966).

The results in table 1(b) that all the six metabolites from the extract of the microsomal incubation system matched six of the twelve U.V. absorbing spots from the urine extract of humans on chloroquine, is also interesting in view of species differences demonstrated on the metabolic fate of certain drugs (Dingell et al , 1964). These data in table 1(b) therefore suggest that chloroquine metabolism at least, in part, follows qualitatively similar pathways in both man and the rat.

The result on the effect of SKF 525A on the in vitro metabolism of ^{14}C - labelled chloroquine is rather puzzling, though, not contradictory to current theories on drug metabolism. In table 2, SKF 525A is apparently activating the metabolism of chloroquine through the formation of both metabolite V, and the 4-amino-7-chloroquinoline. This observation is contradictory to the report made by Gaudette and Coatney (1961) that SKF 525A

caused a 50% inhibition of chloroquine metabolism in their in vitro experiments, in which they incubated chloroquine with the 9,000 x g supernate of rabbit liver microsomes. They assayed their enzyme activity by measuring the disappearance of substrate, chloroquine from an n-heptane extract (PH 9.0) using a spectrophotofluorimeter at peak activation of 330 and 440 nm. It has however been demonstrated in this laboratory that the n-heptane extract (pH 9.0) of rat liver microsomal incubation system is a mixture of the unchanged drug, the secondary amine (SN 13616) and the primary amine (SN 13617). Further, since the authors did not study the effect of SKF 525-A on the metabolic pattern of chloroquine in their incubation system, it would be very difficult to compare their results with the work in this thesis. SKF 525-A is a known inhibitor of most N-dealkylation reactions. The metabolism of a number of compounds such as, the N-dealkylation of N-methylaniline, O-dealkylation of phenacetin, sulfoxidation of chloropromazine and the reduction of aromatic nitro compounds, are however, not inhibited by SKF 525-A (Mannering, 1969). Since in these experiments, the amounts of metabolite V and 4-amino-7-chloroquinoline are shown to accumulate on incubation of the hepatic microsomal fraction with chloroquine in presence of SKF 525-A (Table 2), it is being further proposed that the metabolism of chloroquine in vitro may also follow an alternative pathway through metabolite V to produce the 4-amino-7-chloroquinoline.

It is also suggested that it is probably this latter pathway in the rat which is activated by the presence of SKF 525-A. Further investigations to prove this hypothesis were not possible because of lack of more labelled chloroquine and other experimental materials. The difference between these results and that of Gaudette and Coatney (1961) might be explained as due to species differences in the metabolic fate of chloroquine in the rat and the rabbit; and that probably chloroquine is not metabolized through metabolite V in the rabbit. Rats for example, have been shown to metabolize imipramine by demethylation, whilst rabbit microsomes are reported to metabolize this drug through hydroxyimipramine (Dingel et al, 1964).

