CATECHOLAMINES AND HEPATIC DRUG METABOLISM

A thesis submitted to the
University of Glasgow
in candidature for the degree of
Doctor of Philosophy
in the
Faculty of Science
by

Alan Raymond Boobis, B.Sc.

Department of Pharmacology
Glasgow University

June, 1974

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

'If you really want to pass this barrier, you should feel like drinking a hot iron ball that you can neither swallow nor spit out.'

- Mumon

from Zen Flesh, Zen Bones

ACKNOWLEDGEMENTS

I wish to thank Prof. J.S. Gillespie for the opportunity of carrying out this work in his department over the past three years. His advice and friendly criticism are greatly appreciated.

I am endebted to Dr. G. Powis, my supervisor, who thought of the problem initially for his advice, help and guidance throughout this study. I am particularly grateful for his untiring help in the preparation of this thesis.

I would like to thank Dr. D. Sumner and Mr. A. Russell of the Radioisotope Department, Stobhill Hospital for their advice and assistance in the kinetic analyses of the data.

Miss H. McCaffery prepared the tissues for light microscopy. Dr. H. Elder and Mr. I. Montgomery of the Department of Physiology prepared tissue for electron microscopy and took the micrographs.

Mr. R. Callander and Mr. I. Ramsden prepared the artwork for this thesis and Mr. H. MacDonald took many of the photographs that appear in this thesis.

Dr. Sleigh of the Department of Bacteriology, Western Infirmary performed the bacteriological examination of samples.

The blood gas analyses were carried out with the help of the Department of Anaesthetics, Royal Infirmary.

The Department of Radiography, Western Infirmary, developed the X-radiographs.

Mrs J.S. Grimley provided valuable help in the laboratory.

Miss M. Paton was very considerate in lending me texts for periods far in excess of the stipulated time.

The Western Regional Blood Transfusion Unit kindly supplied the red blood cells used throughout the study.

I would like to accord my sincere gratitude to my sister, Stephanie Boobis, who typed all the drafts, and finally this thesis itself, a considerable achievement in patience and perserverance

Finally, I would like to thank the Medical Research Council for its continued financial support during the course of this work.

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SUMMARY

The thesis embodies work designed to investigate the acute effects of catecholamines on the hepatic metabolism of foreign compounds. Most of the studies were performed on the isolated perfused liver of the rat. The historical development of the techniques used in the perfusion of the liver is reviewed. The literature concerning the properties of the hepatic mixed function system responsible for the metabolism of foreign compounds has been reviewed. Previous work on the effects of catecholamines on drug metabolism is also discussed.

The operative procedure and the technique for the perfusion of the liver were developed to enable rapid transfer of the liver to the perfusion chamber without a period of anoxia, which has characterised many previous attempts at perfusion of the liver. The liver was perfused with a semi-synthetic medium. The viability of the liver was assessed by a variety of tests of biochemical and physiological function and by histological examination. The technique developed allowed the liver to be successfully perfused for periods of up to 6 h, whilst maintaining a functional integrity.

The metabolism of the type I and the type II substrates, hexobarbitone and aniline respectively, were investigated. Hexobarbitone is removed by a first order reaction at a rate comparable to that previously reported for the liver <u>in vivo</u>.

Aniline metabolism, which has not previously been investigated in the perfused liver, was studied in some detail. Aniline is removed biphasically from the perfusion medium.

Half of the aniline removed is converted to an acid-labile conjugate, possibly aniline-N-glucuronide, and a further 25% is converted to p-aminophenol and its conjugates. 3H-aniline added to the perfused liver could be quantitatively accounted for after 3 h perfusion. In agreement with previous findings, it was found that p-aminophenol reacts with haemoglobin, thus making it impossible to detect free p-aminophenol in the perfusion medium. The differences between the pattern of aniline metabolism by the perfused liver and by the whole animal, where aniline is excreted mainly as p-aminophenol and its conjugates, are discussed.

The kinetics of aniline removal have been analysed by curve stripping and model fitting. The mathematics of these procedures are described separately in Appendix II. In the perfused liver aniline is distributed throughout a two-compartment system. The first compartment probably represents the total aqueous phase and aniline is removed from this compartment only. The identity of the second compartment is less certain. Several possibilities are discussed and in view of evidence obtained with SKF 525-A, an inhibitor of drug metabolism, it is suggested that the second compartment may represent the binding of aniline to a non-metabolic site of cytochrome P-450.

Both adrenaline and noradrenaline inhibit the metabolism of hexobarbitone by the perfused liver but neither catecholamine has any effect on the metabolism of aniline. Catecholamines do not inhibit the metabolism of either aniline or hexobarbitone by the microsomal fraction or by liver slices.

It was thus concluded that catecholamines are not inhibiting hexobarbitone removal directly. The possibility that their effect is mediated by cyclic AMP, a compound reported to inhibit hexobarbitone metabolism by the perfused liver, was investigated. Papaverine, an inhibitor of phosphodiesterase, does not potentiate the inhibitory effects of catecholamines on hexobarbitone metabolism, nor does it cause them to have any inhibitory effect on the metabolism of aniline. Cyclic AMP was shown to inhibit drug metabolism by liver slices, whereas catecholamines are without effect. The probability that cyclic AMP is not important in mediating the effects of catecholamines on drug metabolism is discussed in some detail.

Both catecholamines increase the portal pressure in the perfused liver by over 100%. When Ca^{2+} is omitted from the perfusion medium it was found that this pressor effect is almost totally inhibited. Catecholamines also no longer inhibit the metabolism of hexobarbitone. The omission of Ca^{2+} leaves the metabolic effects of catecholamines relatively unaffected. It was thus suggested that catecholamines might be inhibiting hexobarbitone metabolism through an effect on the hepatic vasculature.

In a constant flow system, as used in this study, one way in which such an effect of catecholamines might manifest itself is as a redistribution of perfusate through the liver. It was found that considerable controversy exists as to whether or not catecholamines can cause a redistribution of blood flow within the liver. The available evidence is

assessed separately in Appendix II. The effects of adrenaline on the intrahepatic distribution of perfusate have been investigated by X-radiography, and by perfusing the liver with Indian ink followed by histology. It was concluded from these studies that catecholamines can cause a redistribution of blood flow within the liver, away from the periphery of the lobes towards more central regions.

Several possibilities involving a redistribution of perfusate are considered which might explain the selective inhibition of hexobarbitone metabolism by catecholamines. It was concluded that it probably results from a hypoxia of peripheral cells. This is superimposed on an overall tendancy to hypoxia caused by the increased oxygen consumption that will result from the stimulation of intermediary metabolism by catecholamines. This may then cause an inhibition of hexobarbitone metabolism, which is very sensitive to hypoxia, while having very little effect on aniline metabolism, which is relatively unaffected by even severe hypoxia.

The possible physiological significance of this effect of catecholamines on the metabolism of foreign compounds is discussed.

Rats were also pre-treated with catecholamines to determine their in vivo effects on hepatic drug metabolism. Noradrenaline was found to stimulate the metabolism of both aniline and aminopyrine by the microsomal fraction whereas adrenaline produces an inhibition of the metabolism of both types of substrate. It was found that this is not due to a

direct effect of catecholamines on the components of the microsomal electron transport chain.

The possibility that the effect is manifest during the preparation of the microsomal fraction was studied by washing microsomes, after their original preparation, in solutions of catecholamines. The results obtained were very similar to those found after pre-treating rats with catecholamines. However it was found that the apparent stimulation caused by noradrenaline is in fact a protection against a loss of drug-metabolising activity occurring on washing control microsomes.

The effects of washing microsomes were investigated further. It was found that EDTA also provides protection against the loss of activity caused by washing and even increases the activity compared with unwashed microsomes. Several possible mechanisms were investigated and these are discussed. It was found that one of the main effects of washing the microsomes is to stimulate the non-enzymatic peroxidation of lipid and to reduce the binding of type I substrates to cytochrome P-450. On the other hand, washing in EDTA largely prevents the increase of lipid peroxidation and gives rise to an increased binding of type I substrates to cytochrome P-450.

It was concluded that washing the microsomes stimulates lipid peroxidation, possibly by the removal of an endogenous inhibitor, and this results in the destruction of phosphatidylcholine, known to be necessary for the integrity of the type I binding site. This then results in a decrease in the metabolism

of type I substrates. EDTA, a known inhibitor of lipid peroxidation, prevents these effects.

Washing of the microsomes also almost completely abolishes the enhancement of aniline metabolism by acetone. This can also be prevented by washing with EDTA. It is suggested that the current work supports a previous hypothesis that acetone causes enhancement by promoting the binding of type II substrates to type I sites, thus increasing their metabolism. It is further suggested that washing the microsomes, by destroying type I sites, reduces acetone enhancement.

CHAPTER 1

Introduction

1.1 Historical perspectives of the metabolism of foreign compounds

Between 1840 and 1900 considerable research was carried out on the metabolism of foreign compounds and by the turn of the century most of the major routes of metabolism had been discovered. After 1900 the discovery of new routes of metabolism was slower and most research was concerned with studying the known routes in detail, although some work was directed towards investigating the underlying mechanisms of metabolism.

As research into the metabolism of foreign compounds was progressing during the first few decades of this century simultaneous discoveries were being made on cellular ultrastructure, with the pioneering work of Claude. In 1938 he isolated a fraction of the cell, later called the microsomal fraction (Claude, 1943a), which was shown to be derived from the endoplasmic reticulum (Porter, Claude and Fullam. 1945).

In 1949 Mueller and Miller reported that the hepatic microsomal fraction could metabolise aminoazo dyes by oxidative N-demethylation. This finding stimulated an upsurge in mechanistic interest in metabolic reactions which continues to the present day. However the study of the microsomal metabolism of foreign compounds was put on a firm basis by Brodie who showed that the hepatic microsomal fraction metabolised a wide variety of foreign compounds and established that it contained a group of low specificity enzymes capable of transforming many of the diverse anutrients ingested by the organism (Brodie,

Axelrod, Cooper, Gaudette, La Du, Mitoma and Udenfriend, 1955). Shortly after this classic paper Williams (1959) published an enlarged second edition of his book on detoxication mechanisms which was to become one of the leading sources of information on the subject for many years. He outlined the two phase concept of the metabolism of foreign compounds which rationalised the information available to that time and in the light of which future discoveries were more easily interpreted. In his scheme compounds could undergo either transformation (mainly oxidation) reactions or synthetic (conjugation) reactions or both.

The previous year Klingenberg (1958) and Garfinkel (1958) had discovered a new haemoprotein in the hepatic microsomal fraction, later called cytochrome P-450 (Omura and Sato, 1964). It was soon shown to be essential for microsomal oxidation reactions and with the development of more sophisticated spectrophotometric techniques considerable information became available on the new protein. A scheme for the molecular mechanisms involved in the oxidation of foreign compounds was devised by Gillette (1963) which included a requirement for cytochrome P-450 and an activated oxygen species. Spectrophotometric studies on cytochrome P-450 continued and in 1966 Remmer, Schenkman, Estabrook, Sasame, Gillette, Narasimhulu, Cooper and Rosenthal and Imai and Sato (1966b) found that foreign compounds produced characteristic changes in the difference spectrum of the microsomal fraction. Most foreign compounds producing such a change could be classified into one of two groups, based on the type of spectral change occurring. Subsequent work revealed that the changes were a reflection of

binding to sites on cytochrome P-450 and that the significance of the distinction into two groups went further than changes in the difference spectrum and had ramifications throughout almost all aspects of microsomal oxidative reactions (Gillette, Davis and Sasame, 1972).

During the past 15 years considerable advances have been made towards an understanding of many of the phenomena of microsomal oxidative metabolism. Although induction of drug metabolism was first reported in 1954 by Brown, Miller and Miller the mechanism involved is still not completely understood. Gelboin and Nebert (see review by Gelboin, 1971) have recently been examining the molecular mechanisms involved in induction and the genetic regulation of induction. Recently the importance of induction and metabolism in carcinogenesis has been realised (Creaven and Parke, 1966; Miller, 1970; Lake and Parke, 1972b).

Several groups of workers have recently been investigating the phenomenon of enhancement, an increase in the specific activity of the microsomal mixed function oxidase occurring in vitro. This work stems from findings in the latter half of the last decade by Imai and Sato (1966a) and Anders (1968) that several compounds could enhance drug metabolism by the hepatic microsomal subcellular fraction. Enhancement provides a means whereby the metabolising activity of the liver can be increased quickly and reversibly. At present there is little information available as to the mechanisms of enhancement or whether it may play a physiological role.

The mechanisms of the mixed function oxidases in the

liver have received considerable attention and many attractive reaction schemes have been proposed to explain the observed results (see review by Estabrook, 1971). Although a definitive scheme has yet to be devised some of the more recent schemes explain most of the observed data. Coon and his associates have recently been studying a reconstituted mixed function oxidase system. They have identified the components vital to the functioning of the system which has led to the realisation that cytochrome P-450 and the phospholipid phosphatidylcholine are complexed in some way that is responsible for the activity of the system (Autor, Kaschnitz, Heidema and Coon, 1973). The importance of this association is becoming increasingly apparent and recent evidence, over the past two years, implicates it in many of the unexplained phenomena of the oxidative metabolism of foreign compounds such as substrate-induced changes in the microsomal difference spectrum and the loss of metabolising activity that occurs on storage of the microsomes.

The hepatic microsomal mixed function oxidase is affected by a wide variety of genetic and environmental conditions and this aspect of its activity has received much attention. Another aspect to be investigated in some detail is the role of several hormones in possible regulatory mechanisms. Many steroids can cause an induction of the enzymes of drug metabolism (Conney, 1967) and a sex difference in the activity of the mixed function oxidase in rats has been shown to be due to androgens (Kato and Onoda, 1970). In contrast, high levels of circulating corticosterone result in inhibition of the mixed function oxidases (Radzialowski and Bousquet, 1968). Many other hormones such as growth hormone (Wilson, 1969) growth

factors in pregnancy (Neale and Parke, 1973), ACTH and thyroxine (Kato and Gillette, 1965) and glucagon and cyclic AMP (Weiner, Buterbaugh and Blake, 1972a) have been shown to influence mixed function oxidase activity but the physiological role of such effects has still to be determined.

Early work by Fouts (1962) suggested that an alteration in glycogen levels in the liver affected mixed function oxidase activity but it was later shown that this was not so (Dixon, Rogers and Fouts, 1964). Thus although the effects of many hormones in the long term control of drug-metabolising enzyme levels have been investigated in detail little is known of how the activity of the mixed function oxidase is regulated acutely, or even if such regulation occurs.

1.2 The aims of the investigation

A survey of the literature on the metabolism of foreign compounds revealed that very little is known of the normal physiological mechanisms for the acute regulation of drug metabolism. In view of the known acute regulatory effects of catecholamines upon other metabolic pathways it was thought possible that they might play some role in any such mechanism. The acute interaction of catecholamines and the hepatic mixed function oxidase have therefore been studied.

The effects of catecholamines upon drug metabolism were first studied in the isolated perfused liver of the rat. The perfused liver provided a system that closely resembled the liver *in vivo* while being free of possible complicating factors such as the secondary release of other hormones by catechol-

amines or interaction of the substrate with other organs. The viability of the perfused liver was first established, using several tests of hepatic function. The metabolism of hexobarbitone, a typical type I compound and aniline, a typical type II compound, both hydroxylated by the hepatic microsomal mixed function oxidase system, were studied in the perfused liver. Although the metabolism of hexobarbitone in the perfused liver had been previously reported (Sitzel, Anders and Mannering, 1966) the metabolism of aniline had not previously been investigated. Thus the metabolism of aniline by the perfused liver was first characterised in some detail. The effect of the catecholamines adrenaline and noradrenaline on the metabolism of these two substrates by the perfused liver was studied.

Due to the possible complications of the vasoactive effects of catecholamines upon hepatic mixed function oxidase activity the effects of adrenaline and noradrenaline on the microsomal fraction itself and upon the interaction of substrates with cytochrome P-450 were investigated.

Because it was possible that catecholamines might be acting at the level of the intact cell the investigation was later expanded to include a study of the effects of catecholamines on liver slices, which by their nature are independent of changes in blood flow and hypoxia but retain the organisation and compartmentalisation of the intact cell.

It was considered possible that the effect of catecholamines upon the activity of the mixed function oxidase of the

liver might be explained in terms of haemodynamic effects. Due to the controversy in the literature on the effects of sympathomimetics upon liver blood flow the influence of adrenaline on flow was investigated in the perfused liver by X-radiography and by Indian ink perfusion followed by histology.

The effects of the pre-treatment of rats with catecholamines was investigated to determine the time course of the changes in microsomal metabolism reported by Fouts (1962) and to determine if such changes could be due to the effects observed in the perfused liver.

The results of this investigation raised the possibility that some of the effects of catecholamine pre-treatment were due to changes caused in the microsomal fraction during its preparation.

Since adrenaline was known to be an inhibitor of lipid peroxidation the possible involvement of lipid peroxidation with mixed function oxidase activity was investigated. In the course of this study it was observed that washing microsomes reduced their drug-metabolising activity and also abolished acetone enhancement of this activity. This was further studied using inhibitors of lipid peroxidation.

In all studies the rat was used as an experimental animal. It has been widely used both in studies on the metabolism of foreign compounds and in liver perfusion experiments, and its use is well documented in these areas. It is easily available,

cheap and can be maintained with the minimum of attention. Rat liver is particularly suitable for perfusion since it is of a manageable size. The liver of larger animals presents problems in the adequacy of the oxygen supply and the relative importance of the hepatic artery.

CHAPTER 2

The Metabolism of Foreign Compounds

2.1 Historical introduction to the metabolism of foreign compounds

For over 120 years it has been known that the organism can transform ingested foreign compounds and excrete the products. The earliest recorded observation of such a reaction was made by Rouelle in 1784 who noticed that animals excreted hippuric acid after the ingestion of benzoic acid. However he did not propose any causal relationship and it was not until 1831 that Wöhler put forward the idea that after the ingestion of benzoic acid the organism actively converted it to hippuric acid. It was later shown by Schultzen and Gräbe (1867) that the formation of hippuric acid was a common metabolic pathway for several aromatic acids.

The first reported examples of the oxidation of foreign compounds were the hydroxylation of benzene to phenol and the hydroxylation of toluene to benzoic acid (Schultzen and Naunyn, 1867). From this date to the turn of the century most of the routes of biotransformation were to be discovered.

Ethereal sulphate conjugation was discovered by Baumann in 1876. He observed that these conjugates were much less toxic than the parent compound. This was the first suggestion that biotransformations were other than simple metabolic conversions and from this time the concept of detoxification slowly gathered momentum, though it was not until 1895 that it was explicitly stated when Neumeister (1895) proposed that the process of glucuronide conjugation was one of detoxification.

In 1879 after 25 years of accumulated evidence Schmiedeberg

and Meyer established that glucuronidation was one of the synthetic reactions of the biotransformation of foreign compounds. In the same year Baumann and Preusse (1879) observed that bromobenzene was converted to a mercapturic acid and independently Jaffe (1879) also discovered that halogenated benzenes formed mercapturic acids.

In 1887 Jaffe and Cohn found that furfural, a heterocyclic compound, was converted to pyromucic acid by oxidation, followed by conjugation to furoylglycine and furfuracryluric acid. They also found that although furfural was oxidised to pyromucic acid by hens it was then conjugated with ornithine (Jaffe and Cohn, 1888) confirming an earlier finding of Jaffe (1877) that hens formed ornithine conjugates only.

His in 1887 reported the transformation of a foreign organic compound by methylation, the conversion of pyridine to methylpyridinium hydroxide.

In 1893 Cohn discovered that dogs and rabbits fed nitrobenzaldehyde excreted acetylaminobenzoic acid and hence discovered the acetylation of aromatic compounds.

An interesting observation by Lang (1894) was that some of the hydrocyanic acid fed to dogs was excreted as the thiocyanate. This compound is only 0.5% as toxic as the parent drug so that this reaction may be regarded as a true detoxification mechanism without conjugation.

Discovery of new routes in the metabolism of foreign

compounds became slower after the turn of the century, since most reactions had been described in the previous 25 years. Thierfelder and Sherwin (1914, 1915) observed that phenylacetic acid could be conjugated with glutamine in some primates. Forty years later Myers and Smith (1954) found that phenols were conjugated with glucoside in insects.

Thus, by 1920, almost all of the routes of metabolism had been discovered and over the next three decades most work was concentrated on consolidating these findings. This work culminated in the publication of 'Detoxication Mechanisms' by R.T. Williams in 1947. The mid 1950's witnessed one of the major turning points in the study of drug metabolism. Two important proposals were made that were to transform our understanding of the drug-metabolising system. The first, by Brodie et al in 1955, was that most biotransformation reactions were catalysed by a series of enzymes situated in the endoplasmic reticulum of the liver cell. The full significance of this finding is discussed later. It turned attention towards a specific site of metabolism and in the twenty years that followed, intensive research has provided much insight into the mechanisms operating therein. The second proposal, by Williams in a second edition of his book in 1959, was a scheme for the progressive metabolism of compounds to active or inactive products. Metabolic reactions were divided into phase I and phase II. Phase I reactions consisted of oxidation and reduction involving transformation of existing groups on the molecule whereas phase II reactions consisted of conjugation reactions involving a synthetic process in which an existing molecule was added to a compound. These proposals permitted

a rationalisation of the metabolic processes that had not been possible previously. The different types of biotransformation reactions have been discussed at length in several excellent reviews (see Williams and Parke, 1964; Parke, 1968; Parke and Williams, 1969; La Du, Mandel and Way, 1971; Brodie and Gillette, 1971; Parke, 1972).

2.2 The metabolism of foreign compounds by the hepatic microsomal subcellular fraction

2.2.1 The concept and nature of the microsomal fraction

Brodie et al (1955) showed that the hepatic microsomal fraction was responsible for the metabolism of many foreign compounds. This fraction was first isolated by Claude in 1938 and was found to consist mainly of a lipoprotein complex together with some ribosomal type nucleic acid as well as some other proteins (Claude, 1939, 1940; Feulgen and Bersin, 1939).

Although the fraction was of cellular origin, the form that the elements adopted occurred during their isolation (Claude, 1938, 1939). Claude (1943a) called them 'microsomes' to distinguish them from other, natural, cell components. It was shown by histological methods and preparative ultracentrifugation that the microsomal fraction was derived from an important part of the fundamental substance of the cytoplasm (Claude, 1943b, 1946) and further investigations established that it was derived from the endoplasmic reticulum (Porter et al, 1945; Claude, Porter and Pickels, 1947). Thus in the ten years following its first isolation the microsomal fraction

was characterised as vesicular, with a diameter between 100 and 150μ, derived from the endoplasmic reticulum, and comprising mainly lipoprotein.

2.2.2 The involvement of the microsomal subcellular fraction in the metabolism of foreign compounds

Shortly after the characterisation of the microsomal fraction Mueller and Miller (1949) reported that certain foreign compounds could be metabolised by this system. However it was not until 1955 that Brodie et al published their important paper, with its much wider implications, in which they established that the hepatic microsomal fraction was responsible for the metabolism of a wide range of foreign compounds. Microsomal metabolism was found to have a requirement for NADPH, Mg^{2+} and molecular oxygen (Brodie et al, 1955). This placed the enzyme system within the external mixed function oxidase classification of Mason (1957, 1965) and also in the monooxygenase classification of Hayaishi (1964). It was shown with labeled O_2^{18} and H_2O^{18} that the oxygen utilised in the hydroxylation of acetanilide was derived from molecular oxygen and not from water (Posner, Mitoma, Rothberg and Udenfriend, 1961).

The hepatic microsomal fraction was found to catalyse a wide variety of oxidative reactions. These were deamination, N-, O-, and S- dealkylation, aryl and alkyl hydrocarbon hydroxylation, epoxidation, formation of alkylol derivatives, N-hydroxylation, N- and S- oxidation, and dehalogenation (for review see Gillette, 1966). This bewilderingly diverse series of reactions becomes more comprehensible when viewed in the

light of a suggestion made by Brodie, Gillette and La Du (1958). They pointed out that all of these reactions could be regarded as forms of a hydroxylation reaction and this concept was supported by further work of Gillette (reviewed 1963, 1966). The reactions of the hepatic drug-metabolising enzymes situated in the microsomal fraction have been reviewed many times in the past decade (see Gillette, 1963, 1966, 1969; Mannering, 1968, 1971; Parke, 1968, 1972; Gillette, Conney, Cosmides, Estabrook, Fouts and Mannering, 1969; Brodie and Gillette, 1971; Kratz, 1973).

2.2.3 The components of the hepatic microsomal electron transport system

Brief mention was made in the last section of the involvement of NADPH and molecular oxygen in the metabolism of foreign compounds by the microsomal fraction. They are utilised by an electron transport system responsible for the oxidation of substrates. Chance and Williams (1954) had noticed that more NADPH was required to reduce cytochrome b_5 in the liver microsomal fraction than was theoretically necessary. They postulated that in addition to cytochrome b_5 there was another electron accepting component present. Four years later the development of new spectrophotometric techniques by Chance (1957) enabled a thorough investigation of the cytochrome pigments observed in the microsomal fraction to be carried out. In the following year independently, and almost simultaneously, Klingenberg (1958) and Garfinkel (1958) announced the discovery of a new carbon monoxide-binding cytochrome (haemoprotein) in the microsomal fraction. This was confirmed by Omura and Sato (1962, 1964)

who were first to name the new haemoprotein cytochrome P-450 (Omura and Sato, 1964). Considerable evidence has now accumulated that cytochrome P-450 is vital, and central, to the functioning of the microsomal mixed function oxidase and it has been extensively reviewed recently (Gillette and Gram, 1969; Estabrook and Cohen, 1969; Estabrook, 1971; Mannering, 1971; Gillette et al, 1972). The properties and role of cytochrome P-450 are discussed in Section 2.2.5.

Discrepancies in the response of cytochrome P-450 to a variety of factors led many workers to the conclusion that there could be two forms of cytochrome P-450. A second form was identified and designated cytochrome P_1-450 (Sladek and Mannering, 1966, 1969a, b; Shoeman, Chaplin and Mannering, 1969; Mannering, Sladek, Parli and Shoeman, 1969). This has also been referred to as cytochrome P-448 (Alvares, Schilling, Levin and Kuntzman, 1967) and cytochrome P-446 (Hildebrandt, Remmer and Estabrook, 1968) though all forms are now thought to be identical (Mannering, 1971). However it is not thought to be normally present in the microsomal fraction in any appreciable amount (Mannering, 1971).

Unqualified reference has already been made to another cytochrome, cytochrome b_5. The existence of this compound was first suspected in 1940 by Keilin and Hartree although it was not until 1952 that it was possible to show that the unidentified pigment was associated with the microsomal fraction of several cell types including liver (Strittmatter and Ball, 1952). Two years later it was identified and called cytochrome b_5 (Pappenheimer and Williams, 1954). The studies of Klingenberg (1958) and Omura and Sato (1964) revealed that it was the only

other cytochrome present in the hepatic microsomal fraction apart from cytochrome P-450 and its variants.

Work with the adrenal microsomal fraction, which hydroxylates steroids, led to the discovery that a non-haem iron protein, adrenodoxin, functions as an electron carrier between the flavin enzyme, adrenodoxin reductase, and cytochrome P-450 in the electron transport system (Suzuki and Kimura, 1965). Although very little evidence exists to support the hypothesis, it has been suggested several times that an analogous non-haem iron protein could function in the transport of electrons to cytochrome P-450 in the hepatic microsomal fraction (Estabrook and Cohen, 1969). Recently Diplock and Lucy (1973) repeated this suggestion and reported that they had identified such a protein with selenide in complex with non-haem iron as the active centre. Further studies by the same group (Caygill and Diplock, 1973; Caygill, Diplock and Jeffery, 1973) support this theory and indicate that the protein is normally protected from oxidation in vivo by vitamin E, possibly because the incorporation of selenium into the protein depends on the organisation of the membrane phospholipids.

In 1950 Horecker reported that the enzyme NADPH-cytochrome c reductase could be detected in extracts of whole liver. NADPH-cytochrome c reductase activity was later found in microsomal fractions (Strittmatter and Velick, 1956) and in 1962 the enzyme was conclusively demonstrated to be associated with this fraction (Phillips and Langdon, 1962; Williams and Kamin, 1962). The prosthetic group was identified as flavine adenine dinucleotide, FAD (Williams and Kamin, 1962) so that the enzyme could be

classified as a flavoprotein. The properties of the enzyme have been comprehensively reviewed by Kamin, Masters, Gibson and Williams (1965). Cytochrome c and other electron acceptors not normally present in the microsomal fraction inhibited the oxidation of foreign compounds. It was reasoned that since the enzyme normally catalysed the transport of electrons, and since a diversion of these electrons to electron acceptors such as cytochrome c resulted in an inhibition of metabolism, then a free flow of electrons catalysed by this enzyme must have been a requirement for metabolism. It was thus suggested that NADPH-cytochrome c reductase was a component of the microsomal drug-metabolising system (Gillette, Brodie and La Du, 1957; Gillette, 1966) and was regarded as the most probable agent in the transfer of electrons from NADPH to cytochrome P-450, a role that is now generally accepted (Kratz, 1973).

After the detection of NADPH-cytochrome c reductase a second flavoprotein, NADH-cytochrome b_5 reductase, was isolated from the hepatic microsomal fraction (Strittmatter and Velick, 1957a, b; Strittmatter, 1958). This enzyme has been suggested to be involved in the reduction of cytochrome P-450 by an alternative electron-donor pathway via cytochrome b_5 (Estabrook and Cohen, 1969). Cytochrome b_5 may also be reduced by NADPH via a so-called NADPH-cytochrome b_5 reductase (Strittmatter, 1963). Recently detergent-solubilised NADPH-cytochrome c reductase was shown to reduce cytochrome b_5 (Satake, Imai and Sato, 1972) so that NADPH-cytochrome b_5 reductase is possibly identical to NADPH-cytochrome c reductase.

The rate of oxidation of foreign compounds is more closely

related to the rate of cytochrome P-450 reduction than to the total amount of cytochrome P-450 present or to the rate of cytochrome c reduction (a measure of NADPH-cytochrome c reductase activity) (Gillette and Gram, 1969; Gillette, 1969; Gigon, Gram and Gillette, 1969). This finding led these authors to the conclusion that an 'NADPH-cytochrome P-450 reductase' was rate limiting in the overall oxidative reaction of the microsomal fraction. The existence of such an enzyme has been accepted in theory although little is known about it except how its activity is affected. The enzyme would be responsible for the reduction of oxidised cytochrome P-450.

In a reconstituted microsomal system that hydroxylated foreign compounds there was a requirement for an unidentified lipid component for maximal activity (Lu and Coon, 1968). The factor was heat stable (Lu, Strobel and Coon, 1969) and in some way increased the rate of reduction of cytochrome P-450 (Strobel, Lu, Heidema and Coon, 1970). It has now been shown to be phosphatidylcholine (Strobel et al, 1970; Autor et al, 1973).

2.2.4 The organisation of the microsomal mixed function oxidase system

The metabolism of foreign compounds by the microsomal mixed function oxidase system involves the reduction of some component of the microsomal fraction by NADPH which then reacts with molecular oxygen to form an 'active oxygen' intermediate. The 'active oxygen' is subsequently transferred to the substrate (Gillette, 1963). In such a mechanism equivalent amounts of NADPH, molecular oxygen and substrate would be utilised in the

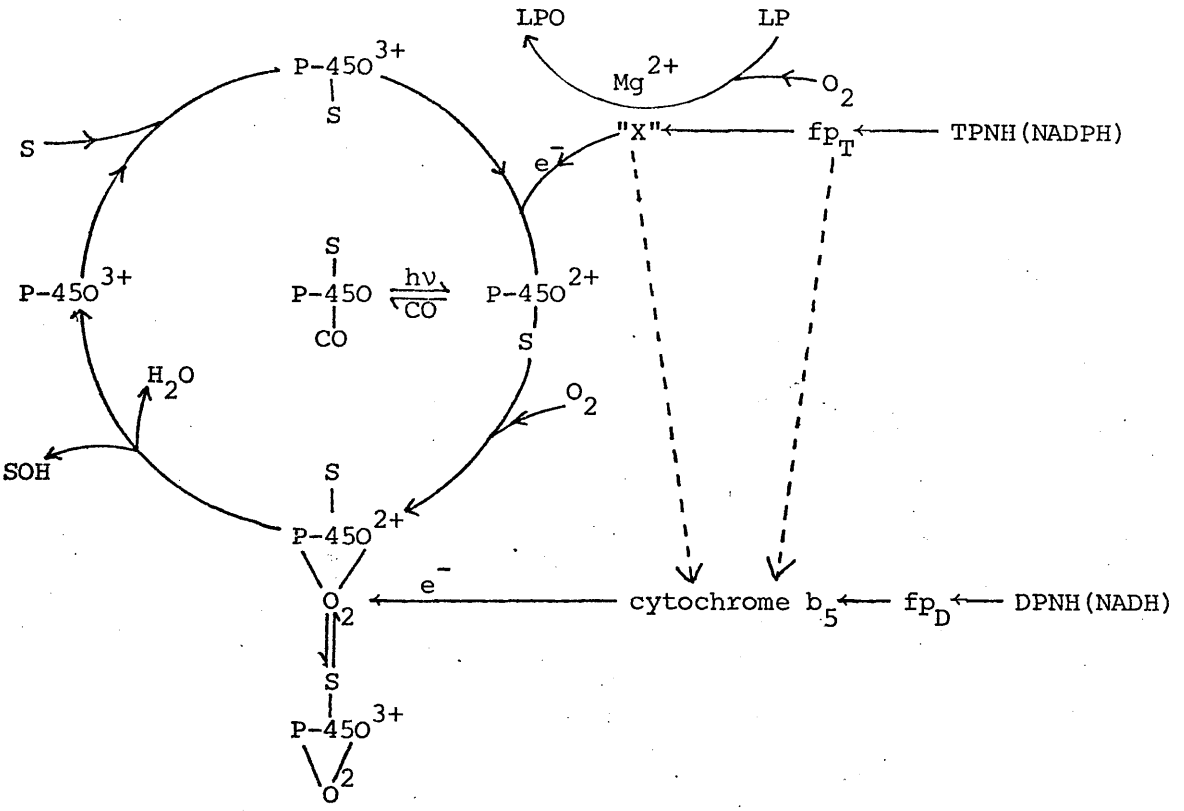


Figure 1 Hypothetical scheme proposed by Estabrook (1971) to explain the mechanism of drug metabolism by the hepatic microsomal mixed function oxidases.

The substrate is designated S. fp_T is the flavoprotein NADPH-cytochrome c reductase and fp_D is the flavoprotein NADH-cytochrome b_5 reductase. "X" represents a postulated non-haem iron protein which is shown to take part in the peroxidation of lipid (LP). The substrate combines with oxidised cytochrome P-450 ($P\text{-}450^{3+}$) which is then reduced by one electron from NADPH, reacts with molecular oxygen and is then further reduced by a second electron from either NADH or NADPH. The reduction of the oxidised substrate cytochrome P-450 complex is thought to be rate limiting.

reaction. A 1:1:1 stoichiometry for the demethylation of aminopyrine was observed (Ernster and Orrenius, 1965) but later work showed that two moles of NADP were formed for every mole of formaldehyde produced (Estabrook and Cohen, 1969). Thus the overall reaction is more complex than in the scheme proposed by Gillette (1963).

In the original description of microsomal metabolism there was a requirement for NADH as well as NADPH (Mueller and Miller, 1949, 1953). From these earliest reports to the present time various investigators have reported that NADH can take part in microsomal oxidative metabolism (Conney, Brown, Miller and Miller, 1957; Nilsson and Johnson, 1963; Ullrich, 1969). In addition 50% more NADPH was found to be oxidised than hexobarbitone was metabolised (Sasame, 1964). These findings were rationalised by Estabrook who suggested a mechanism for the metabolism of foreign compounds that included a role for NADH (Estabrook and Cohen, 1969; Estabrook, 1971).

The more recent scheme (Estabrook, 1971) is illustrated in Fig. 1. Hildebrandt and Estabrook (1971) have suggested that NADPH preferentially donates the first electron in the reduction of cytochrome P-450 and that either NADH or NADPH can donate the second electron, which would be transported via cytochrome b_5. This was confirmed in studies with antibodies to cytochrome b_5 which prevented NADH synergism of oxidative metabolism but did not affect the reaction when NADPH was the only electron donor (Sasame, Mitchell, Thorgeirsson and Gillette, 1973). Recent work by Correia and Mannering (1973a, b) also confirmed these findings and showed that the second electron is donated by NADPH or NADH, both via cytochrome b_5,

with NADH being the more efficient of the two donors of this electron only in the metabolism of type I substrates. Some form of interactive mechanism similar to that proposed by Estabrook (1971) is almost certainly operative in the microsomal fraction.

2.2.5 Properties of cytochrome P-450 and the substrate-induced spectral changes in the microsomal fraction

Cytochrome P-450 was established to be the pigment responsible for the activation of oxygen in microsomal mixed function oxidase reactions in the early 1960's (Estabrook, Cooper and Rosenthal, 1963; Omura, Sato, Cooper, Rosenthal and Estabrook, 1965; Cooper, Levin, Narasimhulu, Rosenthal and Estabrook, 1965). It was also found that the addition of 17-hydroxyprogesterone to the cytochrome P-450-containing fraction of adrenal cortical microsomes caused a characteristic change in the microsomal difference spectrum (Narasimhulu, Cooper and Rosenthal, 1965). Two groups independently found that spectral changes also occurred with suspensions of liver microsomal fraction on the addition of exogenous substrates (Remmer et al, 1966; Imai and Sato, 1966). Subsequent work (see Gillette et al, 1972) established that these changes were due to interactions of the substrates with cytochrome P-450. Both Remmer and his group (1966) and Imai and Sato (1966) classified the different substrates into two groups on the basis of the spectral change produced. The groups were named type I and type II respectively after the so-called type I and type II spectral changes produced. Schenkman, Remmer and Estabrook (1967) confirmed this division and added a third

classification comprising substrates that produced a modified type II spectral change.

The type I spectral change may result from substrate binding causing a modification of the electronegativity of the sixth ligand of the haem-iron moiety attached to the protein of cytochrome P-450 (Schenkman and Sato, 1968). A diverse range of compounds produce a type I spectral change (Schenkman et al, 1967). In contrast only a few compounds cause a type II spectral change, and most are amines (Schenkman et al, 1967). The type II spectral change may be due to ferrihaemochrome formation produced by direct interaction of the haem-iron of cytochrome P-450 with the basic nitrogen of the amine substrate (Schenkman et al, 1967). The modified type II spectral change is the mirror image of the type I spectral change (and is sometimes called a reverse type I). A fairly diverse range of compounds produce a modified type II spectral change (Schenkman et al, 1967). This change may be due to the interaction of a substrate with the bound form of cytochrome P-450 with the same substrate at a site other than the type I site (Schenkman, Cinti, Orrenius, Moldeus and Kraschnitz, 1972).

Digestion of the phospholipids of the membranes of the microsomal fraction by phospholipase C, which degrades phosphatidylcholine (Tzur and Shapiro, 1969) resulted in a loss of the type I spectral change and thus of type I binding (Chaplin and Mannering, 1969, 1970) since it has been shown that a spectral change is synonymous with binding at the corresponding site (Mannering, 1971). The type I binding site is probably localised in the hydrophobic region of

cytochrome P-450 and associated with membrane phospholipids. Although type I binding was destroyed by phospholipase C digestion, the metabolism of some type I compounds was reduced by only 40% (Chaplin and Mannering, 1970). Thus it appears that the binding of type I compounds is not obligatory for their metabolism although it is facilitative. In contrast Jefcoate, Gaylor and Calabrese (1969) have found that type II binding does not appear to have any direct role in the metabolism of type II substrates.

Chaplin and Mannering (1970) observed that a destruction of the type I binding site resulted in an elevation in the magnitude of the spectral change produced by type II compounds. One explanation is that normally there is a type I component in the spectral change produced by type II compounds. In 1970 Schenkman confirmed that this was so. Recently Gorrod and Temple (1973) and Kamataki, Shimokawa and Kitagawa (1973), working independently, also confirmed this and in addition found that the difference spectrum of type I compounds have a type II component.

It has been observed that NADPH-cytochrome P-450 reductase activity is influenced by the nature of the substrate present (Gigon et al, 1968, 1969; Gillette, 1969; Gillette and Gram, 1969). Type II substrates depressed the rate of reduction of the substrate-cytochrome P-450 complex while type I compounds stimulated the reduction of the complex by NADPH-cytochrome P-450 reductase (Gigon et al, 1968). The rate-limiting step in the oxidation of type I substrates may be the rate of reduction of the cytochrome P-450-substrate complex by the first electron so that the rate of reduction of the oxidised cyto-

chrome P-450-substrate (type I) complex is then greater than that of the uncomplexed oxidised cytochrome P-450 (Gigon et al, 1969; Gillette and Gram, 1969). Gillette and Gram (1969) also suggested that the type II substrate-cytochrome P-450 complex may not be reduced at all but that only the uncomplexed cytochrome P-450 would be reduced which could then form a complex with the type II substrate which would undergo subsequent metabolism. Since the type II interaction results in the formation of ferrihaemochrome type II compounds may compete with oxygen at the oxygen binding site of cytochrome P-450 and hence such interactions would not be likely to lead to further metabolism at this stage (Orrenius, Wilson, von Bahr and Schenkman, 1972).

2.3 Microsomal lipid peroxidation and its relationship to the metabolism of foreign compounds by the microsomal fraction

Incubation of the microsomal fraction with NADPH and a pyrophosphate has been found to cause peroxidation of membrane lipid (Hochstein and Ernster, 1963). Lipid peroxidation in the microsomal fraction was not inhibited by carbon monoxide (Nilsson, Orrenius and Ernster, 1964) so that cytochrome P-450 does not appear to be involved in the reaction. Although NADH alone did not support the peroxidation of lipid it enhanced lipid peroxidation in the presence of NADPH (De Matteis and Sparks, 1973). Oxidative metabolism by the microsomal fraction was found to inhibit lipid peroxidation (Orrenius, Dallner and Ernster, 1964) and this led to the conclusion that a common electron transport system was involved in the mixed function oxidase and in lipid peroxidation. The scheme shown in Fig. 2

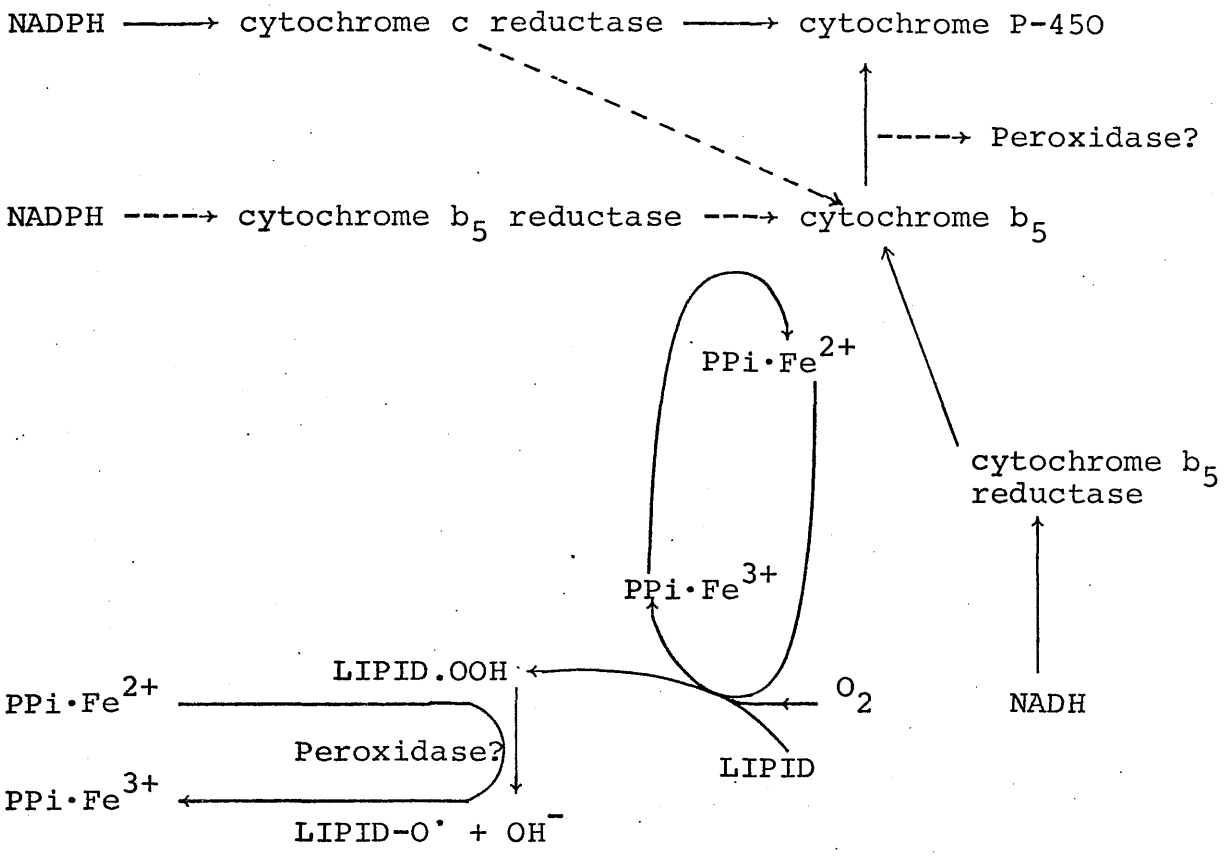


Figure 2 Hypothetical scheme devised by Bidlack et al (1973) to explain the mechanism of hepatic microsomal peroxidation of lipids and its interaction with the mixed function oxidase.

A chelate of a pyrophosphate and Fe^{3+} ($PPi \cdot Fe^{3+}$) was necessary for lipid peroxidation. The initial oxidation of cytochrome b_5 may reflect the reduction of the chelate by NADPH-cytochrome b_5 reductase which is possibly identical with NADPH-cytochrome c reductase. Subsequent activation of oxygen, formation of lipid peroxides and their turnover could be due to a simple Fenton's type reaction. The second oxidation of cytochrome b_5 occurred only during active peroxidation and was inhibited by EDTA. Thus electrons may be drained via peroxides, the iron chelate and peroxidase, the drain occurring either after, or directly off, cytochrome b_5.

has been devised by Bidlack, Okita and Hochstein (1973).

The oxidative metabolism of aniline and aminopyrine was found to inhibit lipid peroxidation (Orrenius et al, 1964) although it was not inhibited by the metabolism of codeine and hexobarbitone (Gram and Fouts, 1966). During incubation at 37°C of the microsomal fraction, as well as an increased lipid peroxidation, there is loss of drug-metabolising activity (Gram and Fouts, 1966), and in the pig liver microsomal fraction inhibition of lipid peroxidation was found to stimulate the epoxidation of aldrin (Lewis, Wilkinson and Ray, 1967). Although aldrin and lipids may compete for a common electron transport system reciprocal inhibition of lipid peroxidation by aldrin could not be shown. In these studies lipid peroxidation in the microsomal fraction was measured in the absence of exogenous Fe^{2+} or pyrophosphate so that sufficient Fe^{2+} may remain in the microsomal pellet to stimulate lipid peroxidation.

Although lipid peroxidation can be inhibited with EDTA there is still loss of drug-metabolising activity during microsomal incubation at 37°C (Gram and Fouts, 1966; Carpenter, 1972). In contrast Kamataki and Kitagawa (1973) have recently reported that inhibition of lipid peroxidation with low concentrations of EDTA prevents the loss of type I and type II drug-metabolising activity that occurs during incubation.

2.4 The effect of catecholamines upon the hepatic microsomal metabolism of foreign compounds

Lamson, Grieg and Hobdy (1951) reported that an injection

of adrenaline into guinea pigs recovering from barbiturate anaesthesia re-induced sleep. In the mouse adrenaline had similar effects although the effects of noradrenaline were less-pronounced (Lamson, Grieg and Williams, 1952). Mazel and Bush (1969) found that noradrenaline was active only at ten times the dose of adrenaline employed. The effect was not due to an increase in brain barbiturate levels nor was it due to anaesthesia induced by adrenaline itself (Mazel and Bush, 1969). Since adrenaline has many effects including the release of ACTH, corticoids, insulin, thyroid hormone and glucose, any of these may be involved in the effect (Mazel and Bush, 1969).

In the early 1960's Fouts investigated the possibility that the effects of catecholamines on drug metabolism were related to their glycogen depleting ability. At first only adrenaline was used (Fouts, 1962) but later he used noradrenaline (Dixon et al, 1964) since Vrij, Cho, De Groot and Weber (1956) had found that it was more specific and more potent in depleting liver glycogen in the rat.

A single injection of either adrenaline (Fouts, 1962) or noradrenaline (Dixon et al, 1964) depleted liver glycogen within 12 h but did not alter the microsomal metabolism of either type I or type II substrates. Pre-treatment with noradrenaline caused no alteration in microsomal 2- or 4-biphenyl hydroxylase activity (Parke and Rahman, 1970). Fouts' (1962) results showed however that there was a significant increase of 35% in the metabolism of type I compounds 1 h after an injection of adrenaline although no mention of

this was made in the text. In the later paper the same author found an increase of 50 to 60% in the metabolism of type I substrates 2 h after an injection of noradrenaline although this increase was not significant (Dixon et al, 1964).

Repeated injections of either catecholamine caused inhibition in the microsomal metabolism of both type I and type II substrates (Fouts, 1962; Dixon et al, 1964). Although glycogen levels returned to control values shortly after the last injection of noradrenaline the metabolism of both type I and type II compounds remained inhibited for some time (Dixon et al , 1964).

Kato and Gillette (1965) reported that adrenaline pre-treatment inhibited the metabolism of only type I substrates in male rats and that drug metabolism was not affected at all in female rats after catecholamine pre-treatment.

It was concluded that when glycogen was depleted such that the amount of smooth-surfaced microsomes in the liver was reduced then drug metabolism was inhibited. A single injection of catecholamine would not deplete glycogen sufficiently to alter the amount of microsomal fraction in the liver so that drug metabolism would not be affected (Fouts, 1962; Dixon et al, 1964). Thus any relationship between the effects of catecholamines on liver glycogen levels per se and inhibition of drug metabolism appears to be indirect at least.

Although adrenergic blocking agents prevented noradrenaline

from depleting liver glycogen they did not prevent it from inhibiting drug metabolism. However some adrenergic blocking agents were also capable of inhibiting drug metabolism on their own (Dixon et al, 1964). Peters (1972) suggested that alprenolol and other β-blockers may inhibit microsomal metabolism by interacting allosterically with the mixed function oxidase.

All adrenergic blocking agents were found to increase hexobarbitone sleeping time in mice although not all inhibited the in vitro microsomal metabolism of foreign compounds (Mullen and Fouts, 1965). Adrenergic blocking agents may increase hexobarbitone sleeping time by lowering body temperature and some may, in addition, directly inhibit the hepatic microsomal metabolism of barbiturates. When the ambient temperature was increased so that the body temperature of hexobarbitone-anaesthetised mice was maintained at normal only those adrenergic blocking agents that directly inhibited microsomal drug metabolism in vitro prolonged hexobarbitone sleeping time (Mullen and Fouts, 1965).

Noradrenaline has been reported to stimulate C-21 hydroxylation of steroids by bovine adrenocortical homogenates but not to affect 11-hydroxylation (Cooper and Rosenthal, 1962a, b). Steroid C-21 hydroxylase is a microsomal enzyme (Pincus, 1958). Lewis et al (1967) suggested that stimulation of C-21 hydroxylation of steroids by catecholamines was due to inhibition of peroxidation of endogenous lipids which may otherwise compete for electrons from NADPH. Adrenaline is known to inhibit the peroxidation of lipid (Privett, 1962).

Recently Gielen and Nebert (1972) have reported that high concentrations of catecholamines (1 mM) induced benzpyrene hydroxylase activity in foetal rat liver cells *in vitro*. The effect was not stereospecific nor was it prevented by α- or β- blocking agents. They speculated that the *in vivo* administration of catecholamines might produce both monooxygenase induction and toxicity, or a marked pharmacological response, so that any stimulation would be masked by the toxic or pharmacological effect (Gielen and Nebert, 1972). They also suggested that the rise in hydroxylase activity occurring during the first week of neonatal life was due to release of biogenic amines through the stress of the birth process.

2.5 Effect of cyclic 3', 5'-adenosine monophosphate (cyclic AMP) on the hepatic microsomal metabolism of foreign compounds

Considerable evidence now exists that pre-treatment of rats with cyclic AMP can inhibit the metabolism by the hepatic microsomal fraction of some substrates (Weiner, Blake and Buterbaugh, 1970; Weiner *et al*, 1972a, b; Weiner, 1973; Ross and Oppelt, 1970, 1971, 1973; Ross, Simrell and Oppelt, 1973). Cyclic AMP also inhibited the metabolism of foreign compounds by the isolated perfused liver (Weiner *et al*, 1970, 1972a) and in liver slices (Weiner *et al*, 1972b), but it was without effect when added directly to the microsomal fraction (Weiner *et al*, 1972b; Ross and Oppelt, 1970).

Cellular integrity appeared to be a pre-requisite for cyclic AMP to exert any inhibitory effect upon the metabolism

of foreign compounds (Weiner et al, 1972b) and the presence of an inhibitor in the post-microsomal supernatant of rats previously treated with cyclic AMP has been shown by Weiner et al (1972b). The inhibitory factor was later found to be dialysable and heat stable so it was concluded that it was unlikely to be a protein (Weiner, 1973). Its molecular weight was estimated at less than 5,000. It has been suggested (Weiner, 1973) that cyclic AMP may be acting by the stimulation of liver lipase to cause the liberation of free fatty acids which would meet the characteristics of the inhibitor as outlined above. It had already been shown that free fatty acids can cause inhibition of the microsomal metabolism of foreign compounds (Di Augustine and Fouts, 1969). This suggestion at present remains an attractive, but unproven, hypothesis for the mode of action of cyclic AMP.

2.6 The enhancement of the microsomal metabolism of foreign compounds

Enhancement is an increase in the specific activity of the mixed function oxidase occurring in vitro in the presence of the enhancing agent. It was first reported by Imai and Sato in 1966 who found that ethyl isocyanide stimulated the in vitro metabolism of aniline by the rabbit liver microsomal fraction. Ethyl isocyanide also stimulates the metabolism of aminopyrine, a property that no other enhancing agent appears to share (Imai and Sato, 1966). Ethyl isocyanide may act by binding to oxidised cytochrome P-450 in competition with aniline, at a non-metabolic site, and the oxidised cytochrome P-450-ethyl isocyanide complex would be reduced by NADPH more rapidly than either un-complexed cytochrome P-450

or the cytochrome P-450-aniline complex (Imai and Sato, 1968).

Acetone also enhances the metabolism of aniline but inhibits the metabolism of type I substrates (Anders, 1968). Only acetone and 2-pentanone of many solvents investigated produced enhancement. Acetone enhancement differed from that of ethyl isocyanide (Anders, 1968). Acetone may act preferentially on one of two postulated aniline hydroxylases to reveal one of higher activity or it may increase the rate of breakdown of enzyme-substrate complex by forming an acetone-enzyme-substrate complex (Anders, 1968). It does not appear to act by inhibiting microsomal lipid peroxidation (Anders, 1969).

Acetone does not produce its effect by altering the relative amounts of cytochrome P-450 and cytochrome P-448 present in the microsomal fraction (Anders, 1972).

In the presence of acetone low concentrations of aniline produced a type I spectral change (Vainio and Hanninen, 1973). Phospholipase C abolished the acetone enhancement of aniline metabolism but reduced the metabolism of aniline in the absence of acetone by only 20% (Vainio and Hanninen, 1972). The enzyme phospholipase C causes degradation of lipids, mainly phosphatidylcholine (Tzur and Shapiro, 1969), a necessary component of the reconstituted mixed function oxidase system (Strobel et al, 1970) and related to the type I binding of substrates (Chaplin and Mannering, 1970). Thus acetone may cause enhancement by promoting the binding of type II substrates to the type I site of cytochrome P-450 since

type I binding has already been shown to produce an increased rate of metabolism (Gigon et al, 1968).

α, α'-bipyridyl enhances the metabolism of aniline in trout liver microsomes (Buhler and Rasmusson, 1968). It also enhances aniline metabolism in the rat liver microsomal fraction (Anders, 1969) where its effect is probably due to a metabolite (Anders, 1972). Like acetone, α, α'-bipyridyl inhibited the metabolism of type I compounds (Anders, 1969). The only other chelator found to enhance metabolism was 1,10-phenanthroline (Anders, 1969). Although α, α'-bipyridyl inhibits lipid peroxidation, Anders (1969) excluded this as a possible mechanism for enhancement. However it has recently been shown that these chelators at low concentrations enhance the metabolism of type I compounds, apparently by inhibiting lipid peroxidation (Kamataki and Kitagawa, 1973). These authors also found that the metabolism of aniline was enhanced. It is not possible at present to evaluate the significance of the results of Kamataki and Kitagawa (1973). It may be that chelators have two distinct effects on drug metabolism, one due to inhibition of lipid peroxidation and the other due to direct interaction with the mixed function oxidase.

In 1969 Leibman reported that metyrapone enhanced the metabolism of type II substrates by the microsomal fraction, and like other enhancing agents inhibited the metabolism of type I compounds. Unlike most other compounds metyrapone binds to the reduced form of cytochrome P-450 (Hildebrandt, Leibman and Estabrook, 1969). Metyrapone could be acting by altering the equilibrium between two forms of cytochrome

P-450 (Hildebrandt et al, 1969). However there is little evidence to support the idea of distinct forms of cytochrome P-450 for each of type I and type II binding.

Several volatile anaesthetics including halothane and methoxyfluorane enhanced the metabolism of aniline while inhibiting that of aminopyrine (Van Dyke and Rikans, 1970). These anaesthetics may be acting by selective inhibition of one of two forms of aniline hydroxylase revealing one of higher activity (Anders, 1971, 1973) or they may bind to the type I site and shift electrons to the type II site (Korten and Van Dyke, 1973). Both hypotheses are poorly supported by the experimental evidence of other work and the volatile anaesthetics may act in the same way as acetone.

Pre-treatment of rats with safrole caused an increase in the metabolism of 2-biphenyl by the microsomal fraction (Parke and Rahman, 1970). Although this is, in part, due to induction (Parke and Rahman, 1970, 1971) there appears to be activation of existing enzyme (Parke and Rahman, 1970; Lake and Parke, 1972a).