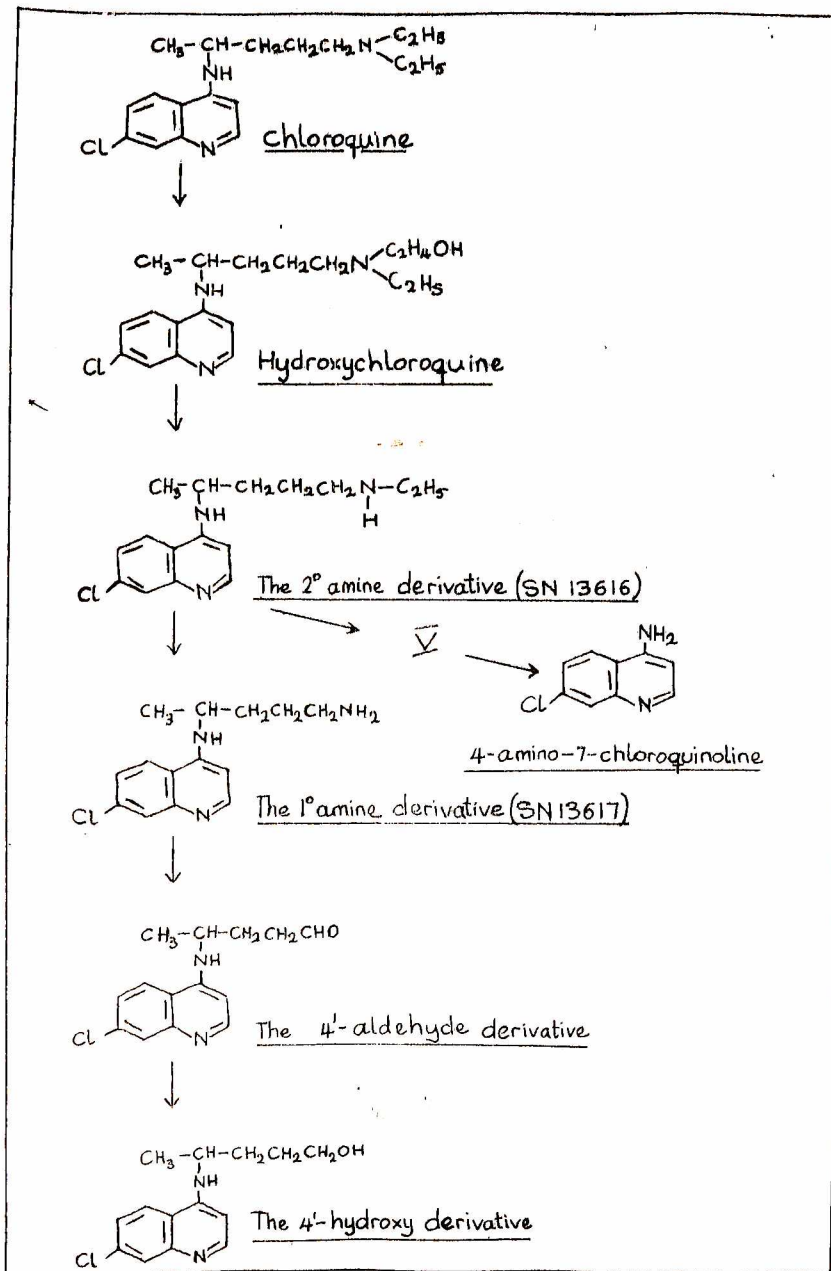
In man, chloroquine is slowly metabolized and excreted; and 600 mg chloroquine base have been shown to provide suppression of malaria for at least 20 days (Gaudette and Coatney 1961). Individuals receiving single doses of 300 mg base for 26 weeks experienced suppression for at least 16 days after the last treatment. Yet in a program where drugs are used for the eradication of malaria, longer parasite-free intervals would be desirable. Chloroquine is known to exert its antimalarial activity directly in vivo (Olatunde, 1971); and a prolongation of this activity might be obtained if its metabolic transformation could be retarded. It is hoped the observations

FIGURE 24

PROPOSED PATHWAY FOR THE METABOLISM OF CHLOROQUINE
BY RAT HEPATIC MICROSOMAL ENZYME SYSTEM

FIGURE 24

130.



and conclusions in this thesis would stimulate further interest into finding sensitive methods for enzymic studies on the metabolism of chloroquine in vitro; and also to map out the probable pathways of chloroquine metabolism in different animal species. In conclusion, the proposed pathways of chloroquine metabolism by rat liver microsomes is summarised in Fig. 24. The metabolic fate of chloroquine in man probably follows the same pathway.

SUGGESTION FOR FURTHER STUDIES

1. Chloroquine is known to exert its antimalarial activity directly in vivo (Olatunde, 1971); and a prolongation of this activity might be obtained if its metabolic transformation could be retarded. This could be done by finding sensitive methods for enzymic studies on the metabolism of chloroquine in vitro.
2. Patients are often given several drugs at the same time (a practice often referred to as 'polypharmacy'); and sometimes the combination results in an undesirable effect because one drug may inhibit or stimulate the metabolism of ~~the~~ ^{another} ~~drug~~. It might therefore be useful to study the metabolic interaction between chloroquine and other drugs in common use. The need for such an investigation arises when one considers the evidence that, the urinary excretion of chloroquine is slow and discontinuous (Zanca and Benetti, 1959); and that three to five years after the last known injection of chloroquine, patients were found to be excreting the drug in the urine.
3. There is also the evidence that long term treatment of rats with microsomal enzyme inducers or inhibitors affect the uterotrophic action of compounds used extensively as oral

contraceptives (Levin et al., 1968). In view of the extensive use to which chloroquine is put against malaria and other diseases, and the fact that it is slowly excreted from the body, it might also be worth the effort to study the effect of chloroquine on the metabolism of estrogens.

One approach to this investigation is to study the effect of chloroquine on the uterine response to estrogens using immature female rats.

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