Recently Schenkman, Ritchie, Cha and Sartorelli (1974) reported that urethane enhances aniline metabolism in rat liver slices. It does not act directly on microsomes so it differs in action from other enhancers. The authors showed that the effect was on phase I metabolism and may be due to inhibition of an alternative pathway of aniline metabolism, permitting p-aminophenol formation to increase.

Paraoxon, a cholinesterase inhibitor, enhances the

metabolism of aniline (Stevens, McPhillips and Stitzel, 1971) and it inhibits the metabolism of type I compounds (Stevens et al, 1972). Its effect is not dependent upon its metabolism (Stevens et al, 1972) and it probably acts by a different mechanism from acetone. Paraoxon could be interacting with aniline aromatic hydroxylase to alter its three-dimensional structure thereby increasing its activity in some undefined way (Stevens et al, 1972).

With the exception of ethyl isocyanide it is not possible with the results available at present to suggest any probable mode of action for any one of the above enhancers on the microsomal metabolism of foreign compounds.

CHAPTER 3

The Technique of Liver Perfusion

3.1 The advantages of the perfused liver

Perfused organs have many advantages over other preparations in several types of investigations. The system may be defined to a very high degree, particularly when using a semi-synthetic perfusion medium, and conditions can be altered to an extent that would not be tolerated by the whole animal. The cells are intact and study can be extended for several hours in stabilised conditions, unlike many other *in vitro* preparations.

The liver was chosen being the major organ involved in the metabolism of foreign compounds. Most previous studies on the metabolism of foreign compounds have been performed either on intact animals or on the hepatic microsomal fraction. *In vivo* the rate of metabolism is affected by the amount of substrate reaching the liver and this depends upon the uptake and distribution of the compound. The perfused liver overcomes these problems providing a system that is functionally integrated and compartmentalised as *in vivo* but which is not influenced by complicating factors such as secondary hormone release or reflex nervous activity. The perfused liver also overcomes the disadvantages of an *in vitro* preparation, such as the microsomal fraction, which operates under optimal co-factor concentrations which may differ considerably from the situation *in vivo*.

An isolated perfused liver system was preferred to an *in situ* system since the former enables more complete control to be maintained over the organ in perfusions lasting several

hours.

3.2 Historical introduction to liver perfusion

During the 17th and 18th centuries it was debated whether or not the organs of the body were capable of independent existence. Willis (1664) maintained that the viscera could only function under control of the cerebellum. It was not until 1753 that von Haller suggested that organs could be maintained extra-corporeally. Le Gallois (1812) took this further and proposed that many organs could be kept functioning indefinitely by artificial perfusion. Forty years later Bernard (1855) suggested the use of isolated organs in metabolic studies.

The earliest attempt to put these theories into practice was that of Loebell (1849) who attempted to perfuse the kidney of the pig. In 1868 Ludwig and Schmidt carried out the first successful isolated perfusion, of a hind limb muscle. Attention then turned to the liver and early experiments involved larger animals which were easier to manage with the available surgical techniques. Luschinger (1875) first perfused a liver and was soon followed by von Frey and Gruber (1885).

Despite the comparatively widespread use of liver perfusion at the start of this century these early experiments are of little scientific value because of the deficiencies in the techniques employed but are of considerable historical importance. One of the most successful attempts was by Grube (1903) who used the apparatus devised by Brodie (1903). This

included two glass-wool filters which almost undoubtedly contributed to the success of these experiments. Grube (1903) perfused cat livers through the portal vein with a mixture of defibrinated cat and sheep blood by gravity-feed. The liver was maintained for 2 h during which time glucose was converted to glycogen. Several comprehensive reviews were published around this time in which the theoretical aspects of liver perfusion expounded are still valid (Skutul, 1908; Baglioni, 1910; Müller, 1910).

Very few experiments with the isolated liver in metabolic studies were reported between 1910 and 1950. One possible explanation (Trowell, 1942) is that researchers had turned to the tissue slice technique of Warburg (1923) which was more reliable, reproducible and economical. It was not until 1941 that Corey and Britton reported attempts to perfuse rat liver, with oxygenated Krebs bicarbonate buffered saline. The immediate extensive glycolysis was probably indicative of the deteriorating condition of the organ. Trowell (1942) perfused the liver retrogradely with buffered saline, to increase the flow rate and maintain adequate oxygenation. Although he claimed that the liver could be maintained for up to 4 h D'Silva and Neil (1954) subsequently found that there was considerable potassium efflux and oedema within 2 h. Various other attempts at perfusion with oxygenated buffered saline have met with only partial success (Brauer and Pessotti, 1949; Dawkins, Judah and Rees, 1959; Bristow and Kerly, 1964; Nestruck and Furneaux, 1972a, b).

In 1951 independently, and almost simultaneously, Brauer,

Pessotti and Pizzolato and Miller, Bly, Watson and Bale successfully perfused the isolated rat liver. The systems were essentially similar, homologous blood diluted with bicarbonate buffered saline being perfused at constant pressure into the portal vein. Outflow was from a cannula in the vena cava. Perfusion medium was oxygenated in a film oxygenator and recycled. Both systems included mesh filters to remove small clots from the medium. The main differences were the aseptic conditions and the addition of antibiotics to the perfusion medium by Brauer et al (1951). However most techniques of isolated liver perfusion have been modelled on that of Miller et al (1951) since such precautions are unnecessary except for long perfusion periods (Bartosek, Guaitani and Garattini, 1972b).

Many modifications of the basic perfusion method of Miller et al (1951) have been introduced. Most notable of these are replacement of homologous blood with washed heterologous erythrocytes in a buffered saline albumin medium and perfusion of the liver in situ at a constant flow rate (Mortimore, 1961; Exton and Park, 1965), an approach that had been attempted earlier by Archdeacon and Brucer (1952). Powis (1970) based a constant flow system for rat liver on that used by Blakely and Brown (1963) for perfusion of the cat spleen. Recently a comprehensive review on the various forms of perfusion technique in use at the present for small animals has been published (Ross, 1972).

3.3 The experimental approaches adopted to liver perfusion

3.3.1 Should the hepatic artery be perfused in addition to the portal vein

No more than 20% of the blood normally reaching the liver is supplied by the hepatic artery (Fischer, 1963). Several workers have attempted to perfuse the rat liver via both the hepatic artery and the portal vein (Brauer, Shill and Krebs, 1959; Ross, Hems, Freedland and Krebs, 1967; Abraham and Dawson, 1967; Powis, 1970). Their results were recently compared (Ross, 1972) with those of Schimassek (1962a, b) and Hems, Ross, Berry and Krebs (1966) who perfused the liver via the portal vein only. No significant difference in the tests of function measured was apparent and, in the rat, perfusion via the portal vein alone is totally adequate.

3.3.2 Choice of perfusion medium

Although whole homologous blood, either fresh or diluted, would appear to be the most suitable perfusion medium its major disadvantage is the 'early outflow vascular block' often observed particularly in larger animals (Miller et al, 1951; Brauer, Leong and Pessotti, 1953; Eiseman, Knipe, Koh, Normell and Spencer, 1963). This is characterised by an increase in hepatic vascular resistance, swelling and cyanosis. Several methods have been partially successful in overcoming this problem by removing the vasoconstrictor substance from the perfusion medium (Felts and Mayes, 1966). Vasoconstriction does not occur when a semi-synthetic perfusion medium is used (Ross, 1972).

There is a requirement for an oxygen carrying constituent

in liver perfusion media (Bock, Fröhling and Schlote, 1972). This is usually fulfilled by haemoglobin and the concentration can be varied from 16 g% to 2.5 g% and still provide adequate oxygenation (Hems et al, 1966). With oxygenated buffered salt solutions there is an inadequate oxygenation and greater loss of cell function (D'Silva and Neil, 1954; Nestruck and Furneaux, 1972a, b).

As an alternative to the whole blood media previously used Schimassek (1962a) devised a semi-synthetic medium of a defined solution of salts, washed bovine erythrocytes and bovine serum albumin. Hems et al (1966) described a similar medium with human erythrocytes in place of bovine red cells, and an altered salt composition. When diluted rat blood medium (Miller et al, 1951) and a semi-synthetic perfusate (Schimassek, 1962a, 1963a, b) were compared the latter maintained the liver in the more viable state as judged by the tissue distribution of the media, glucose metabolism and degree of haemolysis (Meiers, Flammann, Albaum and Staib, 1966). Thus the semi-synthetic media appear more suitable than either haemoglobin-free or whole blood media (Ross, 1972). Most semi-synthetic media include bovine serum albumin between 3 to 5 g% (w/v) as an osmotic colloid although Hems et al (1966) found that varying the concentration between 1.5% and 6% had no deleterious effect. Dextran caused aggregation of the red cells (Hems et al, 1966). A totally synthetic system comprising sonicated fluorocarbons has been described (Brown and Hardison, 1972); first introduced by Sloviter and Kamimoto (1967) for perfusion of brain, but this medium causes hepatic sinusoidal swelling due to flocculation of the fluorocarbons.

3.4 Determination of the functional status and viability of the perfused liver

3.4.1 The appearance of the liver

The macroscopic appearance of the liver provides a useful initial indication of the extent of perfusion (Brauer et al, 1951; Exton and Park, 1967). Inadequacies of perfusion are revealed by the appearance of petachiae on the surface of the liver and less frequently a collapsed smaller lobe (Miller et al, 1951).

Light microscopy of sections of liver taken immediately after the experiment provides evidence of the morphological integrity and viability of the organ after perfusion. Histologically the liver appears indistinguishable from that of freshly killed rat when perfusion is adequate (Miller et al, 1951; Brauer et al, 1951).

Electron microscopy is also a useful means of judging the integrity of the liver after perfusion. After an adequate perfusion electron micrographs of the liver reveal no differences to those of unperfused organs (Bock et al, 1972).

3.4.2 Blood flow and blood pressure of the perfused liver

Blood flow and portal venous pressure are routinely monitored during perfusion. In vivo the portal venous flow rate in the rat is 8 to 12 ml/min or 1 ml/g of liver/min (Brauer et al, 1951). The minimum flow rate is unimportant

if adequate oxygenation is achieved (Ross, 1972). Normal portal pressure is 12 to 14 cm water (Brauer et al, 1951). A pressure of 15 to 25 cm of water is required in constant pressure systems to achieve a flow rate of 1 ml/g/min. In constant flow systems (Mortimore, 1961; Powis, 1970) a pressure of about 12 cm water is observed when the flow is set at 1 ml/g/min. Sudden changes in pressure in a constant flow system may reflect inadequate perfusions due to factors such as emboli.

3.4.3 The production of bile in the perfused liver

Bile production is used as an indication of the functional integrity of the liver due to ease of measurement and its synthetic nature, i.e. the liver must actively produce bile to secrete it, (Brauer et al, 1951). Bile flow in vivo is between 0.5 ml/h (Ostashever, Gray and Graff, 1960) and 1.0 ml/h (Brauer et al, 1951). Within a group of experiments carried out under the same conditions it should be possible to establish a typical bile flow pattern during adequate perfusion, deviation from this would be a sign of abnormal liver function.

3.4.4 The efflux of K^+ during perfusion of the liver

Loss of K^+ may be a sign of cell damage (Krebs, Eggleston and Terner, 1951) and many liver perfusions have included determinations of K^+ efflux into the perfusion medium as a measure of cell viability (Frimmer, Gries and Hegner, 1967).

Mortimore (1961) showed that increases in perfusate K^+ in his experiments were not due to red cell haemolysis although Craig (1966) found that in his experiments the slight net increase in perfusate K^+ could be accounted for almost quantitatively by haemolysis. However increases in perfusate K^+ normally provide some indication of the degree of cell damage and the metabolic activity of the remaining intact liver cells.

3.4.5 Levels of glucose in the perfusate

Glucose is involved in several metabolic reactions in the liver such as gluconeogenesis, glycolysis and glycogenolysis so that determination of free glucose levels in the medium will provide some indication of the metabolic integrity of the liver. The liver sheds glucose into the medium after commencement of perfusion followed by a slow decline in concentration (Ostashever et al, 1960; Exton and Park, 1963; Craig, 1966; Hems et al, 1966; Bartosek et al, 1972). Non-glycolysing cells as were employed in the present study use no glucose (Hems et al, 1966). All the changes in glucose levels are thus attributable to the liver. Contrary to previous belief, shedding of glucose over the initial period of perfusion is a sign of the functional integrity of the liver, not of deteriorating function, and is probably induced by the trauma of isolation (Selkurt, 1964; Shimazu and Amakawa, 1968; Mondon and Burton, 1971; Bartosek et al, 1972b).

3.4.6 The lactate:pyruvate ratio of the perfusion medium

One of the most sensitive measures of liver function is the ratio of lactate to pyruvate in the perfusion medium. Schimassek (1963b) has demonstrated that the functioning liver maintains the ratio of these substrates in a fixed proportion by their interconversion and reflects the balance of NAD to NADH within the liver. After perfusion for 2 h a ratio of 10:1 is found in the perfusate similar to that observed in vivo (Schimassek, 1963b). Although similar values have been reported by other workers (Exton and Park, 1967; Woods and Krebs, 1971; Seglen, 1972) a wider range of values has been taken as within physiological levels. The ratio increases considerably in anoxia (Schimassek, 1963b) or during inadequate perfusion (Woods and Krebs, 1971). This test is a critical test of the redox state of the liver and is a very reliable guide to adequacy of perfusion.

3.4.7 Selection of tests of liver viability during perfusion

The tests selected should be relatively easy to perform and of preference should be carried out on the perfusate rather than the liver tissue itself so that continuous monitoring of the state of the liver can be carried out over the duration of an experiment. By combining several tests, it is possible to obtain an accurate picture of the functional state of the liver.

3.5 The metabolism of foreign compounds by the isolated perfused rat liver

The perfused liver is achieving an increasing use in

investigations of the metabolism of foreign compounds and the influence that modifying agents have on drug metabolism (von Bahr, Alexanderson, Azarnoff, Sjoqvist and Orrenius, 1970). Among substrates that have been investigated are ethanol (Van Harken and Mannering, 1969; Lindros, Vihma and Forsander, 1972; Theorell, Chance, Yonetani and Oshino, 1972), methanol (Van Harken, Tephly and Mannering, 1965), desmethylimipramine (Bickel and Minder, 1970; von Bahr et al, 1970), imipramine (Bickel and Minder, 1970), phenylbutazone, antipyrine, oxotremorine and nortriptyline (von Bahr et al, 1970), pentobarbitone (Kalser, Kelly, Forbes and Randolph, 1969; Bartosek, Guaitani and Donelli, 1972a), erythromycin estolate and other derivatives (Kendler, Anuras, Laborda and Zimmerman, 1972), chlorpramazine and related psychotropic drugs (Cordelli, Ferrari and Savonitto, 1969), diazepam (Kvetina, Marucci and Fanelli, 1968), procarbazine (Baggiolini, Dewald and Aebi, 1969; Dewald, Baggiolini and Aebi, 1969), 2-acetamidofluorene (Miyata, Noguchi and Enomoto, 1972), deptropine methiodide (Lavy, Hespe and Meijer, 1972), 2-PAM (Way, 1962) and halothane (Van Dyke and Wood, 1973).

Many substrates are removed from the perfusion medium following first order kinetics (von Bahr et al, 1970) although some compounds such as propranolol follow complex clearance kinetics (Evans, Wilkinson and Shand, 1973). A detailed study into the effects of hypothermia on the metabolism by the perfused liver of atropine (Kalser, Kelvington, Randolph and Santomenna, 1965), sulphanilamide (Kalser, Kelvington and Randolph, 1968), procaine (Kalser, Kelvington, Kunig and Randolph, 1968), and pentobarbitone (Kalser et al,

1969) has been carried out.

Hexobarbitone metabolism has been widely studied in the perfused liver (Stitzel et al, 1966; Stitzel, Tephly and Mannering, 1968; Cumming and Mannering, 1970). The substrate is removed linearly from the perfusate (Stitzel et al, 1966) and its removal is reduced by hypoxia (Cumming and Mannering, 1970) and perfusion with haemoglobin-free medium (Bock et al, 1972). The studies of Stitzel and his co-workers have played an important role in determining the mode of action of inhibitors of drug metabolism such as SKF 525-A (Stitzel et al, 1966, 1968). It was also shown that some compounds which did not inhibit drug metabolism in vivo, such as morphine, did produce an inhibition in the perfused liver (Stitzel et al, 1968).

In addition to the metabolism of foreign compounds the perfused liver has been used to investigate the metabolism of many endogenous compounds such as calcitonin (Greenberg, Martin, Melick, Jablonski and Watts, 1972), hydrocortisone (Bartosek, Marc, Guaitani and Garattini, 1973), methylprednisolone (Kuster and Woods, 1972), thyroid hormone (Flock and Owen, 1965), androstenedione and progesterone (Eriksson, Gustafsson and Pousette, 1972), mestranol (Bolt and Remmer, 1973), testosterone (Staib, Sonnenschein and Staib, 1970), L-DOPA (Tyce and Owen, 1972), and insulin (Stoll, Touber, Menahan and Williams, 1970).

The isolated perfused liver has played an important part

in elucidating some of the mechanisms of induction of drug metabolism. 3,4-benzpyrene causes induction in the perfused liver with peak activity at the same time as after an *in vivo* injection of the compound (Juchau, Cram, Plaa and Fouts, 1965). Thus enzyme inducers are acting directly on the target cells and need not act through any endogenous compound such as a hormone (von Bahr *et al*, 1970).

The perfused liver also exhibits enhancement of the metabolism of type II substrates by metyrapone (Kahl, Minck and Netter, 1973).

The major components of the microsomal mixed function oxidase, cytochrome P-450, cytochrome b_5 and NADPH-cytochrome c reductase, are stable during perfusion of the liver under optimal conditions (Bock *et al*, 1972). However the metabolism of type I substrates by the 9,000 x G fraction was reduced in livers perfused for 4 h although the metabolism of type II substrates was almost unaffected (Bartosek, Guaitani, Garattini and Simonazzi, 1974).

3.6 The effect of catecholamines upon the isolated perfused liver

Adrenaline decreased glycogen levels in the perfused liver of the dog (Bodo and Marks, 1928) and of the cat (Sokal, Miller and Sarcione, 1958) with a concomitant increase in liver and perfusate glucose. Adrenaline also increased perfusate levels of glucose in the perfused liver of the rat due to an activation of liver phosphorylase (Northrop and Parks, 1964). It

has been suggested that the normal hepatic glycogenolytic activity of adrenaline may be due to the release of glucagon since this hormone is 350 times as potent as adrenaline in the perfused liver at producing glycogenolysis (Ezdinli and Sokal, 1966). Adrenaline also inhibits glycogen synthetase I in the perfused liver thus preventing re-conversion of glucose back to glycogen (Höstmark, 1973).

The glycogenolytic activity of adrenaline in liver has been reviewed in depth by Sutherland and Robison (Sutherland and Robison, 1966; Robison, Butcher and Sutherland, 1968).

Catecholamines stimulate gluconeogenesis in the perfused rat liver, increasing conversion of non-carbohydrate precursors such as lactate, into glucose. Although low doses of adrenaline have no effect, at higher concentrations catecholamines increase glucose production by 100% (Exton and Park, 1968). Glucagon and cyclic AMP have also been reported to stimulate gluconeogenesis from lactate in the perfused liver (Exton and Park, 1968, 1969).

Adrenaline causes a loss of K^+ with a concomitant increase in Na^+ in hepatocytes of perfused liver (Lambotte, 1968). In the dog this caused depolarisation of liver cells (Lambotte, 1970, 1971). Stimulation by α-agonists causes a rapid increase in Na^+ permeability with depolarisation and exchange of Na^+ for K^+. β-receptor stimulation causes a slower decrease in Na^+ permeability and hyperpolarisation (Lambotte, 1971). In contrast stimulation of either α-receptors (Friedmann, Somlyo and Somlyo, 1971; Haylett and Jenkinson, 1972) or β-receptors

(Haylett and Jenkinson, 1972) in the guinea pig liver resulted in hyperpolarisation. Isoprenaline caused hyperpolarisation in the rat liver (Haylett and Jenkinson, 1969).

Noradrenaline can stimulate an hepatic lipase in the isolated perfused liver of the rat to cause increased levels of non-esterfied fatty acids in the perfusion medium (Lemberg, Wikinski, Izurieta, Halperin, Paglione and de Neuman, 1972).

CHAPTER 4

Materials and Methods

TABLE 1

Chemicals used, their sources and quality

Chemical	Supplier	Quality
All inorganic salts	B.D.H.	AnalaR
Calcium chloride $6H_2O$	B.D.H.	Volumetric solution
Magnesium chloride $6H_2O$	Hopkin and Williams	AnalaR
Barium chloride	B.D.H.	AnalaR
Manganese chloride	B.D.H.	AnalaR
Ferrous chloride	B.D.H.	Lab reagent
Hydrochloric acid	Hopkin and Williams	AnalaR
Acetylacetone	B.D.H.	Lab reagent
Isoamyl alcohol	Hopkin and Williams	General Purpose Reagent
Acetone	B.D.H.	AnalaR
Petroleum spirit(60-80^{o}C)	B.D.H.	AnalaR
Liquid paraffin, light	B.D.H.	Lab reagent
Absolute alcohol	James Burrough Ltd.	BPC
Folin and Ciocalteau's Phenol Reagent	B.D.H.	Lab reagent
N-1-(Naphthyl)-ethylene-diamine dihydrochloride	Koch-Light	Pure A.R.
Phenol	B.D.H.	AnalaR
Trichloroacetic acid	Hopkin and Williams	AnalaR
2-Thiobarbituric acid	Sigma	PFS
Trizma buffer	Sigma	Reagent Grade
Semicarbazide	Sigma	PFS
Nicotinamide	Sigma	PFS
Sucrose	Hopkin and Williams	AnalaR
Uranyl acetate	Boehringer	
Hydrazine sulphate	Sigma	PFS
Glycine	Sigma	PFS
Triethanolamine hydrochloride	Boehringer	
Pyruvic acid	Sigma	Standard solution
Lactic acid	Sigma	Standard solution
Perchloric acid	Boehringer	
Glucose	B.D.H.	AnalaR
Bovine Serum Albumin	Miles Lab. Ltd.	Fraction V

TABLE 1 (cont'd)

Chemical	Supplier	Quality
Glucose-6-phosphate dehydrogenase	Boehringer	Grade II
Glucose-6-phosphate disodium salt	Boehringer	
Isocitric dehydrogenase	Sigma	Type IV
DL-isocitric acid trisodium salt	Sigma	Type I
β-NADP disodium salt	Boehringer	
β-NADPH tetrasodium salt	Boehringer	Grade II
β-NAD	Boehringer	Grade II
β-NADH disodium salt	Boehringer	Grade II
Lactate dehydrogenase	Boehringer	Analytical
Biochimica Test Combination; Glucose	Boehringer	
Cyclic 3':5' adenosine monophosphate	Boehringer	
N^{6}-2^{1}-O-dibutyryl cyclic 3':5' adenosine monophosphate monosodium salt	Boehringer	
Cytochrome c	Sigma	Type II-A
Halothane	I.C.I. Ltd.	BP
Aminopyrine	Ralph N. Emanuel	
p-aminophenol	Koch-Light	
Aniline hydrochloride	B.D.H.	Lab reagent
Formaldehyde	B.D.H.	AnalaR
Hexobarbitone sodium	May and Baker	BP
EDTA disodium salt	Hopkin and Williams	AnalaR
D-α-Tocopherol	Sigma	Type II
D-α-Tocopherol acetate	Sigma	Type III
D-α-Tocopherol acid succinate	Sigma	Type IV
Butylated hydroxytoluene	Sigma	PFS
1,2-di (2-aminoethoxy) ethane-N,N,N',N'-tetraacetic acid (EGTA)	B.D.H.	AnalaR
Ascorbic acid	Koch-Light	Puriss
Histamine acid phosphate	B.D.H.	BP

TABLE 1 (cont'd)

Chemical	Supplier	Quality
Oxytocin	Parke-Davis	BP
Papaverine hydrochloride	Merck	
Adrenaline hydrogen tartrate	B.D.H.	Biochemical
Vasopressin	Parke-Davis	BP
Noradrenaline bitartrate	Koch-Light	BP
Angiotensin amide	CIBA	BP
Heparin	Evans Medical Ltd.	BP
SKF 525-A	Smith, Kline and French Labs. Ltd.	
Conray 280	May and Baker	Medicinal Product
Eosin solution	George T. Gurr	
Indian ink	George T. Gurr	Special Micro
3H-aniline	Radiochemical Centre	
3H-noradrenaline	Radiochemical Centre	

4.1 Chemicals used and their sources

The various drugs, reagents and biochemical compounds used in the course of this investigation are listed in Table 1 together with their source and quality. All solutions were made with glass distilled water.

4.2 Composition of biological salt solutions

The composition of the bicarbonate buffered saline used in the preparation of the medium for perfusion of the liver was based on that of Krebs and Henseleit (1932). The composition in mmol/l was Na^+ 148.8; K^+ 4.5; Mg^{2+} 1.0; Ca^{2+} 1.25; Cl^- 127; SO_4^{2-} 1.0; phosphate pH 7.4, 2.0; HCO_3^- 2.5; glucose 11.1. The solution was gassed by bubbling with 95% air + 5% CO_2 for at least 15 min.

The phosphate buffered saline used to incubate the liver slices was based on that of Krebs and Henseleit (1932) and had the following composition in mmol/l: Na^+ 148.6; K^+ 4.5; Mg^{2+} 1.0; Ca^{2+} 1.25; Cl^- 137; SO_4^{2-} 1.0; phosphate pH 7.4, 10. The medium was buffered by the phosphate system and was gassed with 100% oxygen for at least 15 min before use. No glucose was added to the medium.

4.3 The perfused liver

4.3.1 The vascular perfusion medium

The perfusion medium was based on that of Hems et al

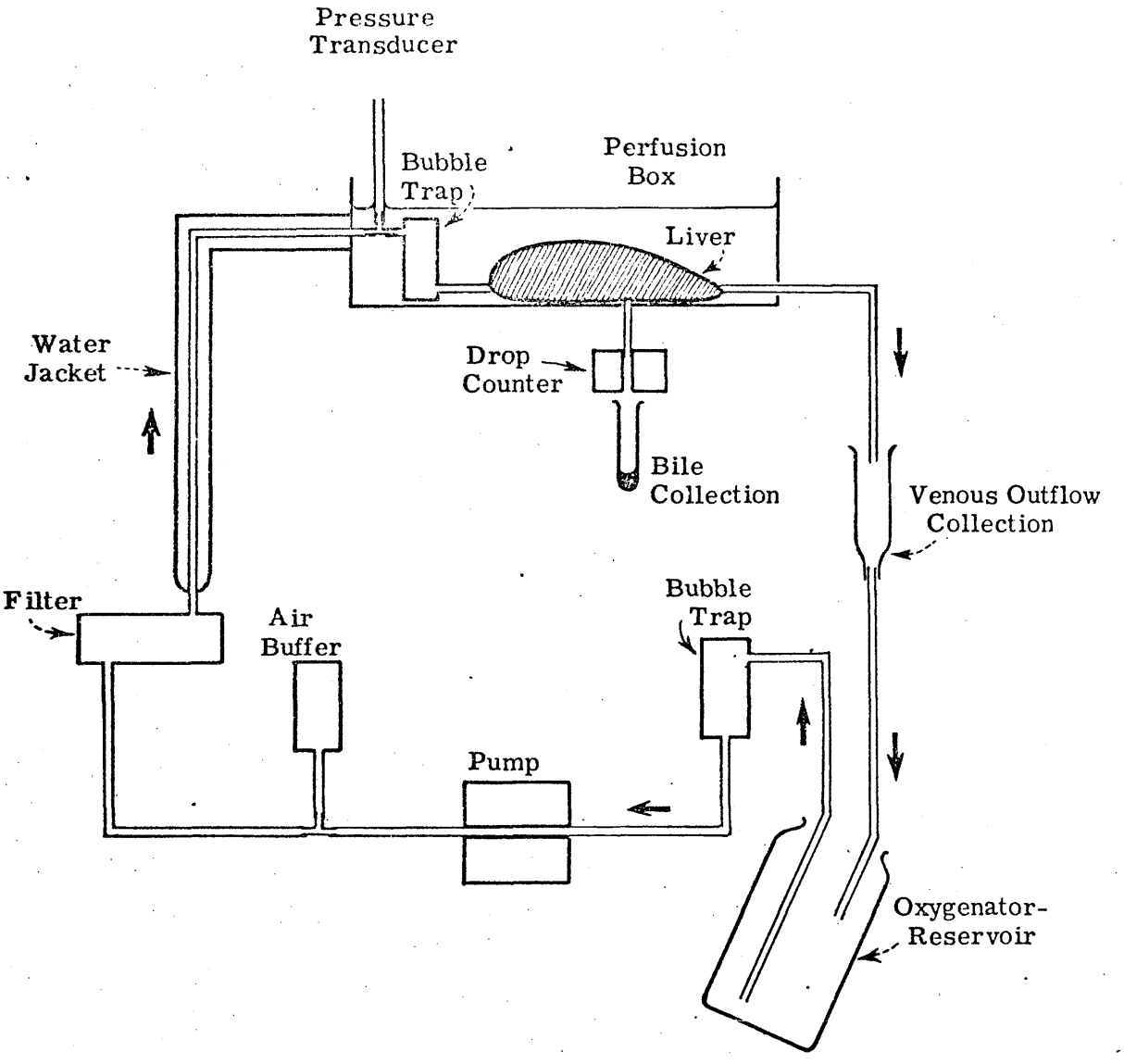


Figure 3

Diagram of the perfusion apparatus. Direction of perfusion medium flow is indicated by the heavy arrows.

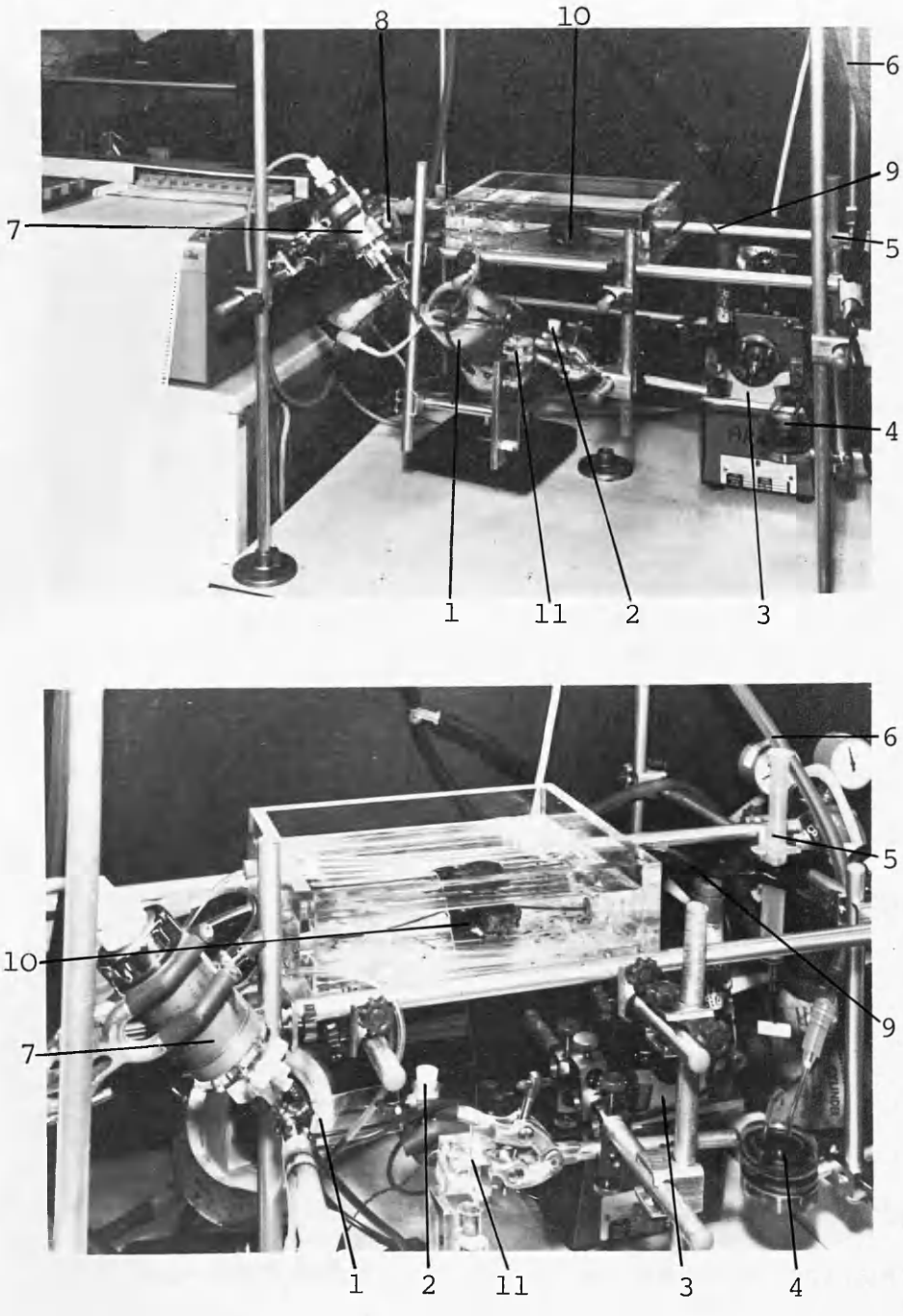


Figure 4

Photograph of the perfusion apparatus. 1, oxygenator-reservoir; 2, bubble trap 5 ml; 3, pump; 4, filter; 5, air buffer 10 ml; 6, heating jacket; 7, pressure transducer 500 mmHg; 8, bubble trap 1 ml; 9, venous outflow; 10, liver; 11, bile drop counter.
Upper plate, low power general view and lower plate, higher power view of the arrangement of the liver.

(1966) although several modifications were introduced, the most significant of which was to increase the haematocrit to 25% (v/v). Oxygen carrying capacity was primarily provided by aged human red cells, stored at 4°C for a minimum of 4 weeks. The cells were washed by suspending in 4 volumes of Krebs bicarbonate buffered saline, pH 7.4, and separated by centrifuging for 8 min at 2,000 x G at 20°C, three times. Bovine serum albumin (Cohn Fraction V powder), 4.5 g, was dissolved in 100 ml of the buffer to give a final concentration of 3 g% (w/v) in 150 ml of perfusion medium. Washed red cells 40 ml, were added to the albumin solution and the volume made up to exactly 150 ml with fresh buffer. The final glucose concentration of the medium was about 0.15 g% (w/v) and the haematocrit was between 20 and 25% (v/v). This semi-synthetic perfusion medium caused no vasoconstriction of the hepatic vasculature.

4.3.2 The perfusion apparatus

The perfusion apparatus was based on that of Powis (1970) which itself was derived from the constant flow perfusion system devised by Blakely and Brown (1963) for the perfusion of the cat spleen. The apparatus is illustrated diagramatically in Fig. 3 and photographically in Fig. 4.

The oxygenator, which also functioned as the reservoir, consisted of a 500 ml polyethylene bottle inclined at an angle of 30° from the horizontal, rotating at 60 rev/min. Perfusion medium from the venous outflow dripped in at the upper end of the oxygenator and filmed out over the wall

where it was equilibrated with a 95% air + 5% CO_2 gas mixture that had been moistened by passage through a flask of 0.9% saline. This gas mixture was used in preference to 95% oxygen + 5% CO_2 since the higher oxygen tensions had been shown to cause degenerative changes due to an increase in lysosomal activity of the liver (Abraham, Dawson, Grasso and Goldberg, 1968). Samples of perfusion medium for analyses were removed from the reservoir and additions to the perfusion medium were made into the reservoir.

Perfusion medium was drawn from the reservoir through a bubble trap consisting of a 5 ml disposable plastic syringe with a hole drilled through the wall at the upper end. The pump was a Watson-Marlow HR constant flow peristaltic roller type (MHRE 22) which compressed a short length of silicone rubber tubing (Silescol TC 156/1, 4.5 x 0.8 mm).

Oscillations in perfusion pressure due to the roller pump were damped by an air buffer consisting of a 10 ml disposable plastic syringe attached to a T-piece next to the pump. The medium then passed through a filter to remove any small clots or debris. The filter consisted of a compressed stack of twenty V75 'Micronyl' nylon photographic water filter discs with a filtration degree of 13-45 μ (Rellumit Ltd.).

The polyethylene tube through which the perfusion medium was flowing then ran for 2 m through a wider P.V.C. tube (10 x 14 mm) carrying a flow of water at 38°C, which served as an efficient heat exchange unit. After passing through a

small bubble trap (1.0 ml) fashioned from Perspex and sealed with a silicone rubber bung the medium entered the liver through a cannula in the portal vein. The portal venous pressure was recorded from a polyethylene Y-piece between the bubble trap and cannula, through a saline filled Tygon tube, by a Pye Ether UP4 pressure transducer (± 500 mm Hg) connected to a potentiometric recorder (Smiths Servoscribe).

The perfusion chamber consisted of a Perspex box with two holes drilled through the wall at one end to carry the tubes containing water from the inflow and outflow of the vinyl heat exchange unit (Baxter, Fenwal plastic blood warming unit) which formed a false floor to the chamber and on which the liver rested. Another two holes, one at each end of the chamber, sealed with rubber stoppers drilled through their centres, carried the polyethylene tubes transporting the perfusion medium to and from the liver. The rubber stoppers permitted adjustment of the cannulae because of the security of grip they provided when the cannulae were set in position. The whole chamber was filled with liquid paraffin at 37^{o}C which kept the liver warm and moist and prevented compression oedema by partially floating the lobes apart.

Bile flow was recorded from a cannula in the bile duct running through an electronic drop counter connected to the potentiometric recorder or, in initial experiments, by timing flow in a capillary tube. Bile was collected at 10 cm negative pressure in a small test tube clamped below the perfusion chamber.

The portal venous circuit could be extended by making use of the length of Tygon tubing normally connected to the pressure transducer. Thus perfusion of the liver could be started while the organ was still in the animal on the operating table below the perfusion chamber. After transfer of the liver to the perfusion chamber this extension tube could be re-connected to the pressure transducer and the cannula in the portal vein attached to a shorter tube from the Y-piece.

Venous blood drained from the liver at 5.0 cm negative pressure through a cannula in the inferior vena cava into a polycarbonate collecting tube and then back into the reservoir for re-oxygenation and re-cycling.

Portex polyethylene tubing was used throughout for the perfusion circuit (PP 270, 2 x 3 mm) and for the bile duct cannula (PP 10, 0.28 x 0.61 mm). The portal vein cannula consisted of a short length of polyethylene tubing (PP 270) drawn out over a bunsen flame to a diameter of 2 mm and cut square at the tip. The venous outflow cannula was of a similar design except that a 30^{o} bevel at the end was necessary to pierce the right atrium of the heart. All connections were push fit or by means of short lengths of silicone rubber tubing (Silescol). All tubing inside the perfusion chamber was of S-50-HL Tygon (2.5 x 4 mm) which provided flexibility without risk of compression. The total dead space of the circuit was 14.0 ml.

The perfusion medium came into contact with only

polyethylene, nylon, silicone rubber, Tygon and Perspex. It was thus unnecessary to siliconise any exposed surface as is necessary with rubber or glass. The filter elements, bubble traps and T-pieces were washed overnight in RBS 25 non-ionic detergent (Chemical Concentrates Ltd.) after each perfusion and then rinsed in several volumes of distilled water before re-assembly. These parts of the apparatus were replaced periodically or if showing signs of wear. All polyethylene and silicone rubber tubing was discarded after each perfusion and replaced with fresh material.

4.3.3 Operative procedure

Male albino Wistar rats (220-300 g) were allowed free access to a standard laboratory diet (Diet 41, Herbert C. Styles) and water prior to the operation. Anaesthesia was induced by Halothane (I.C.I.) 3% in an O_2:N_2O mixture (1:3) and was maintained on 1% Halothane in the same gas mixture delivered through a cannula in the trachea. A midline incision was made from the pubic symphisis to the xiphoid process along the bloodless linea alba and the skin and abdominal muscle coats were retracted to either side. The intestines were displaced to the left of the rat between layers of tissue kept moist with 0.9% saline preheated to 37^{o}C. The superior mesenteric artery was identified and ligated and the inferior mesenteric artery was tied off by ligating the colon proximal to its junction with the rectum. The superior mesenteric vein was ligated by tying off the duodenum above the entry of the bile duct at the sphincter of Oddi. At this stage of the operation 250 I.U. heparin were injected into the animal

through the ventral penile vein. The bile duct was cannulated with a length of polyethylene tubing cut to a sharp point, which was inserted to a point distal to the fusion of the left and right bile ducts to form the common bile duct. Bile flow commenced immediately upon insertion of the cannula into the duct.

The viscera and intestines were then reflected to the right so that the abdominal aorta could be ligated below the coeliac axis. After again reflecting the viscera to the left, the inferior vena cava was dissected free of the connective tissue and tied below the liver above the right renal vein. The superior pancreatico-duodenal vein was cleared and cut between ties where it entered the portal vein. Loose ligatures were placed around the hepatic artery and the portal vein, just distal to their points of entry into the liver tissue. Further loose ties were placed around the splenic vein at its point of entry into the portal vein and around the portal vein distal to the splenic vein. The middle colic vein was ligated close to the portal vein which was then cleared between this tie and the splenic vein. The portal vein was ligated distal to the splenic vein.

The rat was then transferred to the perfusion table below the perfusion chamber. The liver was at this point receiving blood from the hepatic artery and the splenic vein via the portal vein. The splenic vein was then ligated. The portal vein was cut proximal to the middle colic vein and a saline-filled cannula inserted and tied into the vein. After all air bubbles had been removed from the cannula the perfusion circuit, via its extension tube, was attached and perfusion

commenced at 2 to 3 ml/min. The hepatic artery was then ligated.

The thorax was opened and two loose ties placed around the inferior vena cava above the diaphragm. The vena cava was cannulated by piercing the wall of the right atrium with a sharp-pointed cannula and guiding this down the length of the vein to its point of passage through the diaphragm. The cannula was then tied in place. At this stage all of the blood from the rat itself had been cleared from the liver by perfusion with the semi-synthetic medium. Collection of the venous outflow after cannulation of the vena cava thus enabled the volume of circulating artificial medium to be maintained without contamination by rat red blood cells.

The liver was now totally isolated from the circulation of the rat but was still attached physically. The colon was cut below the ligature around it, the bile duct and portal vein dissected free from the surrounding mesentery and the hepatic artery cut distal to its tie. The stomach and spleen were carefully cut free of their attached ligaments and the oesophagus cut near the stomach. The inferior vena cava was cut above the right renal vein but below the ligature around it. The intestines, stomach and spleen could then be dissected out of the rat leaving the liver attached only to the diaphragm by several ligaments and the vena cava. The inferior vena cava with attached cannula was cut free from the heart and the diaphragm dissected round the vein to leave a cuff of diaphragmatic tissue. The remaining ligaments to the liver were cut and the liver transferred to the perfusion chamber.

The venous outflow was adjusted to provide optimum flow, at a slight negative pressure. The tubing attached to the portal vein was disconnected from the cannula and attached to the pressure transducer while the previously sealed shorter tube from the Y-piece at the pressure transducer was filled with perfusion medium, connected to the cannula and opened to permit perfusion to continue. The perfusion flow rate was adjusted to 10 ml/min (approximately 1 ml/g of liver/min) and the position of the portal cannula adjusted to give the lowest pressure reading indicating that the inflow was not occluded. The pressure was normally 8 to 12 cm H_2O. The end of the bile duct cannula was positioned over the collection vessel and bile collection commenced. The chamber was filled with liquid paraffin previously warmed to 37^{o}C. The perfusion medium collected between cannulation of the vena cava and transfer of the liver was then returned to the reservoir.

4.3.4 Operative protocol

The entire operative procedure took 30 min. Half-way through the operation the apparatus was primed with the perfusion medium, care being taken to expel all air bubbles, and the medium slowly re-cycled through the apparatus, being oxygenated in the process. After isolation of the liver it could be attached to the perfusion circuit by means of an extension tube of Tygon as described above, so that transfer of the liver from the operating table to the perfusion chamber could be carried out without any interruption in blood flow to the organ. The liver was normally left to equilibrate for 60 min except in viability studies.

4.3.5 Experimental procedures

4.3.5.1 Assessment of viability

After a few minutes equilibration 3 ml aliquots of perfusion medium were removed from the reservoir at 15 min intervals over the first 90 min and every 30 min thereafter up to 6 h, for the determination of glucose, lactate and pyruvate or the degree of haemolysis and K^+ levels. Bile flow rate was determined by timing the period taken to fill a 20 µl capillary tube. In some perfusions 5 ml aliquots of perfusion medium were taken for the determination of the level of bacterial contamination, pH or oxygen content. Similar samples were taken in control perfusions in which perfusion medium was re-cycled in the circuit in the absence of a liver. Any medium leaking from the liver was collected and measured at 15 min intervals.

After completion of perfusion the appearance of the liver was noted and the liver weight recorded. In some instances sections of liver were removed for electron microscopy together with sections cut from the livers of freshly killed rats.

In calculating the removal or appearance of compounds in the perfusion medium a correction both for removal of the compound during sampling and for loss occurring from leakage of medium from the liver vasculature, normally 0 to 2 ml/h, has been made.

4.3.5.2 Hexobarbitone metabolism

Livers were equilibrated for 60 min after transfer to the perfusion chamber to permit a constant rate of conversion of substrates of intermediary metabolism to be established (Ross, 1972). Hexobarbitone sodium, dissolved in 0.9% saline was added to the reservoir to give an initial concentration of 1 µmol/ml. The two pulse protocol of Stitzel et al (1966) was followed in which, 90 min after the addition of the first pulse of hexobarbitone, a second similar pulse of the substrate was added. Aliquots of perfusion medium, 1 ml, were removed at 15 min intervals. Noradrenaline or adrenaline bitartrate, dissolved in saline, was added to the reservoir 85 min after the first pulse of hexobarbitone and 5 min before the second pulse to give an initial concentration of 6.0 µM.

4.3.5.3 Aniline metabolism

Livers were equilibrated for 60 min and aniline hydrochloride dissolved in saline added to the reservoir to give an initial concentration of 1 µmol/ml. Aliquots of perfusion medium, up to 2.6 ml, were removed at 15 or 30 min intervals up to 4 h and assayed for free aniline and acid-labile aniline conjugate. In some perfusions free and conjugated p-aminophenol were also measured. Aliquots of bile were collected over 1 h periods and also assayed for aniline and its metabolites. Adrenaline or noradrenaline bitartrate dissolved in saline was added 60 min after the addition of aniline to give an initial concentration of 6.0 µM.

4.3.5.4 Blood pressure studies

The vasoactive properties of a variety of compounds were tested in the perfused liver. Ba^{2+}, K^{+}, vasopressin, histamine, papaverine and angiotensin II were administered directly into the reservoir and angiotensin, Ba^{2+}, vasopressin and K^{+} infused in saline at a constant flow rate of between 11 µl/min and 44 µl/min into the portal venous bubble trap.

4.3.5.5 X-radiography of the liver

After equilibration for 60 min the liver was perfused for 3 min with a 10% (v/v) solution of the radio-opaque contrast medium Conray 280 in perfusion medium by a separate perfusion circuit which allowed an almost square wave pulse of contrast medium to be delivered. X-radiographs were taken with a Newton Victor Model K, Type 5 X-ray Machine placed 32 cm above the liver at 30 KeV, with 3 sec exposure, on to 3M type S X-ray film placed 3 cm below the liver. During perfusion of the contrast medium and for 5 to 10 min afterwards the perfusion medium was not re-circulated. 30 min later the liver was perfused with medium containing adrenaline, 6.0 µM and when the increased portal pressure had reached a plateau perfusion with medium containing Conray 280 as well as adrenaline was commenced and X-radiographs were taken as before.

4.3.5.6 Perfusion of the liver with Indian ink

After 30 min equilibration the liver was perfused with medium containing Indian ink, particle size < 3 µ, 10% (v/v)

through the portal vein for 2 min. Perfusion was stopped and sections of liver immediately removed from deep central and peripheral regions of the lobes and placed in Helly's fluid (Disbrey and Rack, 1970) as a fixative. In another group of livers after the equilibration period the medium was changed to one containing 6.0 µM adrenaline and perfusion was continued until the portal pressure response reached a plateau when Indian ink 10% (v/v) was added to the medium and sections taken as above.

4.3.6 Chemical and biochemical analytical techniques

4.3.6.1 Determination of L (+)-lactate

The concentration of lactate in the perfusion medium was determined by the lactate dehydrogenase method of Hohorst (1963). Aliquots of perfusion medium were deproteinised with 2 volumes of ice cold 0.6 M perchloric acid immediately after sampling and the deproteinised supernatants assayed after storage overnight at -20°C. The only significant modification of the assay was to increase the amount of enzyme by 50%. The accuracy of the assay was ± 0.014 µmol/ml.

4.3.6.2 Determination of pyruvate

Pyruvate in the perfusion medium was assayed by a method based on the lactate dehydrogenase method of Bücher, Czok, Lamprecht and Latzko (1963), modified by Bücher (1970). Aliquots of perfusion medium were deproteinised with an equal volume of ice cold 1 M perchloric acid immediately upon collection and the deproteinised supernatants stored overnight at -20°C. The

accuracy of the assay was ± 0.002 µmol/ml.

4.3.6.3 Determination of glucose

The total blood glucose of the perfusion medium was determined by the GOD-Perid method of Werner, Rey and Wielinger (1970) with a commercially available test combination (Boehringer), the manufacturer's instructions being followed exactly. Samples were deproteinised and stored overnight at -20°C. The accuracy of the assay was ± 0.25 µmol/ml (± 5 mg%).

4.3.6.4 Estimation of haemolysis

The extent of haemolysis in samples of perfusion medium was determined on duplicate samples of the synthetic plasma obtained by centrifugation of the medium at 2,000 x G for 10 min. The plasma was stored overnight at -20°C prior to assay. The plasma samples were diluted with 3 volumes of glass distilled water and the absorbance at 416 mµ measured. An aliquot of perfusion medium haemolysed by the addition of 100 volumes of distilled water served as a reference standard. Haemolysis was expressed as a percentage of the haemolysed standard.

4.3.6.5 Determination of haematocrit

The haematocrit of the perfusion medium was determined on samples of medium before starting perfusion of the liver. The samples were centrifuged in glass Wintrobe tubes at 2,000

x G for 15 min and the haematocrit expressed as per cent packed cell volume in the sample.

4.3.6.6 Determination of serum potassium

The concentration of K^+ was determined in cell free samples of perfusion medium prepared by centrifugation at 2,5000 x G for 15 min and stored overnight at -20°C. Samples were diluted with 3 volumes of distilled water and K^+ content determined on an EEL Model 100 direct reading flame photometer. Duplicate samples were sprayed in a randomised order with a standard solution after every third sample to allow for any drift of the photocell. The accuracy of the assay was ± 0.0025 µM/ml.

4.3.6.7 Determination of pH and oxygen content of the perfusion medium

Samples of perfusion medium were collected under liquid paraffin and the pH at 37°C determined immediately on a Metrohm direct reading pH meter, model E 512, with a saturated KCL combination glass electrode (Russell). In later determinations the perfusion medium samples were collected and sealed in plastic syringes and the pH determined on a Radiometer blood gas analyser. The perfusate P_{CO_2} and P_{O_2} were determined on the same samples as were used for the pH determination on the blood gas analyser. Total oxygen content of the medium was estimated in samples sealed in glass syringes and assayed on a Lex-O_2-Con total oxygen analyser.

4.3.6.8 Determination of bacterial contamination of the perfusion medium

Aliquots of perfusion medium were removed at hourly intervals and sealed in glass tubes. Possible bacterial contamination was checked by culturing the sample on both solid and liquid media, using routine microbiological techniques for the identification of any bacterial growth (Cruikshank, 1968).

4.3.6.9 Preparation of tissue for electron microscopy

Sections of liver taken for electron microscopy were fixed for 1 h in a glutaraldehyde-cacodylate fixative consisting of 0.1 M sodium cacodylate with 2% glutaraldehyde, 1.7% (w/v) sucrose and 1 mM $CaCl_2$, pH 7.2 and osmolarity 400 mosmol/l. After a minimum of a 2 h wash in 5.9% (w/v) sucrose in 0.1 M sodium cacodylate, pH 7.2 the sections were post-fixed for 1 h in osmium cacodylate fixative. This was 0.1 M sodium cacodylate with 1% (w/v) O_sO_4, and 5.1% sucrose, pH 7.2 and osmolarity 400 mosmol/l. After fixing, the sections were dehydrated in a series of graded alcohols, embedded in araldite and 400 Å sections cut on a LKB ultramicrotome. These sections were stained with saturated alcoholic uranyl acetate and Reynolds' lead citrate (Reynolds, 1963). The stained sections were examined with an AEI EM6B electron microscope and micrographs taken on Ilford EM4 plates.

4.3.6.10 Preparation of tissue for histological examination

Small sections of tissue were removed and fixed for 24 h in Helly's fluid (Disbrey and Rack, 1970) and then dehydrated using a Histokinette automatic tissue processor. The sections were finally embedded in paraffin wax (Paraplast) and blocked out. Sections 6 μ thick were cut on a Jung microtome and mounted on glass slides which were dried overnight. The slides were cleared of wax in xylene and were examined either unstained or after light background staining in aqueous eosin. Examination was carried out under both low and high power objectives of a Leitz (Wetzlar) Orthoplan microscope and sections were photographed with FP4 film (Kodak).

4.3.6.11 Protocol for the estimation of aniline and its metabolites

Samples of perfusion medium were haemolysed by storage overnight at -20°C. Bile samples collected over periods of 1 h were also stored at -20°C. 1 ml aliquots of the medium and all of the bile in a sample were extracted with 2 x 5 ml light petroleum ether and 1.5% isoamyl alcohol and the free aniline thus removed extracted back into 2 ml of 2N HCl and assayed by the method of Bratton and Marshall (1939). The efficiency of the extraction was 95%. The extracted residue was deproteinised by the addition of half a volume of ice cold 20% (w/v) TCA and centrifuged at 2,000 x G for 10 min. The supernatant was assayed for aniline liberated from acid-labile conjugates by the method of Bratton and Marshall (1939).

A second aliquot of medium or the previously extracted bile sample was deproteinised with a half volume of ice cold 20% (w/v) TCA and centrifuged at 2,000 x G for 10 min. The

supernatant was assayed for free p-aminophenol by the method of Schenkman et al (1967). An aliquot of supernatant was hydrolysed with an equal volume of 1N HCl in a boiling water bath for 60 min. These conditions were found to be optimal for the liberation of p-aminophenol with least degradation. Higher concentrations of acid or more prolonged periods of hydrolysis both resulted in a lower recovery of p-aminophenol. The acid hydrolysate was assayed for total p-aminophenol and for total aniline. The accuracy of the aniline assay was ± 0.001 µmol/ml and of the p-aminophenol assay ± 0.0005 µmol/ml.

4.3.6.12 Assay of hexobarbitone

Aliquots of perfusion medium were haemolysed by storage overnight at -20°C. The samples were then assayed for unmetabolised hexobarbitone by the method of Brodie, Burns, Mark, Lief, Bernstein and Papper (1953). The efficiency of the extraction was 94% and the accuracy of the assay was ± 0.003 µmol/ml.

4.4 Liver slices

4.4.1 Preparation of liver slices

Slices were prepared from the livers of adult male Wistar rats (200-300 g) by a modification of the method of Umbreit, Burris and Stauffer (1964). Rats were killed by decapitation and exsanguinated. The livers were quickly removed and placed in ice cold 0.9% saline. Slices of liver (0.4 to 0.6 mm) were cut by hand between a ground glass plate and a frosted

glass microscope slide with a razor blade. The slices were cut on ice and kept in ice cold saline at all times.

4.4.2 Incubation of liver slices

The incubation conditions were based on those of Cooper, Axelrod and Brodie (1954) and consisted of 4 µmol of hexobarbitone or 2 µmol of aniline in Krebs phosphate-buffered saline in a total incubation volume of 5 ml. The media were pre-warmed to 37°C for 5 min and the reaction started by the addition of approximately 0.5 g of liver slices which had been blotted and weighed accurately. Incubation was at 37°C in a shaking water bath in an atmosphere of 100% oxygen for 3 h. Aliquots of incubation medium, 0.5 ml, were removed at various time intervals and stored at -20°C. Unmetabolised aniline was determined, after extraction, by the method of Bratton and Marshall (1939) and unmetabolised hexobarbitone by the method of Brodie et al (1953).

4.5 Metabolism by the microsomal subcellular fraction

4.5.1 Preparation of the microsomal fraction

The microsomal fraction was prepared from the livers of adult Wistar rats (150-200 g) by a method based on that of Ernster, Siekevitz and Palade (1962). Rats were at all times permitted free access to standard laboratory rat food (Diet 41, Herbert C. Styles) and water.

Rats were killed by decapitation and exsanguinated,

between 9.30 am and 10.00 am, to avoid diurnal variation in drug metabolism (Jori, Di Salle and Santini, 1971). Livers were removed and immediately placed in ice cold 0.9% saline. The livers were then homogenised in 4 volumes of 0.25 M sucrose and 0.05 M Tris buffer, pH 7.4 in a 55 ml Thomas glass homogenising vessel fitted with a Tri-R teflon pestle providing a clearance of 0.40 to 0.45 mm. Homogenisation was for 12 strokes at 6,000 rpm on a Tri-R homogeniser (model S 63C). The microsomal fraction was prepared as described by Ernster et al (1962), using a hand operated 4 ml teflon and glass homogeniser, clearance 0.08 to 0.13 mm (Thomas & Co.) to resuspend the microsomes in sucrose.

4.5.2 Incubation systems

4.5.2.1 Incubation system used in studies on the effects of catecholamines

The incubation system employed in these studies was based on that of Orrenius (1965) and consisted of 150 µmol Tris buffer, pH 7.4 at 37°C, 15 µmol $MgCl_2$, 50 µmol nicotinamide, 20 µmol DL-isocitrate, 2 units of isocitrate dehydrogenase, 1 µmol NADP, and 0.2 µmol aniline, 0.6 µmol hexobarbitone or 10 µmol aminopyrine and 10 µmol semicarbazide. The medium was pre-incubated for 10 min at 37°C to start the generation of NADPH. The reaction was started by the addition of 4 to 5 mg microsomal protein to make the final incubation volume 2 ml.

Samples were incubated in a shaking water bath at 37°C

for 30 min in an atmosphere of air. The reactions were stopped by the addition of 0.5 ml ice cold 25% (w/v) $ZnSO_4$ followed by 0.5 ml saturated $Ba(OH)_2$ when aminopyrine was the substrate and either 3 ml or 5 ml of light petroleum ether and 1.5% isoamyl alcohol when hexobarbitone and aniline were the substrates respectively.

4.5.2.2 Incubation systems used in studies on the effects of washing the microsomal fraction

4.5.2.2.1 Incubation system employing NADPH-generating system

This incubation system was based on that of Kato and Gillette (1965) and consisted of 75 µmol Tris buffer, pH 7.4 at 37°C, 15 µmol $MgCl_2$, 50 µmol nicotinamide, 1 µmol NADP, 26.2 µmol glucose-6-phosphate, 1.4 units of glucose-6-phosphate dehydrogenase and 5 µmol aniline or 10 µmol aminopyrine with 10 µmol semicarbazide. These reagents were in a volume of 1 ml. A further 0.5 ml of distilled water was added, in which could be dissolved any test substances.

The samples were pre-incubated as before and the reaction started by the addition of 4 to 5 mg microsomal protein, the final incubation volume being 2 ml. Incubations were carried out at 37°C as before. The reaction was stopped by the addition of 1 ml of ice cold 20% TCA or 0.5 ml ice cold 25% $ZnSO_4$ followed by 0.5 ml saturated $Ba(OH)_2$ when aniline and aminopyrine respectively were substrates.

4.5.2.2.2 Incubation system employing chemically reduced NADPH

In some experiments the effects of chelators on microsomal drug metabolism were investigated and it was not possible to use an ion-dependent enzyme system for the generation of NADPH. Instead, chemically reduced NADPH was added directly to the incubation system which consisted of 200 µmol Tris buffer, pH 7.4 at 37°C, 5 µmol aniline or 10 µmol aminopyrine with 10 µmol semicarbazide, three separate additions of 2 µmol NADPH at 0 min, 10 min and 20 min and 4 to 5 mg microsomal protein. The total incubation volume after the final addition of NADPH was always 1.8 ml. The reaction was started by the addition of the first 2 µmol of NADPH and the samples were incubated at 37°C for 30 min in a shaking water bath under air. The reactions were stopped as described in Section 4.5.2.2.1.

4.5.3 Washing the microsomes

The microsomal pellet was obtained as described in Section 4.5.1. The pellet was rinsed with 3 x 3 ml of the sucrose buffer, drained briefly, re-suspended in 12.5 ml using a hand operated 4 ml teflon homogeniser (Thomas & Co.) and incubated at 2°C for 30 min then re-centrifuged at 105,000 x G for a further 65 min at 2°C. The washed microsomal pellet obtained was again rinsed in 3 x 3 ml sucrose and re-suspended in 3 ml of the sucrose as before. This overall procedure is hereafter referred to as washing of the microsomes. All assays were carried out on the same day as preparation.

4.5.4 Chemical and biochemical assays

4.5.4.1 Assay of aniline and its metabolite p-aminophenol

Catecholamines were found to inhibit the indo-phenol reaction used for the detection of p-aminophenol. Thus when catecholamines were included in the incubation medium, microsomal aniline metabolism was measured by determining the amount of free substrate remaining. Aniline was assayed colourimetrically by the method of Bratton and Marshall (1939).

In other experiments aniline metabolism was followed by measuring the formation of its hydroxylated metabolite p-aminophenol. The assay used was that of Schenkman et al (1967). Standards were prepared in 6.7% TCA.

4.5.4.2 Assay of formaldehyde

Aminopyrine metabolism was determined by measuring the formation of one of the metabolites, formaldehyde, which was trapped in solution as a semicarbazone. The assay used was the Cochin and Axelrod (1959) modification of the method of Nash (1953). The accuracy of the assay was ± 0.0002 µmol/ml.

4.5.4.3 Assay of hexobarbitone

Hexobarbitone was assayed by the method of Brodie et al (1953).

4.5.4.4 Determination of protein

Microsomal protein was determined by the method of Lowry,

Rosebrough, Farr and Randall (1951). Crystalline bovine serum albumin (Cohn Fraction V) in concentrations of 0 to 100 µg/ml was used as the standard. The accuracy of the assay was ± 0.0002 µg/ml.

4.5.4.5 Assay of thiobarbituric acid reacting substance (malonaldehyde)

Lipid peroxidation was measured by determining the formation of a thiobarbituric acid reacting substance recently shown to be malonaldehyde (Bidlack and Tappel, 1973). The assay was that described by Bidlack et al (1973). Results have been expressed as absorption change per mg microsomal protein.

4.5.4.6 Determination of cytochrome b_5 and cytochrome P-450 content of the microsomal fraction

The microsomal content of cytochrome b_5 and cytochrome P-450 was determined on the same sample by the method of Dallner (1966). The microsomal fraction was diluted to a protein concentration of 2 mg/ml in 0.05 M Tris buffer pH 7.4 and 5 mM $MgCl_2$.

The extinction coefficient of cytochrome b_5 between 500 mµ and 423 mµ was taken as 171 $cm^2 \mu mol^{-1}$ and of cytochrome P-450 between 450 mµ and 480 mµ as 91 $cm^2 \mu mol^{-1}$. Cytochrome content has been expressed in mµmol/mg protein.

4.5.4.7 Assay of microsomal activity of NADPH-cytochrome c reductase

The NADPH-cytochrome c reductase activity of the microsomal fraction was determined by the method of Mazel (1971). The extinction coefficient of cytochrome c at 550 mμ was taken as 19.1 $cm^2 \mu mol^{-1}$ and activity has been expressed as mμmol cytochrome c reduced/mg protein/min.

4.5.4.8 Assay of microsomal activity of NADPH-cytochrome P-450 reductase

The NADPH-cytochrome P-450 activity of the microsomal fraction was determined by the method of Gigon et al (1969). The microsomal suspension was diluted to 5 mg protein/ml in 0.02 M Tris buffer pH 7.4, and the NADPH-generating system consisted of 1.5 μmol $MgCl_2$, 40 μmol isocitrate and 0.02 units isocitrate dehydrogenase in 0.02 M Tris buffer pH 7.4.

The change in absorbance was calculated for the initial rapid phase of cytochrome P-450 reduction over the first minute. The activity of NADPH-cytochrome P-450 reductase has been expressed as mμmol cytochrome P-450 reduced/mg protein/min, using an extinction coefficient for cytochrome P-450 at 450 mμ of 91 $cm^2 \mu mol^{-1}$.

4.5.4.9 Determination of the substrate-induced difference spectrum of the microsomal fraction

The change in the difference spectrum of the microsomal fraction caused by the addition of substrate was measured by the method of Schenkman et al (1967) on a Pye Unicam SP 8000 dual beam recording spectrophotometer. The microsomal fraction was diluted to 3 mg protein/ml with 0.10 M Tris buffer pH 7.4.

4.6 In vivo studies

Male Wistar rats (150-200 g) were pre-treated with either adrenaline or noradrenaline (1 mg/Kg) dissolved in 0.9% saline, administered by i.p. injection. Animals were killed at various times after administration. Liver microsomal fraction was prepared as described in Section 4.5.1 and the metabolism of aniline and aminopyrine determined as described previously. The microsomal content of cytochromes b_5, and P-450 and the activity of the enzyme NADPH-cytochrome c reductase, were determined as previously described.

4.7 Treatment of results

In studies with the perfused liver, and with liver slices, substrate half-lives have been calculated with the aid of a Fortran programme. This calculated gradient, m and intercept, C of the regression line of a plot of logarithm amount of free substrate against time of sampling. The half-life was then calculated from the expression:

$$t_{\frac{1}{2}} = \frac{\log_{10} 10^{C/2} - C}{m}$$

Comparison of results was carried out by means of Student's t test for samples of unequal size (Snedecor and Cochran, 1968) and were regarded as differing significantly if the P-value was less than 5%.

CHAPTER 5

Results

5.1 Viability of the perfused liver

5.1.1 Macroscopic appearance

Within a few minutes of commencing perfusion, the liver became uniformly coloured a dark red, very similar to the colour observed *in vivo*. At higher oxygen tensions in the perfusion medium the liver became abnormally light and more pink in colour. After 6 h of perfusion the liver was almost invariably evenly coloured, although occasionally paler unperfused patches became apparent, particularly at the edge of the lobes. Sometimes a clear exudate was visible on the surface of the lobes, particularly at higher perfusion pressures.

5.1.2 Perfusion pressure

The perfusion medium flow rate was constant throughout an experiment and varied between 0.8 and 1.2 ml/g/min (mean 1.0 ml/g/min) over twenty perfusions. The difference was due to the variations in liver weight since the flow rate was initially set at 10 ml/min and could only be corrected for liver weight at the end of the experiment. The perfusion pressure corresponding to these flow rates was 8 to 12 cm H_2O (mean 10 cm H_2O). The pressure increased proportionally with the flow rate.

The perfusion pressure did not normally change significantly over the duration of an experiment. A typical record of the portal pressure during a control perfusion is

shown in Fig. 16. In a few perfusions the pressure started to increase steadily, at any time after commencing perfusion, finally resulting in an increase of up to 6 cm H_2O. Such perfusions were normally characterised by the appearance of unperfused areas at the edges of the lobes. These perfusions were rejected.

5.1.3 Adequacy of perfusion

In several livers the introduction of Indian ink (particle size < 3μ) into the perfusion medium after up to 6 h of perfusion revealed an even distribution of carbon granules throughout the liver within 2 min, indicating that the medium was perfusing all areas of the organ.

X-radiographs of livers perfused with the radio-opaque contrast medium Conray 280, at a concentration of 10% (v/v), also revealed an even perfusion of the liver, as shown in Fig. 24. The contrast medium was evenly distributed throughout the organ, the edges being evenly outlined. Many fine branches of the portal venous tree were visible and the background was evenly dense, indicative of uniform sinusoidal filling.

5.1.4 Extent of haemolysis

The amount of haemoglobin detectable in the cell free fraction of the perfusion medium stayed constant over the first 30 min of perfusion and thereafter increased almost linearly with time at a rate corresponding to an average rate of

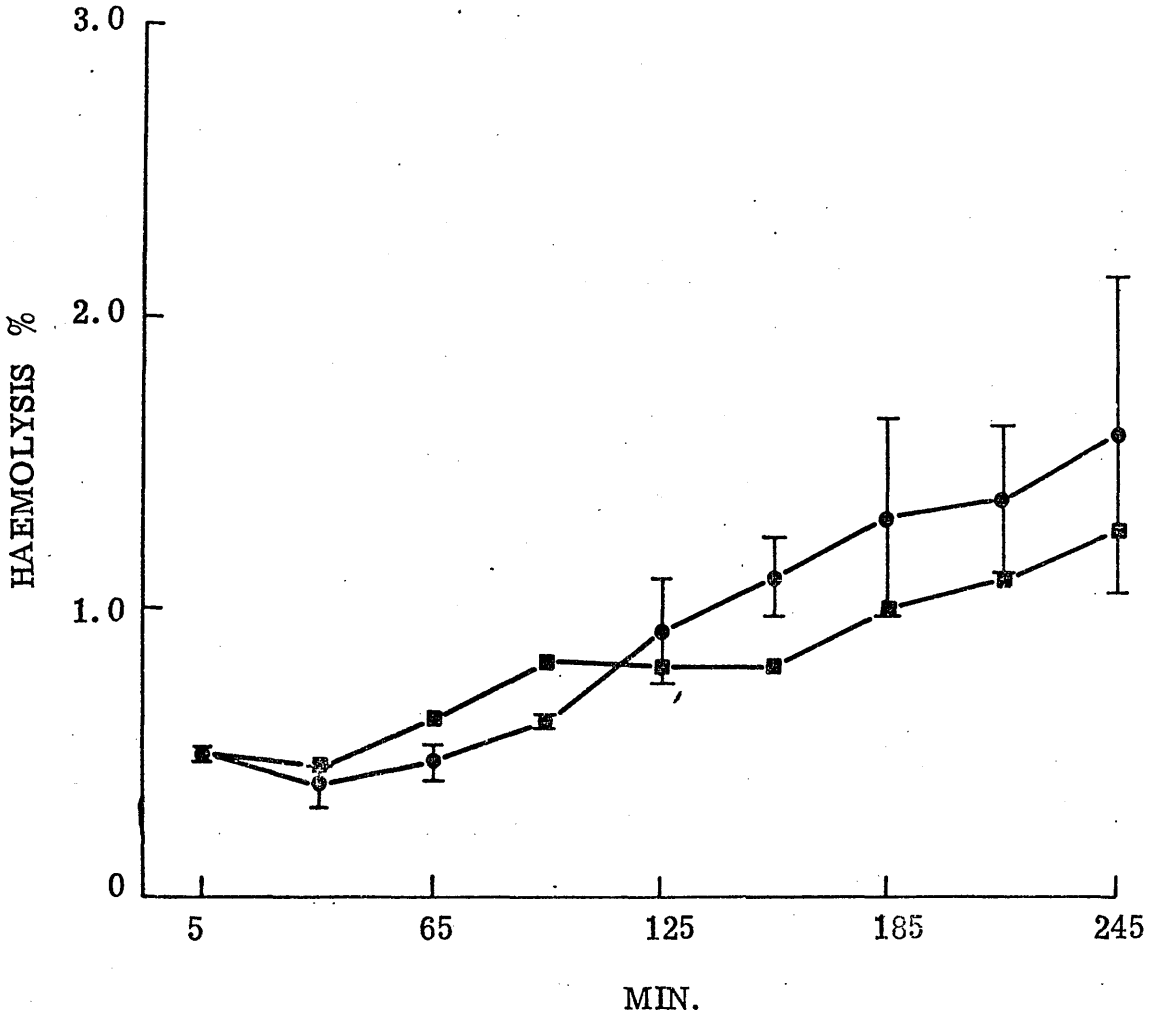


Figure 5

Extent of haemolysis in the perfusion medium during perfusion of the isolated rat liver. Measurements were started 5 min after transfer of the liver to the perfusion chamber. Values obtained with a liver in the circuit (●) are mean of four perfusions. Bars represent S.E. of mean. Values obtained with no liver in the circuit (■) are mean of two perfusions.

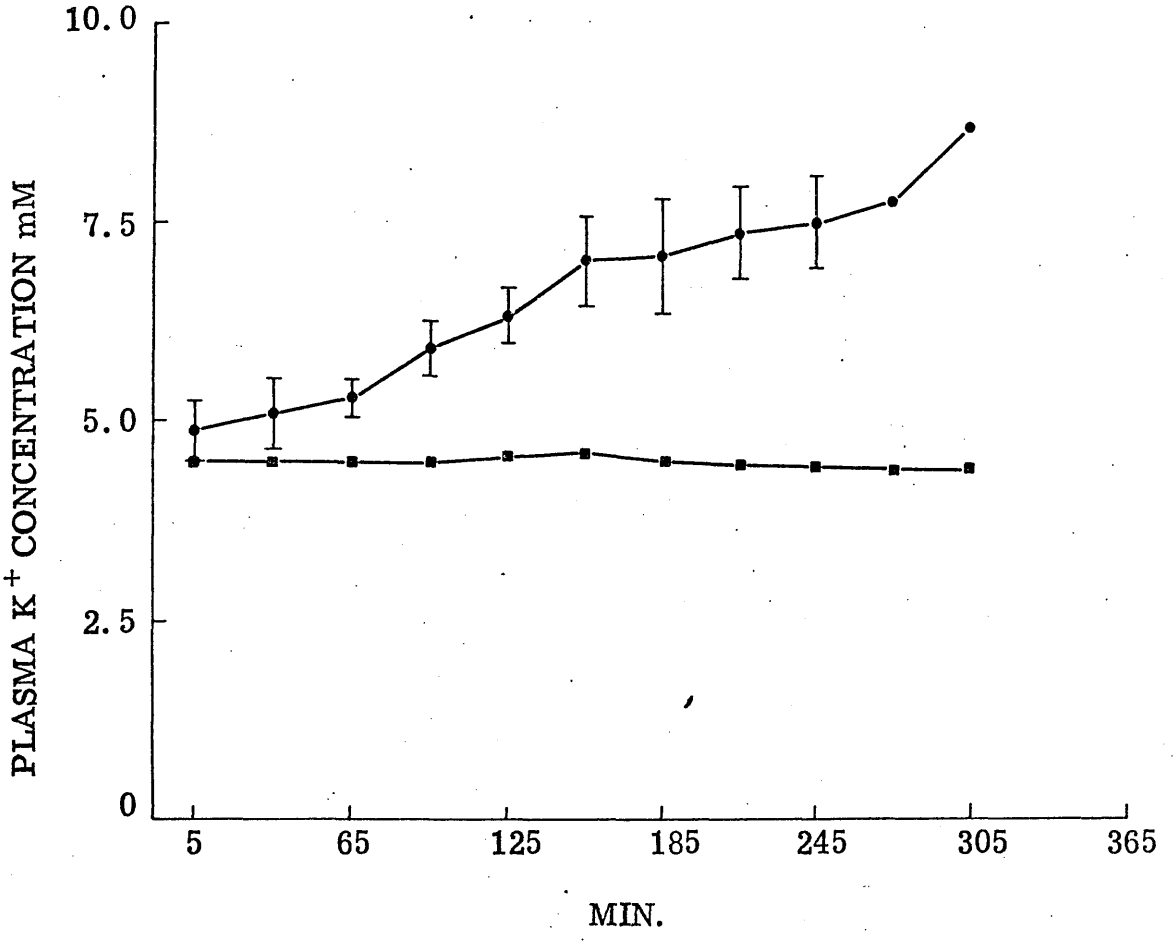


Figure 6

Increase in plasma K^+ concentration during perfusion of the isolated liver. Measurements were started 5 min after transfer of the liver to the perfusion chamber. Values obtained with a liver in the circuit (●) are mean (± S.E.M.) of four perfusions. Values obtained with medium alone (■) are mean of two perfusions.

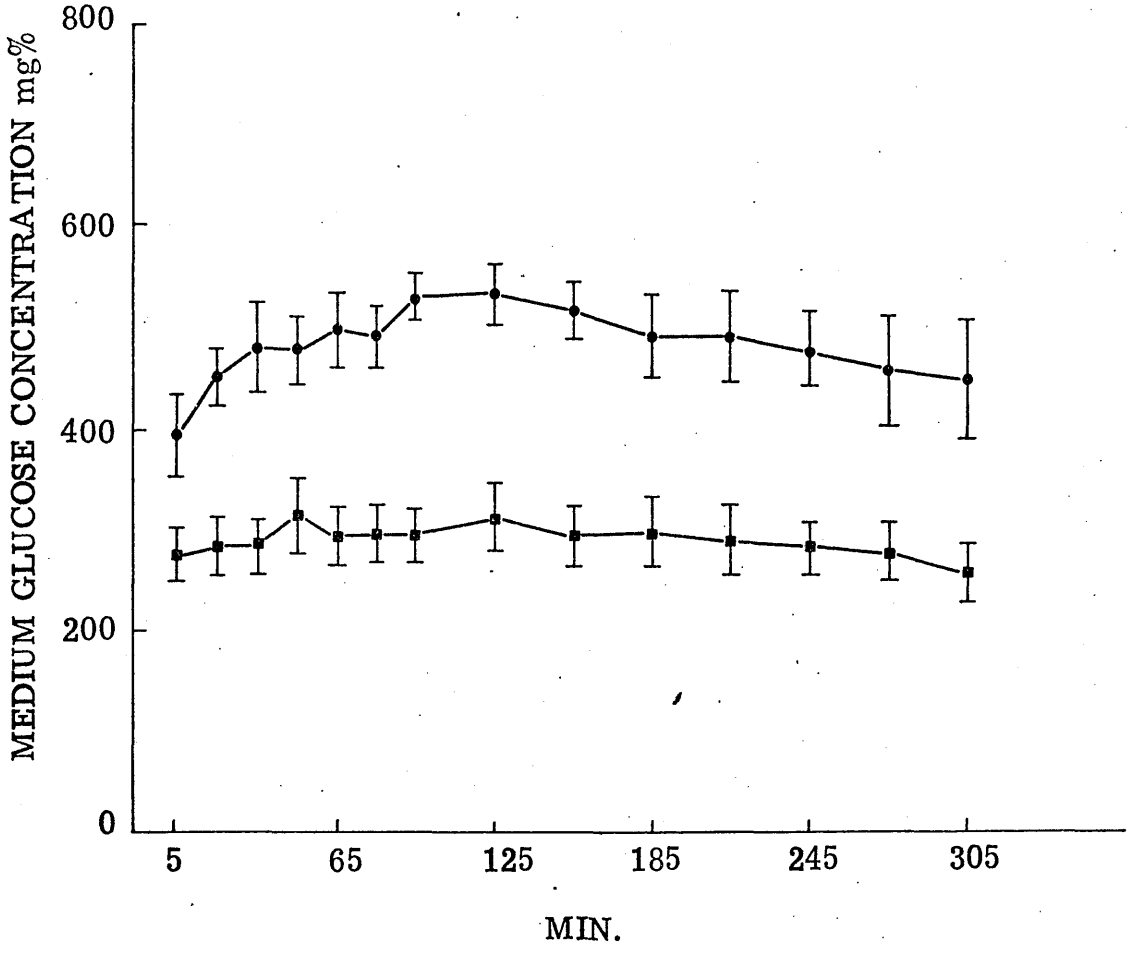


Figure 7

Perfusion medium glucose concentration during perfusion of the isolated rat liver. Measurements were started 5 min after transfer of the liver to the perfusion chamber. Values obtained with a liver in the circuit (●) and with no liver in the circuit (■) are means (± S.E.M.) of four perfusions.

haemolysis of 0.2%/h (Fig. 5). Haemolysis occurred at the same rate whether or not there was a liver in the circuit. Thus it would appear that almost all the rupture of red cells was caused by the physical characteristics of the perfusion system, probably by the roller pump. However in view of reported values of haemolysis in the perfused liver, varying between 0%/h (Exton and Park, 1967) and 2%/h (Craig, 1966) this low level of haemolysis was regarded as acceptable.

5.1.5 Accumulation of K^+ in the medium

With no liver in the circuit the concentration of K^+ in the perfusion medium plasma did not change appreciably over 6 h as shown in Fig. 6. In view of the low level of haemolysis reported above and the fact that non-glycolysing red cells were employed an increase in plasma K^+ would not be expected.

The inclusion of a liver in the circuit resulted in an increase in plasma K^+ levels, which was found to average 10 μmol/g/h. The increase in plasma K^+ was almost linear with time up to 6 h.

5.1.6 Changes in perfusion medium glucose levels

As shown in Fig. 7, in the absence of a liver in the circuit glucose levels in the medium were not altered significantly over 5 h of perfusion. Thus aged red cells do not appear to be capable of glycolysis, in agreement with the findings of Hems et al (1966). When rat liver was perfused it was found to shed large amounts of glucose during

the first 60 min after isolation. Blood glucose levels plateaued after 2 h and thereafter declined up to 5 h, the longest period over which measurements were made. This represents an average rate of glucose utilisation over the latter part of the perfusion of 22 μmol/g/h.

5.1.7 Perfusion medium pH and oxygen content

It was found that when the liver was perfused for periods of up to 6 h the pH of the perfusion medium remained between 7.3 and 7.4. When gassed with 95% air + 5% CO_2 the P_{O_2} of the perfusion medium was 187 mm Hg. The total oxygen content of the medium was 8.6 vol%.

5.1.8 Bacterial contamination of the medium

In several perfusions samples of medium were cultured for possible bacterial contamination. In all such tests no significant bacterial contamination was detectable for the 3 h after commencing perfusion. In one perfusion no bacteria were found for a further hour, the longest period over which sampling had been carried out for that experiment. However in two other perfusions a few bacteria were found after 3 h of perfusion, most being the species S. faecalis. It was concluded that since perfusions were to be carried out for a maximum of 4 h the inclusion of antibiotics in the medium to combat the slight contamination usually present at this time was unnecessary.

5.1.9 Perfusion medium ratio of lactate:pyruvate

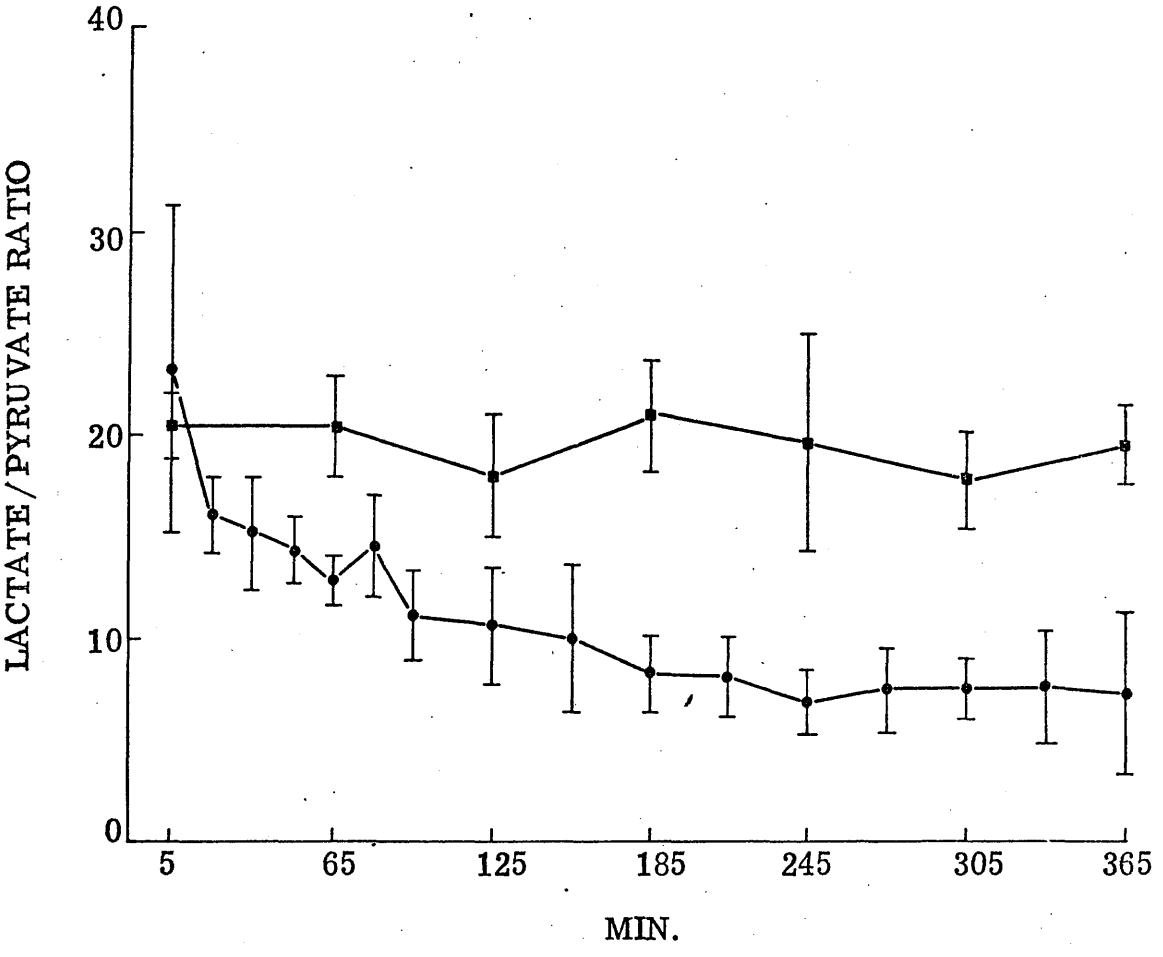


Figure 8

Lactate:Pyruvate ratio in the perfusion medium of the isolated perfused rat liver. Measurements were started 5 min after transfer of the liver to the perfusion chamber. Values obtained with a liver in the circuit (●) are mean (± S.E.M.) of four perfusions. Values obtained in the absence of a liver (■) are mean (± S.E.M.) of three perfusions.

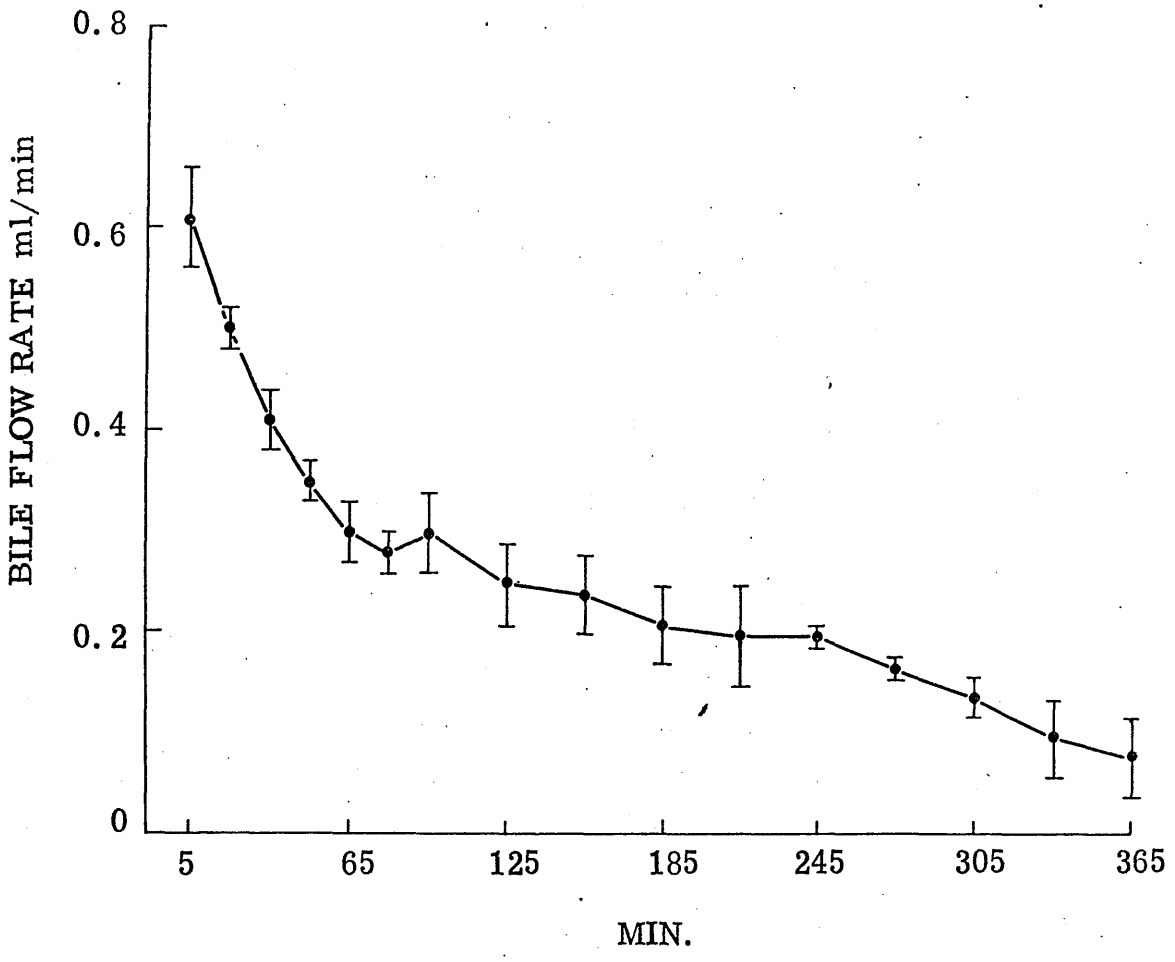


Figure 9

Rate of bile production by the isolated perfused rat liver. Measurements were started 5 min after transfer of the liver to the perfusion chamber. Values are mean (± S.E.M.) of four perfusions.

Electron micrographs of section of liver removed from deep central regions of the lobes of livers. Upper plate, liver of animal sacrificed just prior to fixation and lower plate, that had been perfused for 4 h. Nucleus, N; nuclear membrane, NM; glycogen, G; endoplasmic reticulum, ER; mitochondrion, M; bile cannuliculus, BC; cell junction, CJ. Magnification 20,000x.

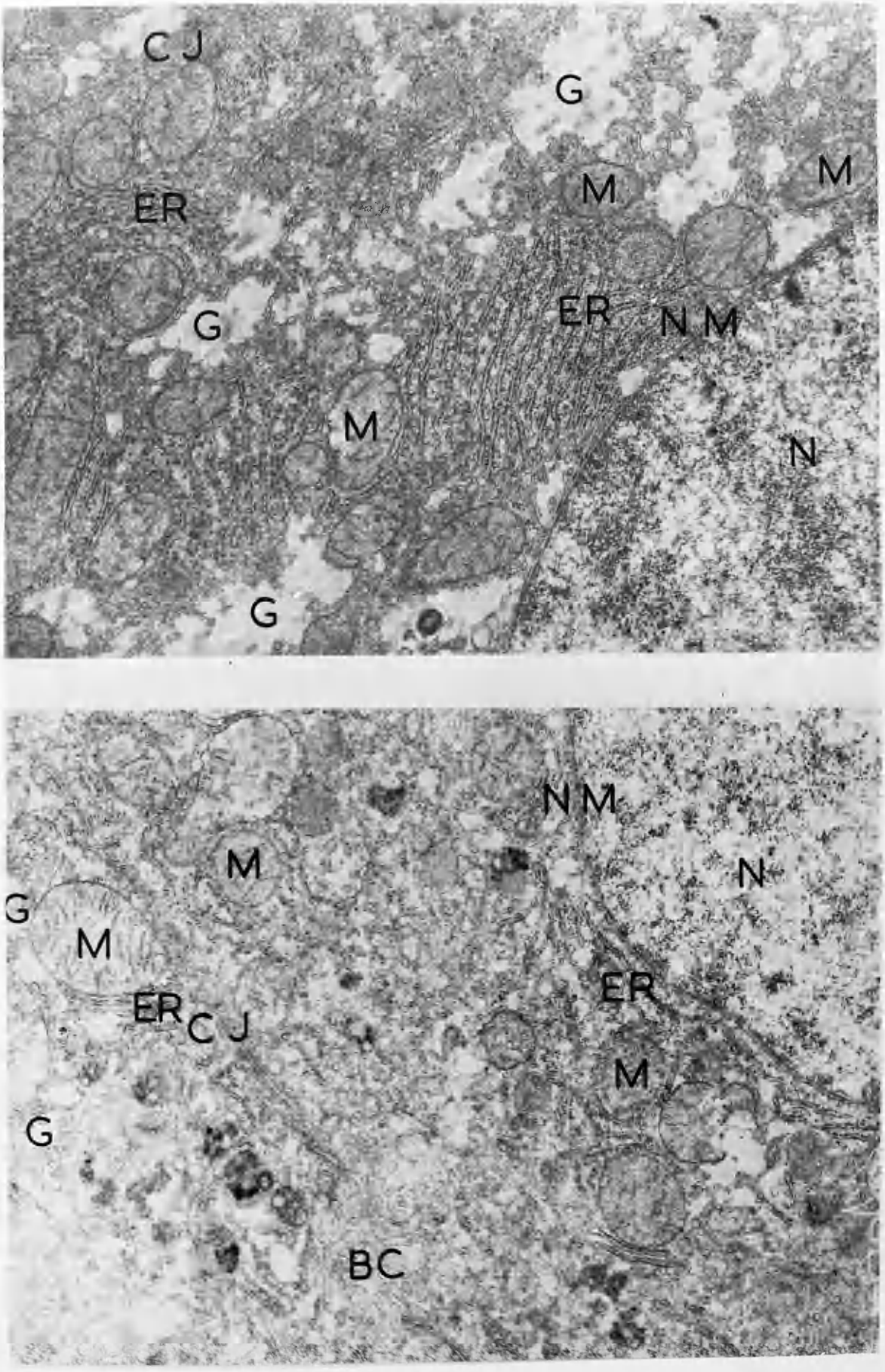


Figure 10

Electron micrographs of sections of liver removed from peripheral regions of the lobes of livers. Upper plate, liver of animal sacrificed just prior to fixation and lower plate, that had been perfused for 4 h. Nucleus N; nuclear membrane, NM: glycogen, G; endoplasmic reticulum, ER; mitochondrion, M: bile cannuliculus, BC; cell junction, CJ. Magnification 20,000x.

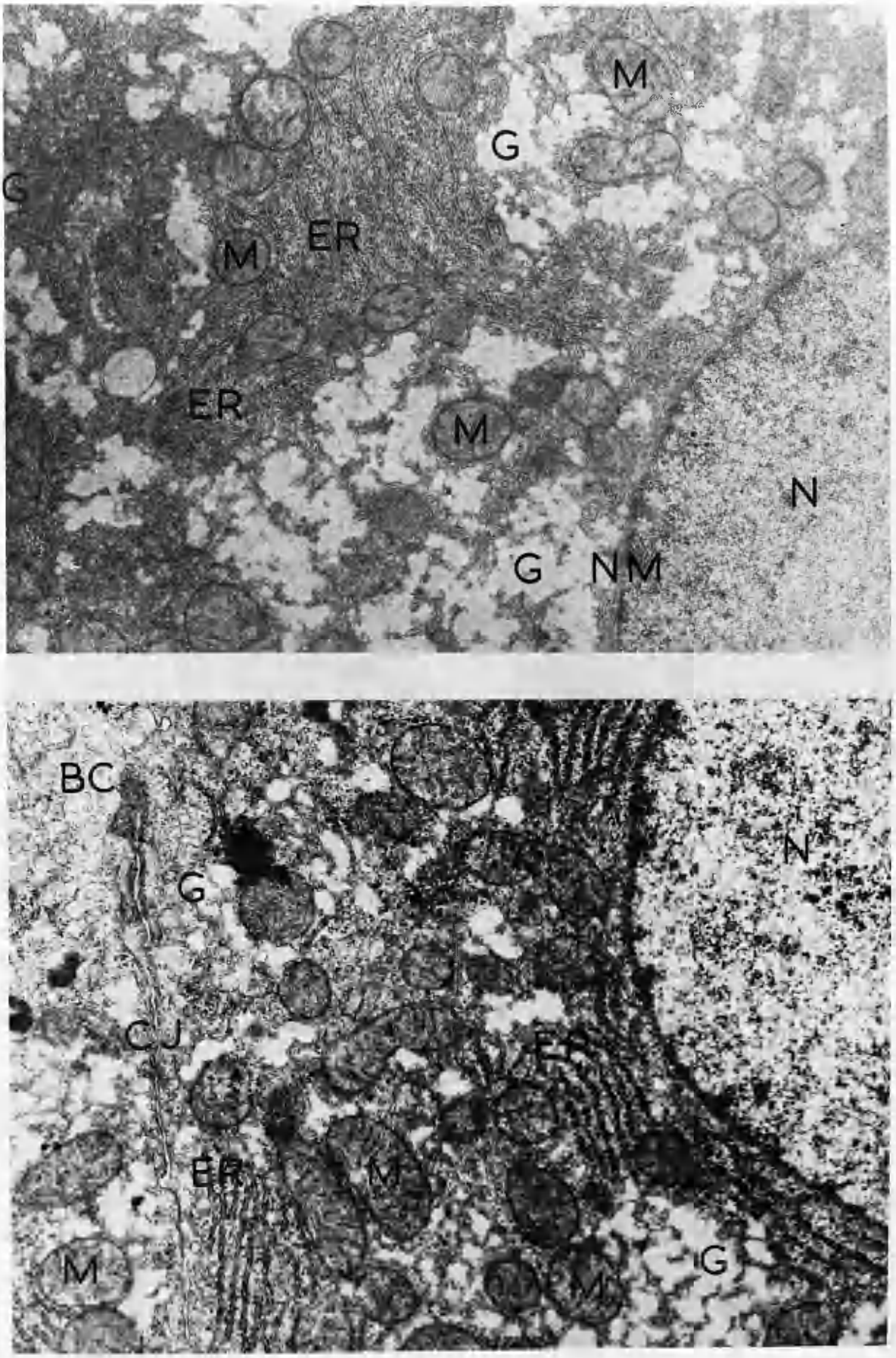


Figure 11

Fig. 8 shows that the perfusion medium contained both lactate and pyruvate, initially in the ratio 20:1. With no liver present this ratio remained unaltered over 6 h of perfusion. However when a liver was included in the system the ratio decreased to 13 within 60 min and was maintained between 8 and 12 for the remainder of the experiment. Thus the liver appears to act to reduce the lactate:pyruvate ratio from its initial high values and to maintain it at a value of around 10. This is the physiological ratio of these two substrates, which the liver maintains in the blood *in vivo* (Schimassek, 1963b).

5.1.10 Bile flow

After commencing perfusion, the bile flow rate was 0.7 ml/h. However, as may be seen from Fig. 9, the flow rate fell rapidly over the first 60 min and then plateaued between 0.2 and 0.3 ml/h over the next 4 h. After this the rate of bile flow again declined, and was less than 0.1 ml/h after 6 h of perfusion.

5.1.11 Electron microscopy of the perfused liver

Electron micrographs of liver from freshly killed rats and livers that were perfused for 4 h are shown in Figs. 10 and 11. Tissue was taken from both the centre and the periphery of the lobes. Comparison of the micrographs reveals no signs of necrosis or cell degeneration after perfusion. Characteristic signs of degeneration are disruption of the nuclear membrane, distortion of cell junctions, depletion of liver glycogen, excessive lysosomal activity and swelling of the

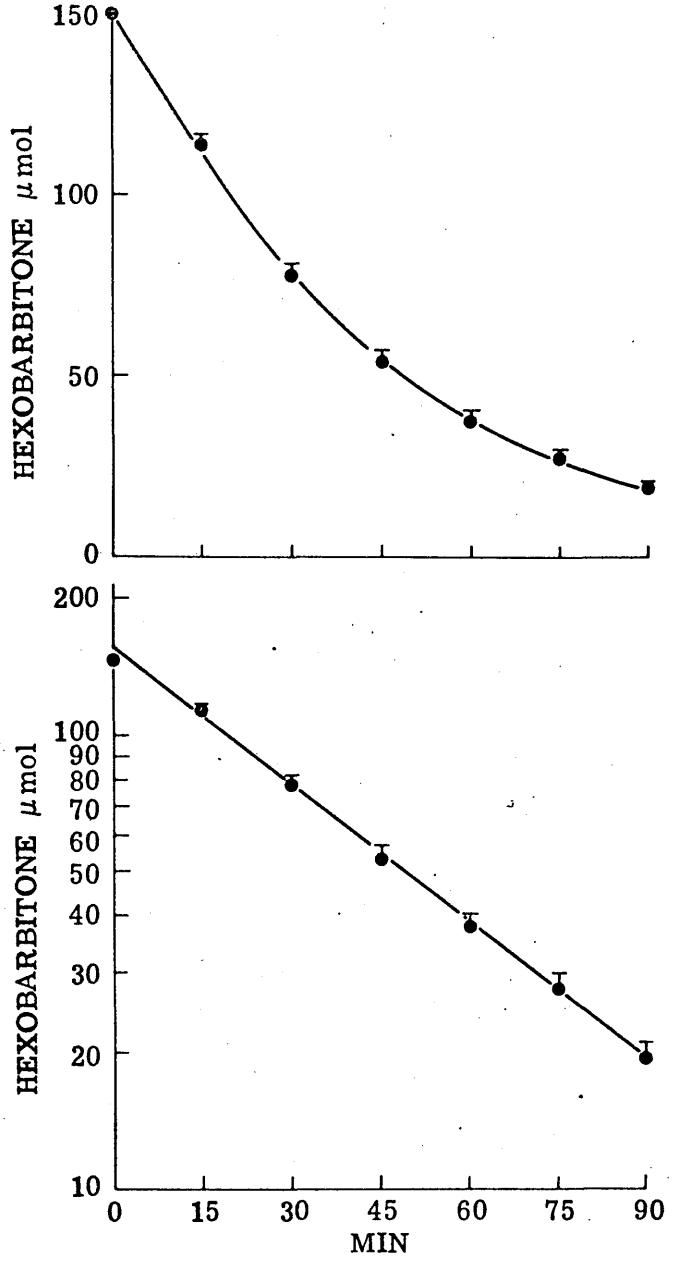


Figure 12

Hexobarbitone metabolism by the isolated perfused rat liver. Removal of hexobarbitone during the first pulse of control perfusions. The upper panel shows the amount of hexobarbitone remaining in the perfusion medium, and the lower panel the same values plotted logarithmically. Values are mean (± S.E.M.) of twelve perfusions.

TABLE 2

Effect of catecholamines on the half-life of hexobarbitone in the perfused liver

Hexobarbitone, 150 μmol, was added to the perfusion medium at the start of the first and second pulses. Catecholamines were added 5 min prior to the second pulse of hexobarbitone. - No drug added. Values are mean ± S.E.M.

*P < 0.05 **P < 0.01 compared with the corresponding first or second pulse of the control.

		Half-life, min		
	n	First Pulse		Second Pulse
Control				
-	5	29.7 ± 2.0	-	39.6 ± 4.3
Drugs				
-	4	32.6 ± 1.6	Adrenaline, 6μM	87.1 ± 8.0**
-	4	30.4 ± 2.3	Noradrenaline, 6μM	72.4 ± 7.8**
Papaverine, 80μM	3	82.5 ± 36.8*	Papaverine, 80μM	152.1 ± 51.9*
0 Ca^{2+}	3	37.4 ± 2.4*	0 Ca^{2+}, Adrenaline, 6μM	55.2 ± 11.0

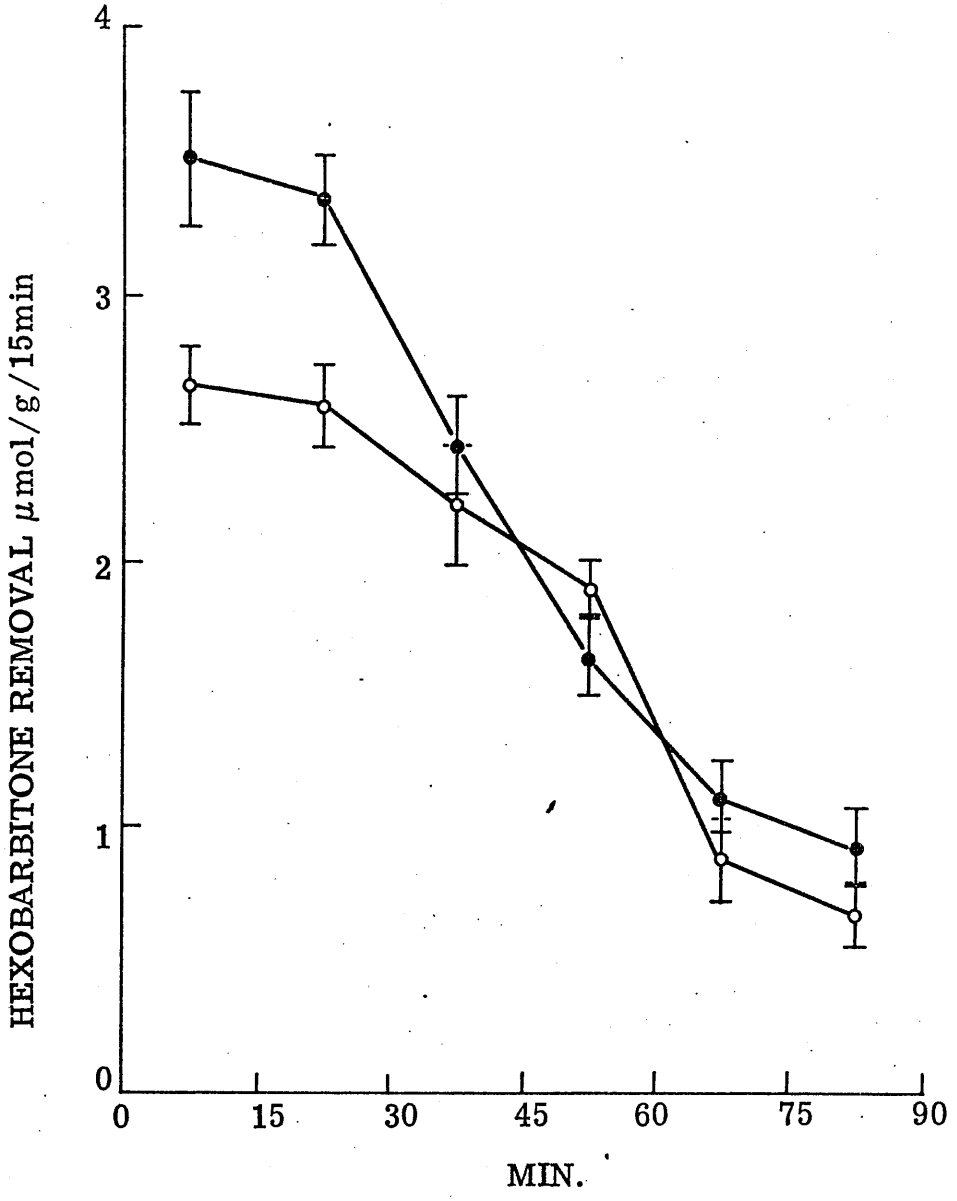


Figure 13

The rate of hexobarbitone metabolism by the isolated perfused rat liver. Values are mean (± S.E.M.) of four perfusions for the first (●) and second (o) pulses.

endoplasmic reticulum (Abraham et al, 1968; Bock et al, 1972). One of the earliest signs of inadequate perfusion or hypoxia is swelling of the mitochondria (Schimassek, 1962a; Bock et al, 1972). However no such swelling was visible after 4 h of perfusion.

5.2 Metabolism of drugs by the perfused liver

5.2.1 The metabolism of hexobarbitone

Hexobarbitone was removed by the liver from the perfusion medium and the amount fell exponentially after the first dose of substrate (Fig. 12). Plotting these results logarithmically reveals that the reaction is first order. The removal of hexobarbitone during the second pulse of substrate in control perfusions was also first order. The half-life of hexobarbitone during the first pulse was 29.7 min (Table 2) and during the second pulse it was increased significantly by 32% ($P < 0.05$).

The rate of hexobarbitone removal was initially 3.51 ± 0.25 µmol/g/15 min and 2.66 ± 0.14 µmol/g/15 min during the first and second pulse respectively, a reduction of 24% during the second pulse. This may be due to depletion of some important co-factor from the liver. In both cases the rates decreased almost linearly with time as the substrate concentration decreased, in accordance with first order kinetics (Fig. 13).

Although hexobarbitone could be detected in the bile

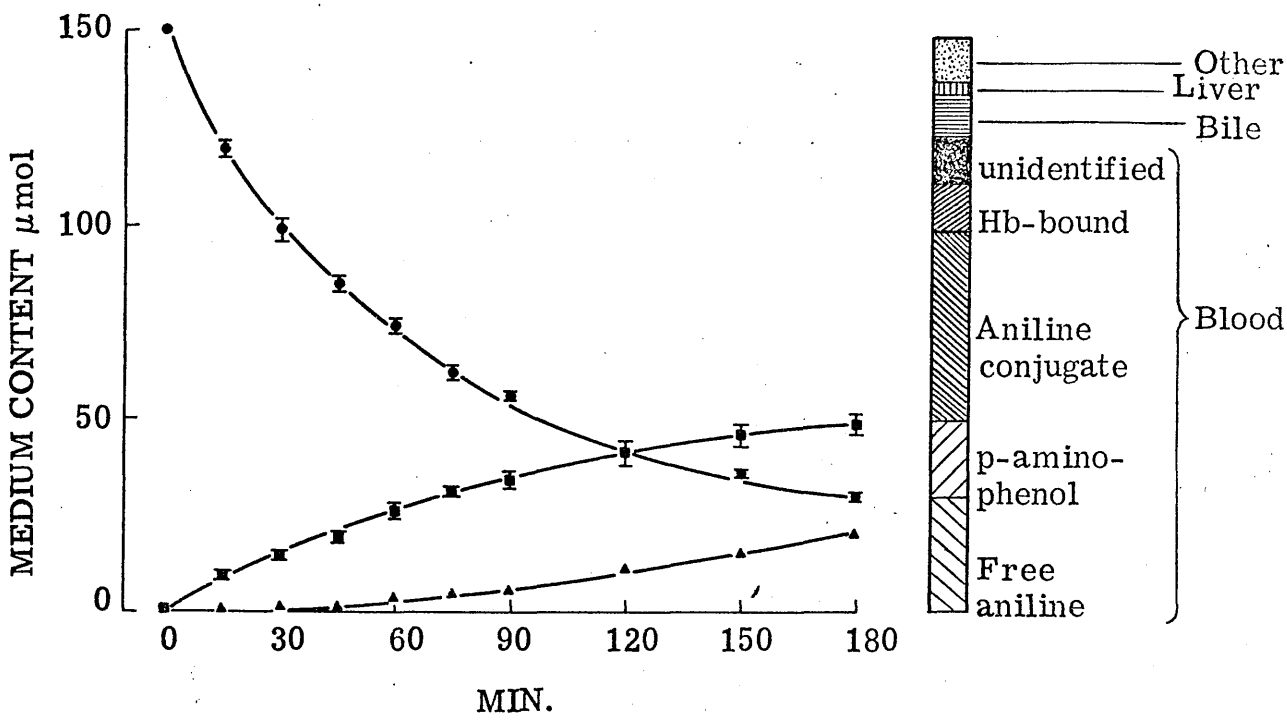


Figure 14

Aniline metabolism by the isolated perfused rat liver. Amount of free aniline (●), acid-labile aniline conjugate (■) and p-aminophenol conjugate (▲) formed during perfusion of the isolated rat liver. Values are mean (± S.E.M.) of four perfusions. The histogram shows the distribution of label amongst aniline and its metabolites 3 h after the addition of 150 µmol of 3H-aniline (two experiments).

after 3 h of perfusion it accounted for only 0.40 ± 0.03% (n = 5) of the substrate added to the medium.

5.2.2 The metabolism of aniline

Fig. 14 shows that the amount of aniline in the medium in control perfusions followed an exponential decline. Also shown in this figure is the accumulation of a p-aminophenol conjugate, which did not appear in the medium in significant quantities until 60 min after the addition of aniline. An acid-labile conjugate of aniline was found from the start of the experiment and its formation levelled off after 150 min.

In perfusions with no liver in the circuit there was almost no change in the amount of aniline in the medium so that aniline was not being lost from the circuit, for example by adsorption. It was very quickly found that no free p-aminophenol could be detected in the medium, even after 4 h of perfusion. This was surprising since the major normal product of aniline metabolism *in vivo* is p-aminophenol (Parke, 1960). Acid hydrolysis of the protein-free medium supernatant was found to liberate p-aminophenol, presumably from some conjugated form. These findings led to a more detailed investigation into the measurement and detection of p-aminophenol. The outcome of the study was the finding that some component of the red cells prevented p-aminophenol from being measured at low concentrations. At high concentrations there was a reduction in the amount of p-aminophenol that could be assayed in a suspension of red cells when compared with the assayable concentration in a solution of bovine serum albumin.

TABLE 3

Aniline and its metabolites accumulated in bile by the perfused liver

Values are the amount of aniline and its metabolites accumulated in the bile after 3 h perfusion. Initial amount of aniline added was 150 µmol. Figures are mean ± S.E.M. of four perfusions.

	µmol	%total
Free aniline	1.92 ± 0.25	20.49 ± 3.28
Labile aniline conjugate	5.38 ± 1.36	47.25 ± 5.05
Other aniline conjugates	2.68 ± 0.40	26.46 ± 4.38
Free p-aminophenol	0.03 ± 0.01	0.31 ± 0.23
p-aminophenol conjugates	0.56 ± 0.09	5.49 ± 1.38
Total	10.57 ± 1.78	100

It was finally concluded that haemoglobin could interact with p-aminophenol to form a complex that was not detected by the methods used in this study.

Most of the aniline removed from the perfusion medium could be detected as an acid-labile complex, probably aniline-N-glucuronide. This acid-labile conjugate was not formed in the perfusion medium when there was no liver present.

The accumulation of aniline and its metabolites in bile is shown in Table 3. As in the perfusion medium most of the aniline was recovered as the acid-labile conjugate. A trace of free p-aminophenol was also detectable, supporting the hypothesis that some free p-aminophenol was being formed but which, in the blood, interacted with haemoglobin.

In two experiments aniline was replaced by 3H-aniline so that the distribution of label could be measured. The results are shown in the histogram in Fig. 14 which was obtained after 3 h of perfusion in the presence of labelled aniline. After this time 30 μmol of aniline remained unmetabolised, 49 μmol of the acid-labile conjugate of aniline had been formed and only 19 μmol of p-aminophenol conjugate. A further 12 μmol of label was bound to haemoglobin, probably from p-aminophenol as discussed previously. There was still 12 μmol of label unidentified in the blood, possibly as other more stable conjugates or metabolites of aniline. In total 122 μmol of label was recovered from the medium. A further 3.5 μmol was in the homogenised liver, which had been flushed clear of

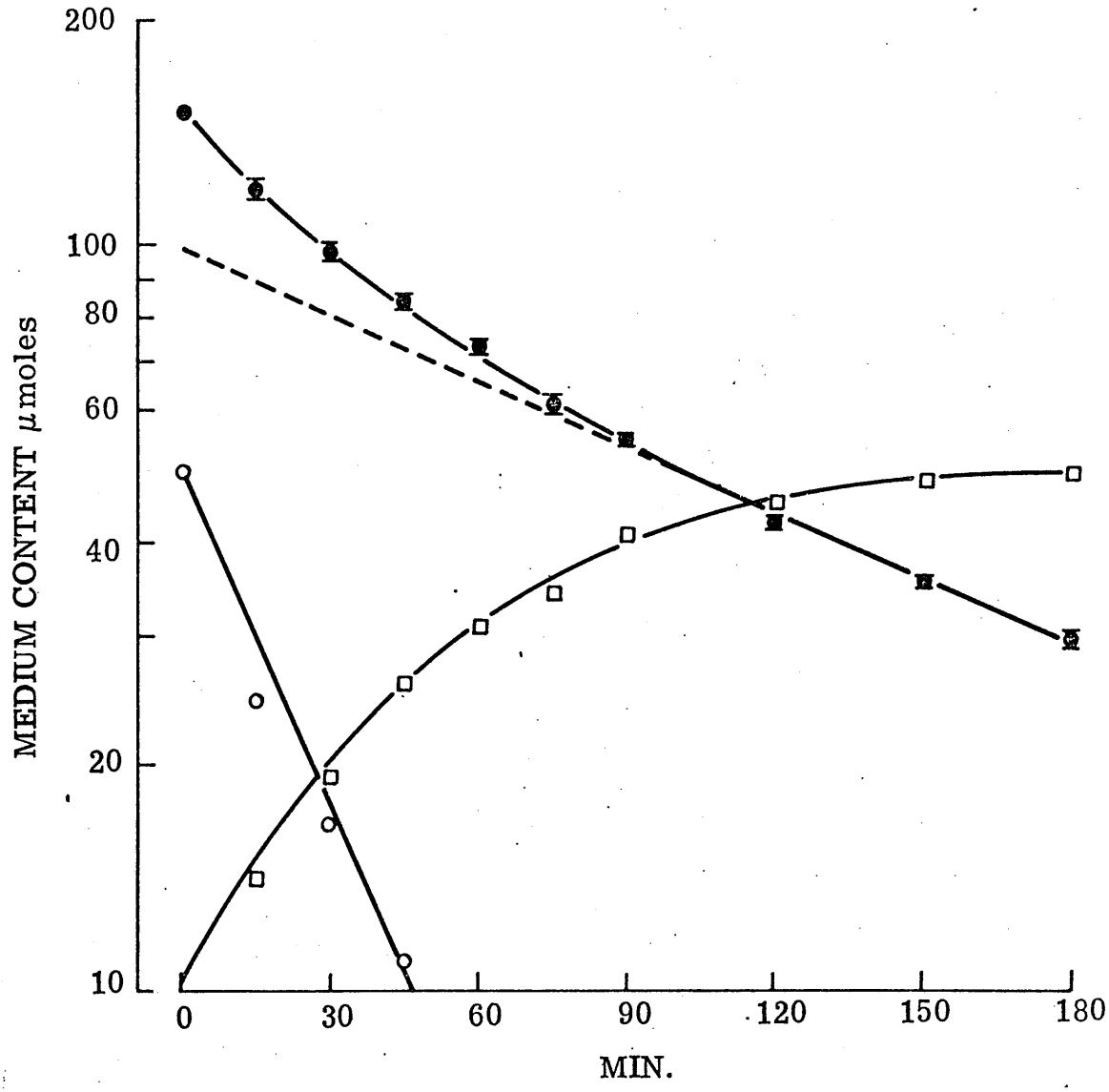


Figure 15

Aniline metabolism by the isolated perfused rat liver. Results are plotted semi-logarithmically. Free aniline (●). The residual curve (o) is obtained after stripping the second exponential, shown by the broken line, from the combined curve as described in Appendix I. Acid-labile aniline conjugate (□). Values are mean (± S.E.M.) of four perfusions.

TABLE 4

Half-life of aniline in the perfused liver

Aniline was added in an initial amount of 150 μmol, and drugs at the concentrations shown, added as described in the text. The half-lives of aniline were calculated by curve stripping as described in Appendix I. Subscript 2 refers to the slow phase of removal and subscript 1 to the faster phase. Figures are mean ± S.E.M. of at least three determinations.

*P < 0.05 **P < 0.01 compared to control perfusions.

	$Half\text{-}life^{2}$ min	$Half\text{-}life^{1}$ min
Control	108.7 ± 3.0	23.4 ± 3.1
Noradrenaline, 6μM	100.5 ± 5.8	20.3 ± 1.9
Adrenaline, 6μM	101.6 ± 6.1	20.6 ± 3.9
Papaverine, 80μM adrenaline, 6μM	122.1 ± 8.7	11.3 ± 0.8**
SKF 525-A, 200μM	383.8 ± 120.2**	19.9 ± 5.3

perfusate. A significant proportion of the label was excreted into the bile, 11 μmol, and a further 12 μmol could be recovered from other sources, 8 μmol surprisingly from the liquid paraffin in the perfusion chamber. When the polyethylene and silicone rubber of the circuit were extracted at 37°C for 5 days with Soluene a total of 1 μmol of label could be recovered, so that neither aniline nor its metabolites were adsorbed on to the surface of the material used in the perfusion system to any extent. The rest of the label, 4 μmol, was detected in the pipettes and glassware used in the collection of perfusion medium samples. A total of 149.5 μmol of the label could be accounted for.

When the aniline remaining in the perfusion medium was plotted semi-logarithmically as in Fig. 15 it was found that its removal was biphasic. The initial phase was obtained after curve stripping of the combined plot as described in Appendix I. The formation of acid-labile conjugate of aniline was non-linear on the semi-log plot. In the initial phase aniline had a half-life of 23.4 ± 3.1 min (Table 4). This phase of removal was almost complete after the first 60 min. In the second phase of removal aniline had a half-life of 108.7 ± 3.0 min. This was a continuous process throughout the experiment but during the initial 60 min it was masked by the faster first phase of removal. SKF 525-A 200 μM, an inhibitor of drug metabolism (Cooper et al, 1954), increased the half-life of aniline during the second phase to 383.8 ± 12.2 min ($P < 0.001$) (Table 4). However it did not affect the half-life of aniline during the first phase. The first phase of aniline removal may thus be a non-metabolic process and thus catecholamines were not added in these perfusions

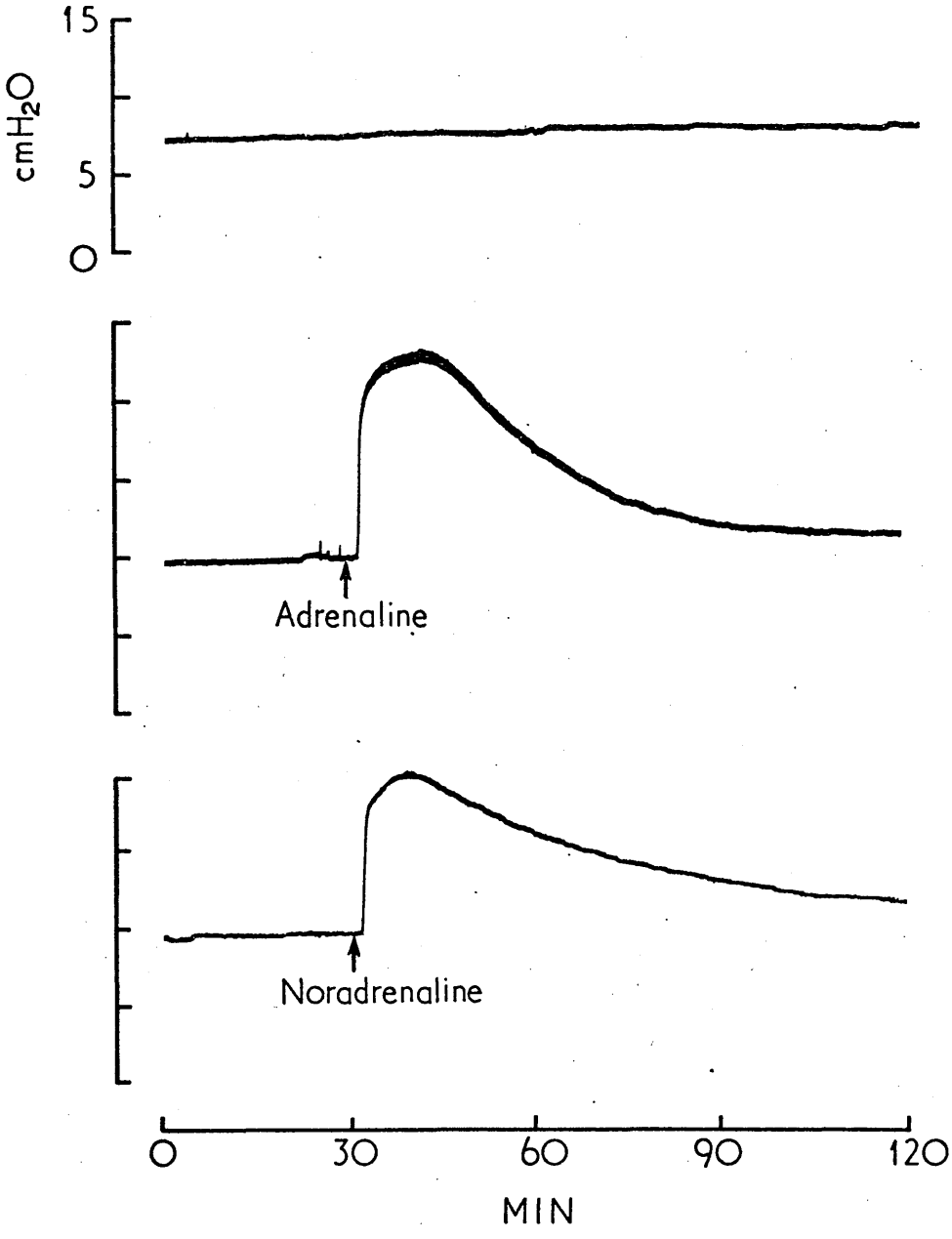


Figure 16

Effect of catecholamines on the portal pressure of the isolated perfused rat liver. The traces shown represent typical results. Adrenaline or noradrenaline, 6 μM, was added at the point indicated (↑).

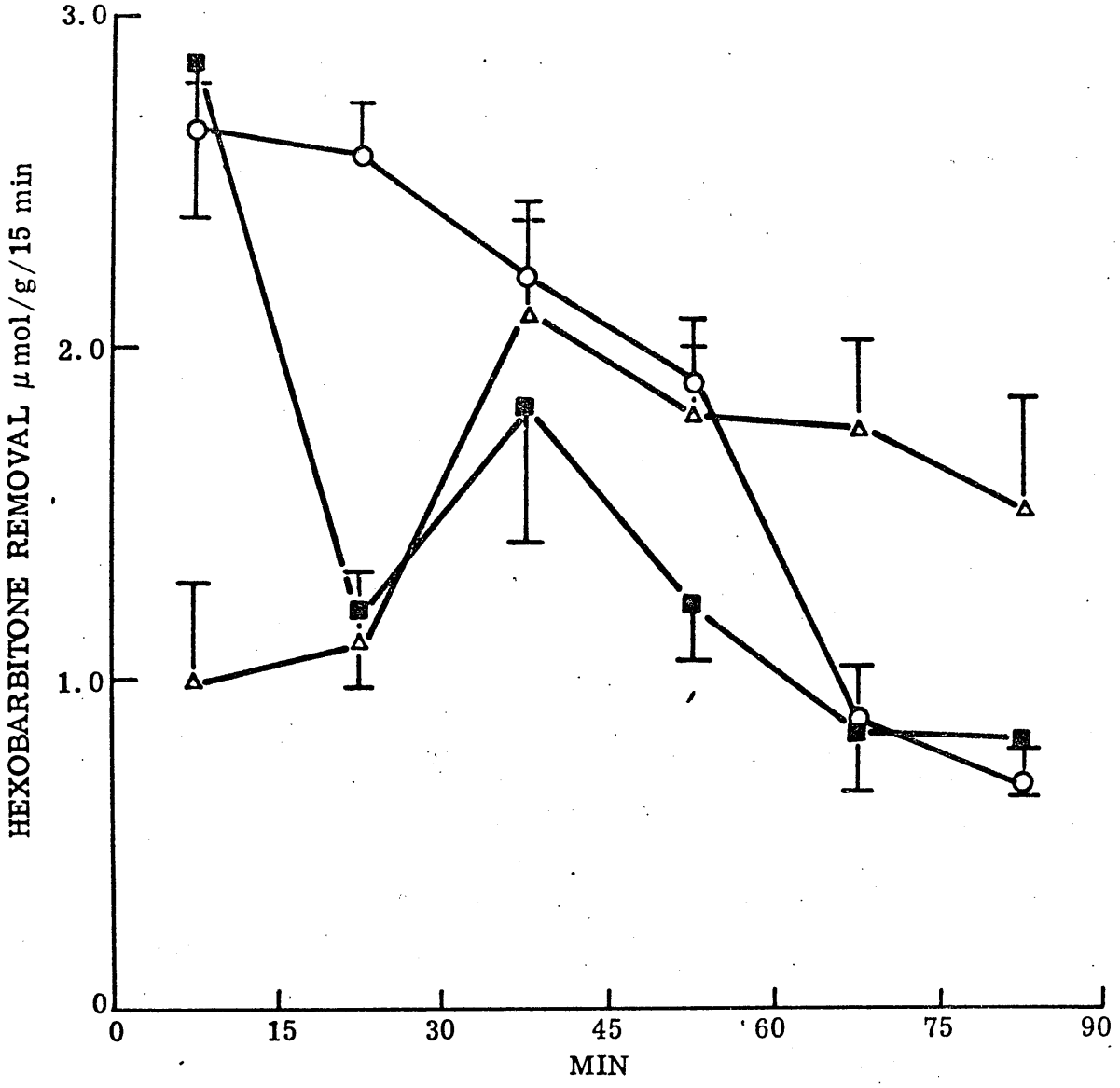


Figure 17

Effect of catecholamines on hexobarbitone metabolism by the isolated perfused rat liver. Adrenaline, 6 μM (Δ) or noradrenaline, 6 μM (■) was added at 0 min. Control perfusions (o). All the results are of second pulses. Values are mean (± S.E.M.) of four perfusions in each case.

until 60 min after the addition of substrate so that their effects upon the metabolic component of aniline removal could be more clearly studied.

5.3 Effect of catecholamines in the perfused liver

5.3.1 Effect on perfusion pressure

In control perfusions the portal pressure remained relatively constant for the duration of the experiment with a value of 10 ± 2 cm H_2O. Fig. 16 shows that the addition of either adrenaline or noradrenaline to the perfusion medium at an initial concentration of 6 μM, which was supramaximal, caused a rapid rise, within 2 min, in the perfusion pressure of 13.7 ± 1.2 cm H_2O (n = 3) and 11.4 ± 0.4 cm H_2O (n = 3) respectively. The pressure then fell towards control levels, the half-life of the fall after adrenaline being 16.1 ± 1.2 min and with noradrenaline 23.5 ± 5.2 min; these values not differing from each other significantly at the 5% level. This fall in pressure is probably a reflection of the rapid metabolism of catecholamines known to occur in the perfused liver (Lightman and Hems, 1973).

5.3.2 Effect on hexobarbitone metabolism

The effects of adrenaline and noradrenaline, at an initial concentration of 6 μM, on the rate of hexobarbitone metabolism by the perfused liver are shown in Fig. 17. For clarity only the second pulse, in the presence of catecholamine, is shown and is compared with the second pulse from control perfusions.

TABLE 5

Effect of catecholamines on excretion of hexobarbitone into bile

Hexobarbitone accumulation was measured in the bile of livers perfused for 3 h, after two additions of 150 µmol hexobarbitone at 0 min and 90 min respectively. Catecholamines were added at 85 min, initial concentration 6µM. Figures are mean ± S.E.M. of three perfusions.

*P < 0.05 compared to control perfusions.

	Hexobarbitone µmol	
	Total	Per g liver
Control	1.187 ± 0.115	0.100 ± 0.014
Adrenaline 6µM	1.473 ± 0.082	0.167 ± 0.032
Noradrenaline 6µM	1.085 ± 0.053	0.110 ± 0.010

Both catecholamines inhibited hexobarbitone metabolism within the first 30 min, by a maximum of 62% ($P < 0.005$) with adrenaline and 54% ($P < 0.005$) with noradrenaline. Inhibition with adrenaline was apparent within 15 min. Since adrenaline markedly inhibited the metabolism of hexobarbitone over the first 30 min there was a higher concentration of hexobarbitone remaining after 60 min in these perfusions compared with the concentration in corresponding control perfusions. The rate of metabolism after 60 min when the effects of adrenaline had worn off was thus higher than in the control perfusions.

The half-life of hexobarbitone in the perfusion medium was calculated between 0 min and 90 min. In perfusions in which catecholamines were added this method of calculating half-lives will result in a slight underestimate of the true effect of catecholamines because it includes a period when the effect of the catecholamines had worn off. In control perfusions the half-life of hexobarbitone was 39.6 ± 4.3 min (Table 2). Adrenaline increased this by 167% ($P < 0.005$) and noradrenaline increased the half-life by 138% ($P < 0.005$). However the half-life of hexobarbitone in the presence of adrenaline was not significantly different from that in the presence of noradrenaline.

Neither adrenaline nor noradrenaline had any significant effect upon the amount of hexobarbitone accumulated in the bile although adrenaline did produce a 67% increase in the amount removed in this way (Table 5).

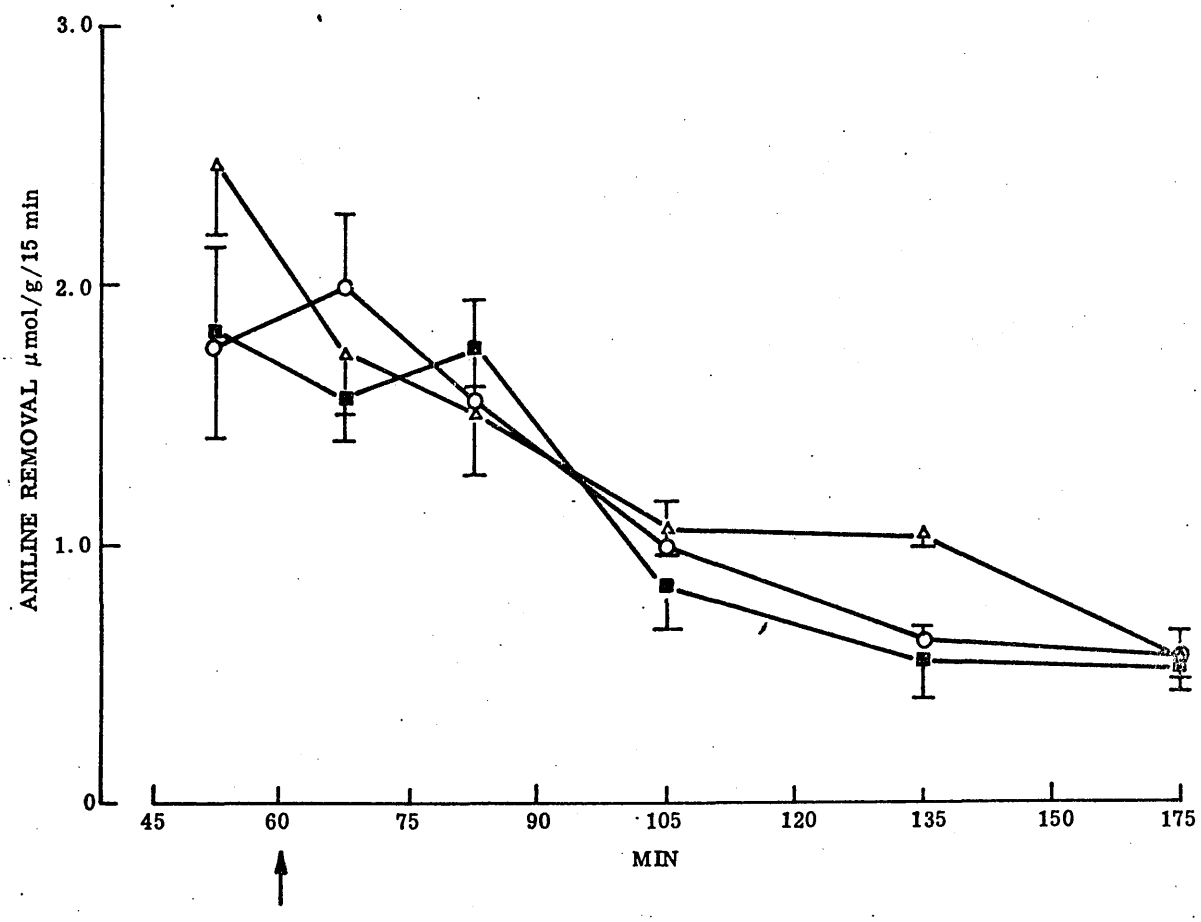


Figure 18

Effect of catecholamines on aniline metabolism by the isolated perfused rat liver. Adrenaline, 6 µM (Δ) or noradrenaline, 6 µM (■) was added 60 min after the addition of aniline as indicated (↑). Control perfusions (o). Values are mean (±S.E.M.) of four perfusions.

5.3.3 Effect on aniline metabolism

Neither adrenaline nor noradrenaline, at initial concentrations of 6 μM, caused any significant alteration in the rate of aniline metabolism in the 60 min following their addition (Fig. 18). At no time did the rate of aniline metabolism change significantly from the control in the presence of noradrenaline. However with adrenaline there was a 62% ($P < 0.01$) increase in the rate of aniline metabolism at one point 75 min after the addition of the catecholamine. Catecholamines did not affect the rate of formation of aniline metabolites or their accumulation in the bile (results not shown).

In control perfusions the half-life of removal during the second phase was 108.7 ± 3.0 min ($n = 6$) and in the presence of noradrenaline or adrenaline it was decreased only slightly (Table 4). With both catecholamines the probability of the results not differing significantly from the control was greater than 20%.

5.3.4 Effect of papaverine on drug metabolism

Papaverine is a potent inhibitor of phosphodiesterase (Goren and Rosen, 1972) and it should potentiate the effects of any cyclic AMP liberated by catecholamines. It should thus be possible to determine if the effects of catecholamines on hexobarbitone metabolism in the perfused liver were due to cyclic AMP, which itself has been reported to inhibit

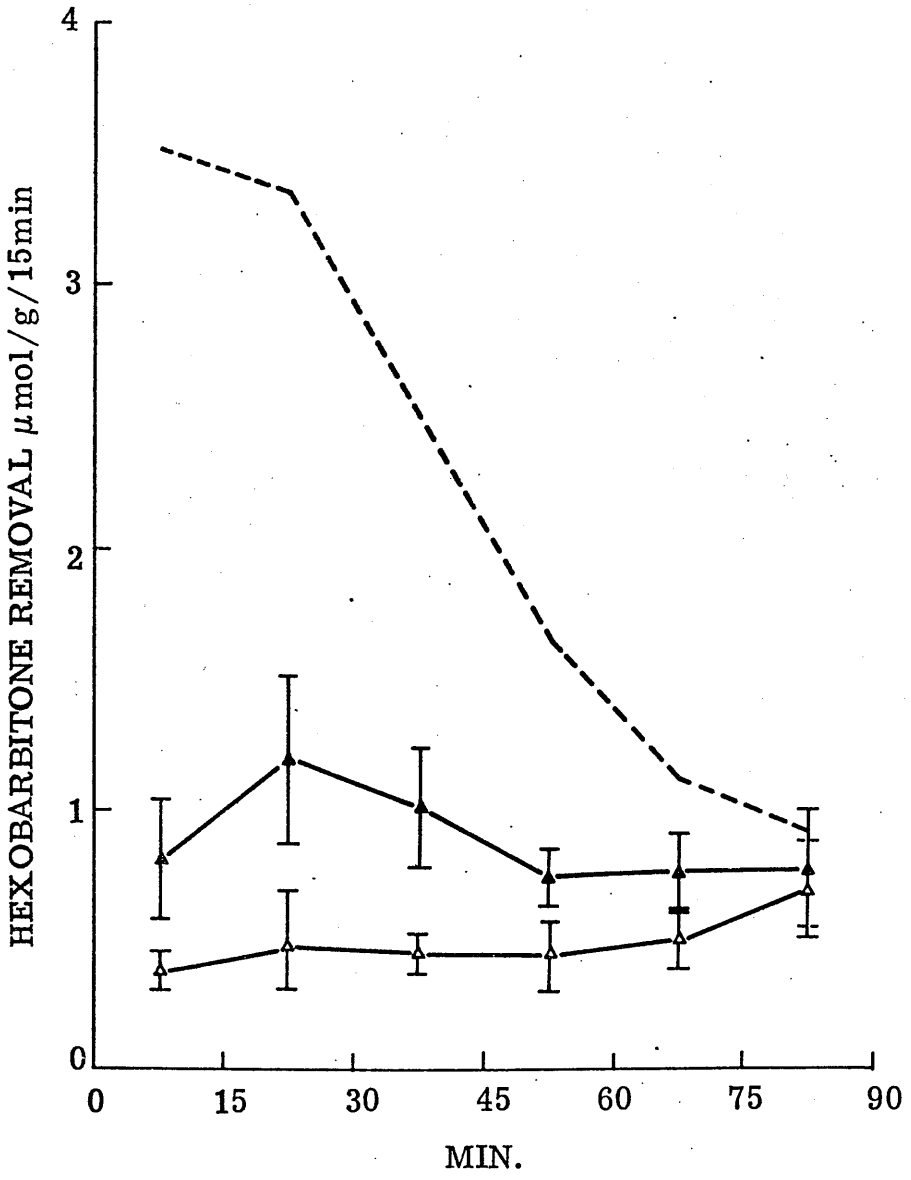


Figure 19

Effect of adrenaline in the presence of papaverine on hexobarbitone metabolism by the isolated perfused rat liver. During the first pulse papaverine, 80 μM (▲) and during the second pulse papaverine, 80 μM and adrenaline, 6 μM (Δ) were added at 0 min. Values are mean (± S.E.M.) of three perfusions. The broken line is the mean of the first pulse of 11 control perfusions and is included for comparison.

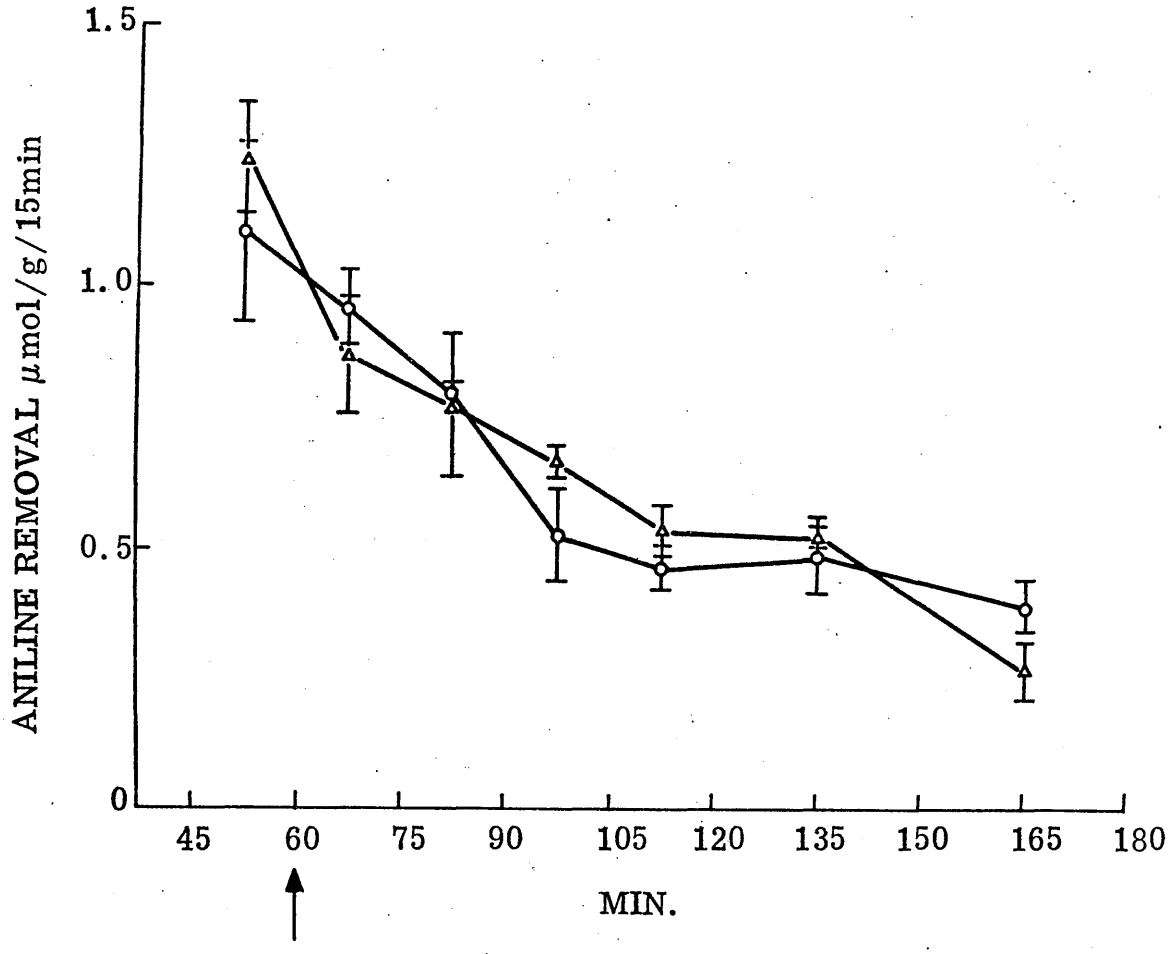


Figure 20

Effect of adrenaline in the presence of papaverine on aniline metabolism by the isolated perfused rat liver. Adrenaline, 6 μM (Δ) or adrenaline, 6 μM and papaverine 80 μM (o) were added to the perfusion medium, adrenaline 60 min after the addition of aniline as indicated, and papaverine at the same time as the addition of aniline. Values are mean (± S.E.M.) of three experiments.

hexobarbitone metabolism in the perfused liver (Weiner et al, 1972a).

During the first pulse papaverine itself, at an initial concentration of 80 μM, inhibited the rate of hexobarbitone metabolism (Fig. 19), maximally at 15 min by 77 ± 3%. Overall the half-life of hexobarbitone was increased by 178% ($P < 0.05$) (Table 2). Adrenaline at an initial concentration of 6 μM, during the second pulse inhibited hexobarbitone metabolism in the presence of papaverine even further. At 30 min the rate of hexobarbitone metabolism was reduced by 61% compared with the rate during the first pulse in the presence of papaverine alone. This was a similar magnitude of inhibition as that observed when adrenaline was added alone compared with the appropriate control. The half-life of hexobarbitone was also increased by adrenaline to a similar extent as previously (Table 2). Thus papaverine appeared not to potentiate the inhibitory effects of adrenaline upon hexobarbitone removal.

In the presence of papaverine, 80 μM, adrenaline at an initial concentration of 6 μM did not cause any significant change in the rate of aniline removal as compared to the rate of removal in the presence of adrenaline alone as shown in Fig. 20 or as compared to the control perfusions.

5.3.5 Effect of vasoactive compounds in the perfused liver

In order to investigate whether the effects of catecholamines on hexobarbitone metabolism in the perfused liver were due to an effect on blood flow, rather than a direct metabolic

effect, several compounds were studied with a view to finding one that would produce a similar pressor response as catecholamines, but by a non-adrenergic mechanism. In this way it was hoped to produce vasoconstriction in the perfused liver by a compound lacking the metabolic effects of catecholamines.

Neither oxytocin nor vasopressin, added directly to the reservoir to give initial concentrations of up to 0.14 U/ml, had any effect upon the perfusion pressure. The addition of $BaCl_2$, to give initial concentrations from 0.004 μM to 27.3 μM, produced, at most, a maximum increase in the perfusion pressure of 0.5 cm H_2O and even infusion of $BaCl_2$ directly into the portal vein, at a rate of 0.18 μmol/min, increased the portal pressure by only 0.2 cm H_2O. Thus vasopressin, oxytocin and Ba^{2+} were unsuitable.

Infusion of KCl into the portal vein, at 44 μmol/min, caused an increase of the portal pressure of only 0.5 cm H_2O. When KCl was added to the reservoir, to produce an initial concentration of 5,000 μM, the portal pressure increased by 10 cm H_2O and returned to 50% above the control value within 10 min. In view of the high concentration of K^+ necessary to produce this response, because of its short duration of action and since catecholamines are known to cause alterations in hepatic cells by the release of K^+ (Haylett and Jenkinson, 1972) it was decided that KCl was unsuitable.

The addition of angiotensin II to the reservoir, to give

an initial concentration of 1.9 μM, caused an increase in portal pressure of 11.0 cm H_2O, although this increase in pressure was very short-lived. However infusions of angiotensin directly into the portal vein at rates as low as 0.007 μmol/ml caused increases in perfusion pressure of 3.5 cm H_2O. As the rate of infusion increased the pressure increased proportionately, to an increase of 9.0 cm H_2O at an infusion rate of 0.031 μmol/min. However even at these higher rates of angiotensin infusion, at which the magnitude of the pressor response was similar to that obtained with catecholamines, the increase was not maintained for more than 5 min and even when the rate of infusion was increased to 0.122 μmol/min the increase in portal pressure could not be maintained. When infusion was stopped for 10 min and then recommenced, the perfusion pressure was immediately increased by 11.0 cm H_2O, but again this was not maintained. Thus angiotensin was not suitable for providing a maintained pressor response in place of catecholamines in the perfused liver.

This approach was then abandoned in favour of finding a compound that would inhibit the pressor effects of catecholamines while not affecting their metabolic effects.

5.3.6 Inhibition of the pressor effects of catecholamines in the perfused liver

It was not possible to use either α- or β-adrenergic blockers since these compounds have been shown to inhibit drug metabolism themselves (Mullen and Fouts, 1965).

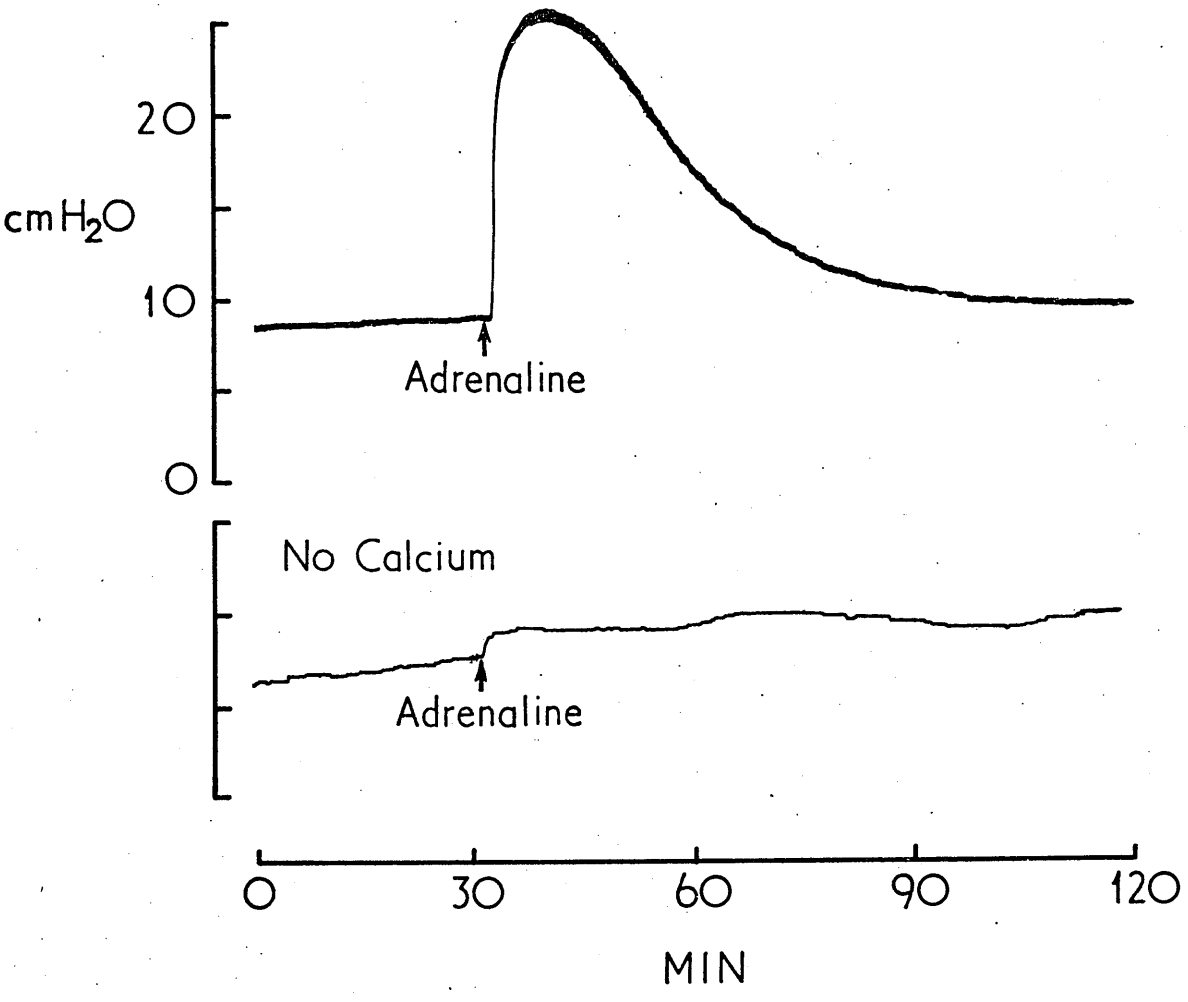


Figure 21

Effect of adrenaline on portal pressure in the absence of Ca^{2+} in the isolated perfused rat liver. Traces are typical results. Adrenaline, 6 μM was added at the time indicated (↑). The upper panel shows the effect of adrenaline in the presence of Ca^{2+}, 1.25 mM and the lower panel the effect of adrenaline when the perfusion medium was prepared without Ca^{2+}.

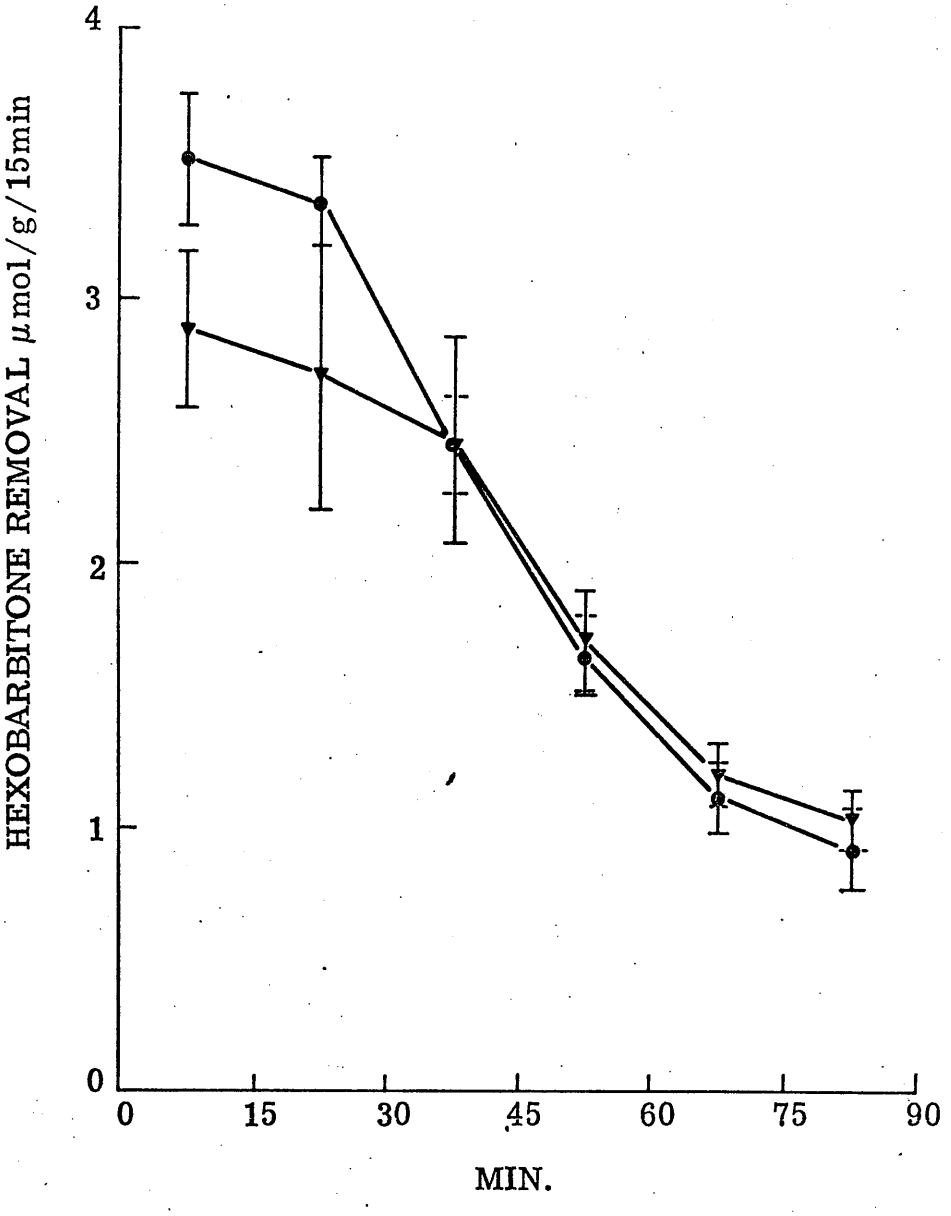


Figure 22

Effect of an absence of Ca^{2+} on hexobarbitone metabolism by the isolated perfused rat liver. First pulse of perfusions with medium containing normal calcium, 1.25 mM (●) and with no calcium (▼). Values are mean (± S.E.M.) of four perfusions.

Papaverine, as well as an inhibitor of phosphodiesterase, is a smooth muscle relaxant (Daniel, 1964). However rather than antagonise vasoconstriction caused by catecholamines, papaverine, at initial concentrations of 80 μM, was found to prolong the pressor response of adrenaline and noradrenaline.

Histamine, added to the reservoir to give an initial concentration of 10.9 μM, reduced the portal pressure, previously increased by adrenaline, by 1.5 cm H_2O. Even at higher concentrations of histamine no pronounced antagonism to vasoconstriction by adrenaline could be produced. Neither papaverine nor histamine was thus suitable as an antagonist of vasoconstrictor activity in the perfused liver.

When the perfusion medium was prepared with Krebs bicarbonate buffer in which all Ca^{2+} had been replaced by Mg^{2+} the pressor effect of adrenaline was almost completely abolished as may be seen in Fig. 21. Thus the removal of Ca^{2+} provided a method whereby the metabolic effects of catecholamines could be investigated in the absence of possible complications caused by changes in hepatic vascular resistance.

5.3.7 Effect of adrenaline on hexobarbitone metabolism in the absence of Ca^{2+}

In perfusions in which the perfusion medium Ca^{2+} had been omitted the rate of hexobarbitone metabolism was 19% less over the first 30 min than in the corresponding control (Fig. 22) and the half-life of hexobarbitone in these

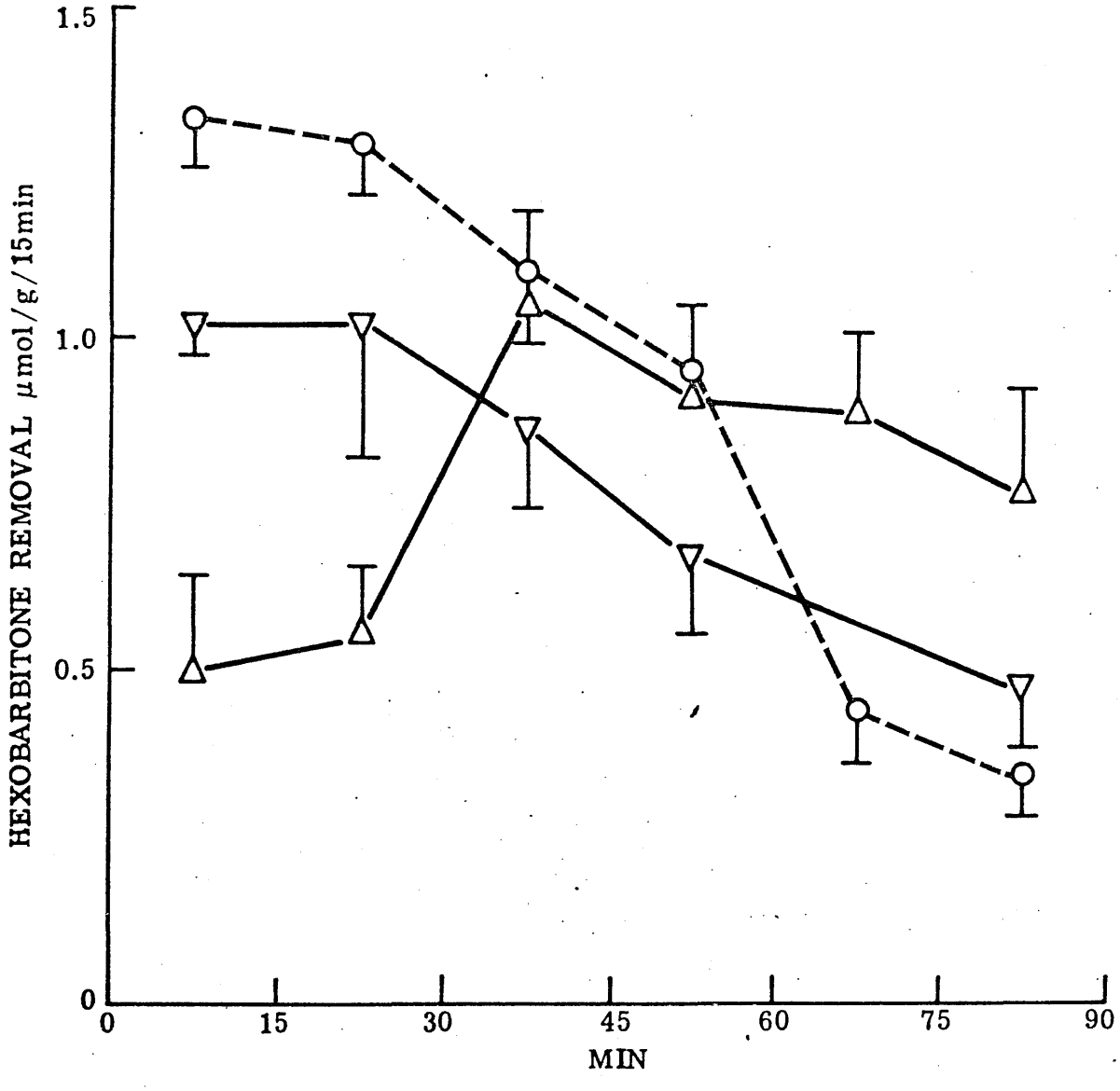


Figure 23

Effect of adrenaline on hexobarbitone metabolism, in the absence of Ca^{2+}, by the isolated perfused rat liver. Only the second pulses are shown for clarity. Adrenaline, 6 µM was added at 0 min in the presence of Ca^{2+}, 1.25 mM (Δ) or in the absence of Ca^{2+} (∇). Control perfusions (o) are shown by the broken line for comparison. Values are mean (± S.E.M.) of four perfusions.

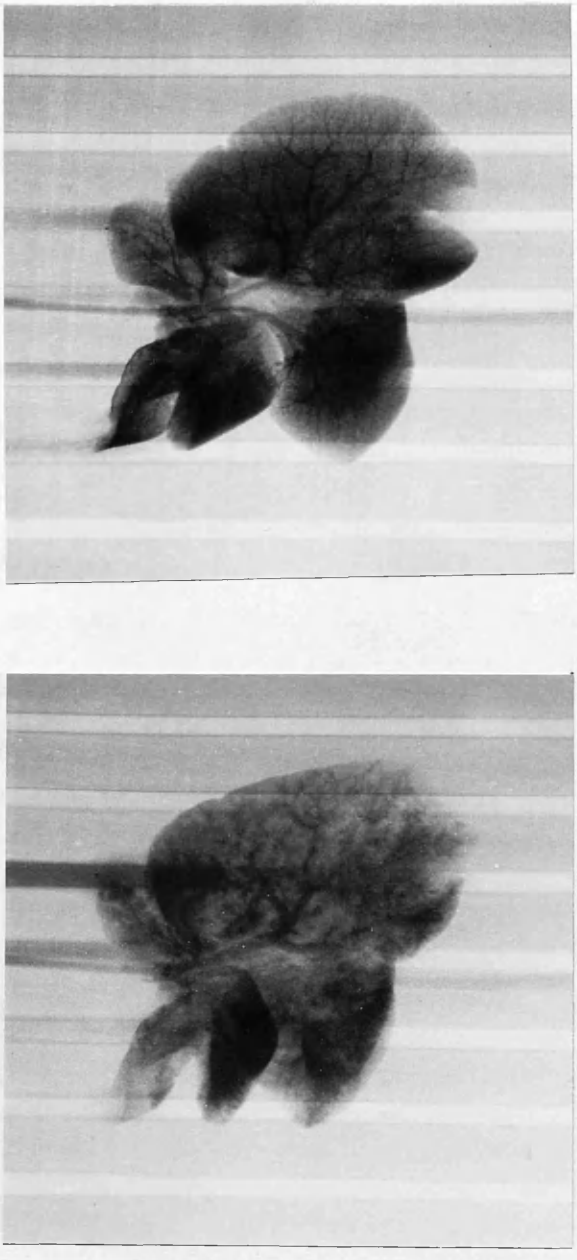


Figure 24

X-radiographs of the isolated rat liver perfused with adrenaline. Photographs are typical of results obtained. Weight of liver 9.3 g. The upper plate shows a liver after control perfusion with 10% (v/v) Conray 280 in the perfusion medium. The lower plate shows a liver after adrenaline, 6 μM was perfused until the pressure reached a plateau then Conray 280 was perfused with adrenaline. The lines in the background are due to the opacity of the heat-exchange unit.

perfusions was increased by 26% ($P < 0.05$) (Table 2).

As shown in Fig. 23 when adrenaline was added in the absence of Ca^{2+} the rate of hexobarbitone metabolism was no longer reduced by the 62% normally observed after 30 min but was only reduced by 21%. Even this decrease can be accounted for entirely in terms of a lack of Ca^{2+}.

In the absence of Ca^{2+} the half-life of hexobarbitone, after the addition of adrenaline, 6 μM, was increased by 39% compared with the second pulse of control perfusions ($P > 0.05$). This increase is of the same magnitude as that observed in the absence of Ca^{2+} when compared with the first pulse of control perfusions. Thus in the absence of Ca^{2+} adrenaline had no significant effect upon either the individual rates of hexobarbitone metabolism or its half-life over 90 min in the perfused liver.

5.3.8 X-radiography of the liver

From the previous section it was apparent that catecholamines may have been producing an inhibition of hexobarbitone metabolism through their effect on hepatic vascular resistance.

When livers were perfused with contrast medium, at the peak of the pressure response to adrenaline, 6 μM, X-radiographs similar to that shown in Fig. 24 were obtained. Similar results were obtained in each of four separate perfusions. The liver was no longer evenly dense, many lighter areas being apparent, probably due to incomplete sinusoidal filling.

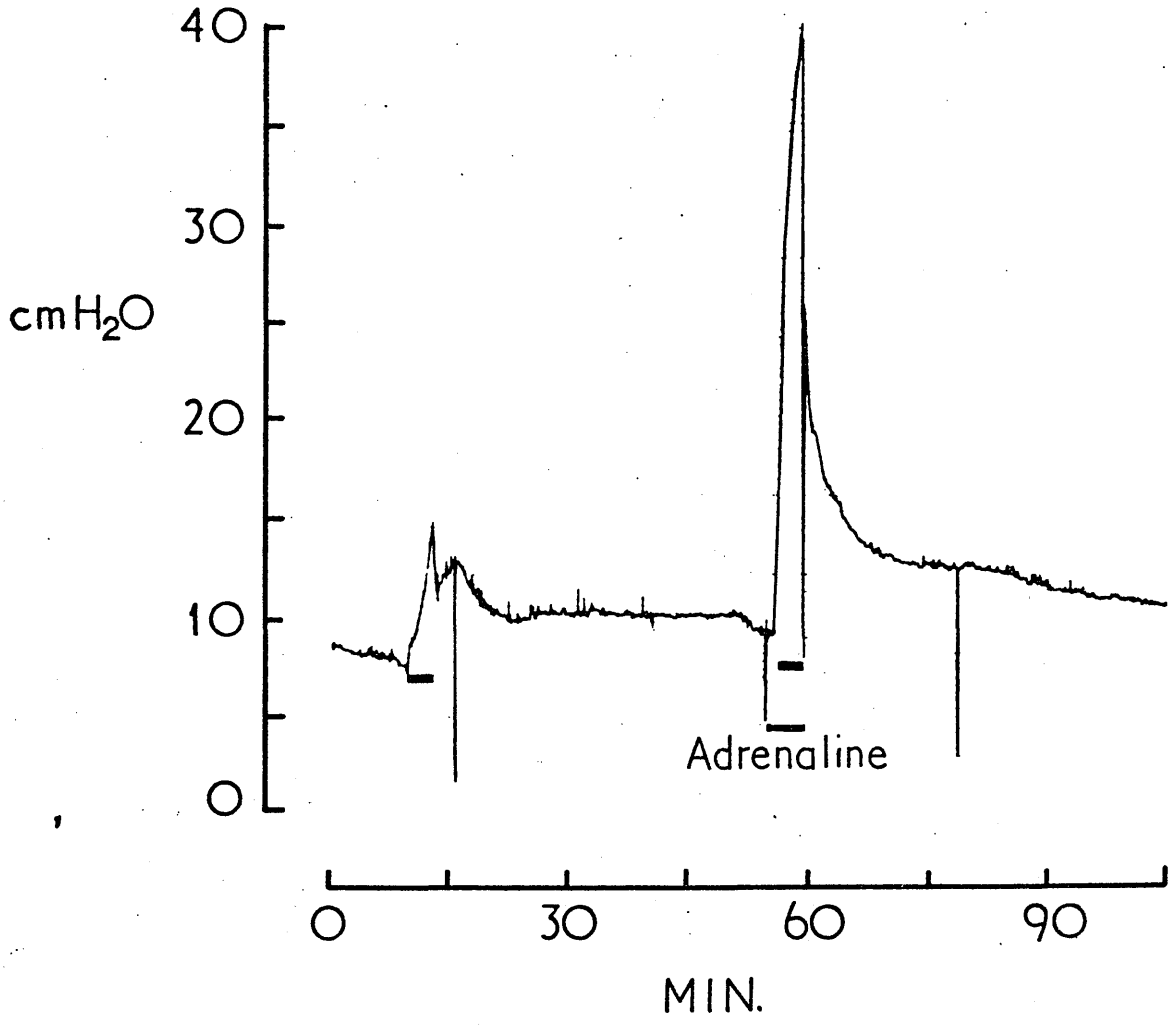


Figure 25

Effect of Conray 280 on the portal pressure in isolated perfused rat liver. Weight of liver 8.4 g. Unmarked bar; perfusion with Conray 280, 10% (v/v) in the perfusion medium. Adrenaline, 6 µM perfused where shown.

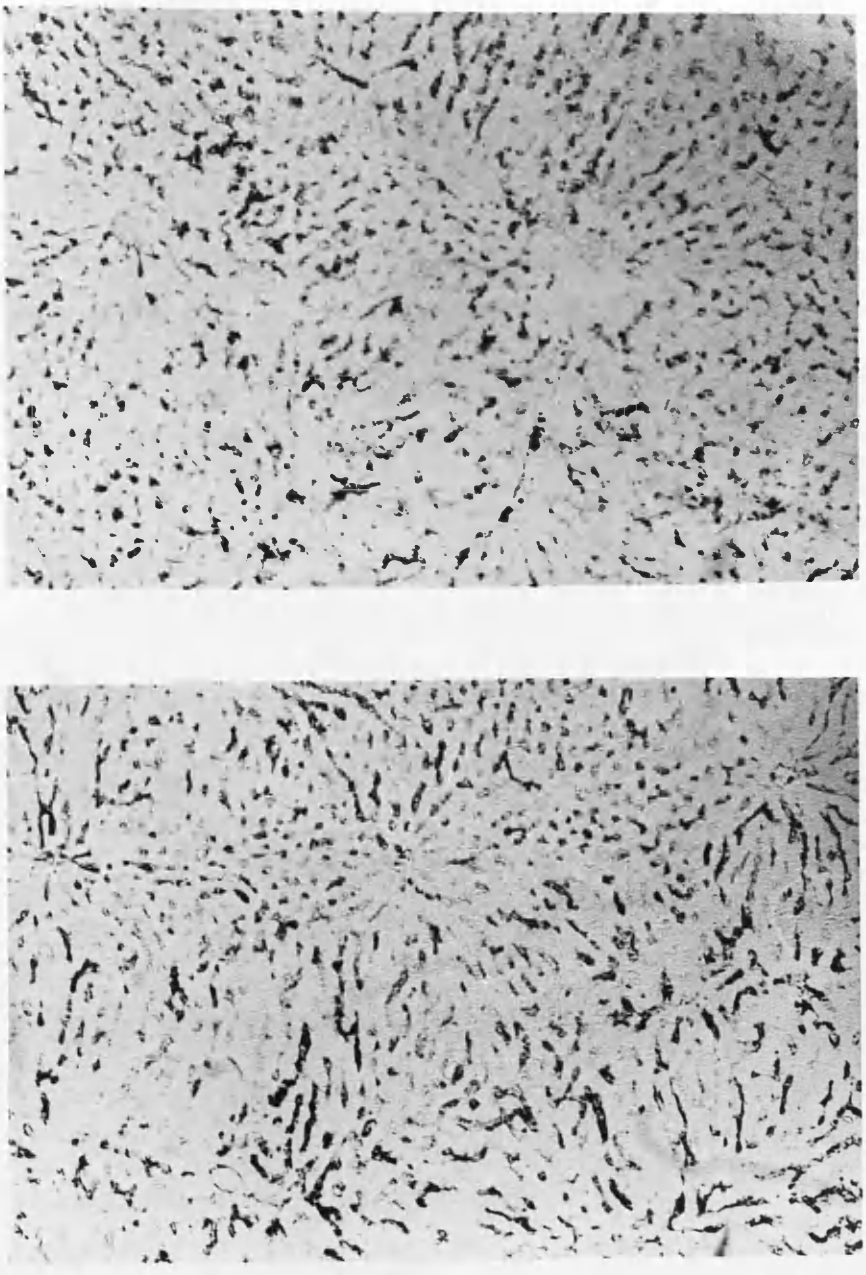


Figure 26

Distribution of carbon particles after perfusion of the isolated rat liver with Indian ink, 10% (v/v) in the perfusion medium. A typical result. Upper plate, sections from deep central and lower plate, from peripheral regions of the lobes were taken from livers perfused for 2 min with 10% (v/v) Indian ink in the perfusion medium. Magnification 100x.

The edges of the lobes were not as clearly defined as before and some of the smaller lobes were under-perfused by up to 50%. Many of the fine branches of the portal venous tree previously visible could no longer be seen.

Unfortunately the contrast medium itself had some vasoconstrictor activity as shown in Fig. 25. Conray 280, 10% (v/v) caused an increase in perfusion pressure of 4 to 6 cm H_2O, both alone and superimposed upon the maximum vasoconstriction caused by adrenaline, 6 μM. Thus is was difficult to interpret the X-radiographs unequivocably, particularly at the lobular level. For this reason further studies on portal vasoconstriction by adrenaline were instituted involving perfusion of the liver with Indian ink with subsequent histology.

5.3.9 Vasoconstrictor effect of adrenaline revealed by Indian ink

In control perfusions, shown in Fig. 26, distribution of carbon granules from Indian ink was even, both within small areas of the liver and between sections taken from the periphery and centre of lobes. Although only one micrograph is shown from each area at least three separate tissue sections were examined from peripheral and central areas of each liver. Perfusions were carried out on three separate control and three adrenaline perfused livers. In control perfusions many small blood vessels were visible and the lobular pattern of the liver was discernable.

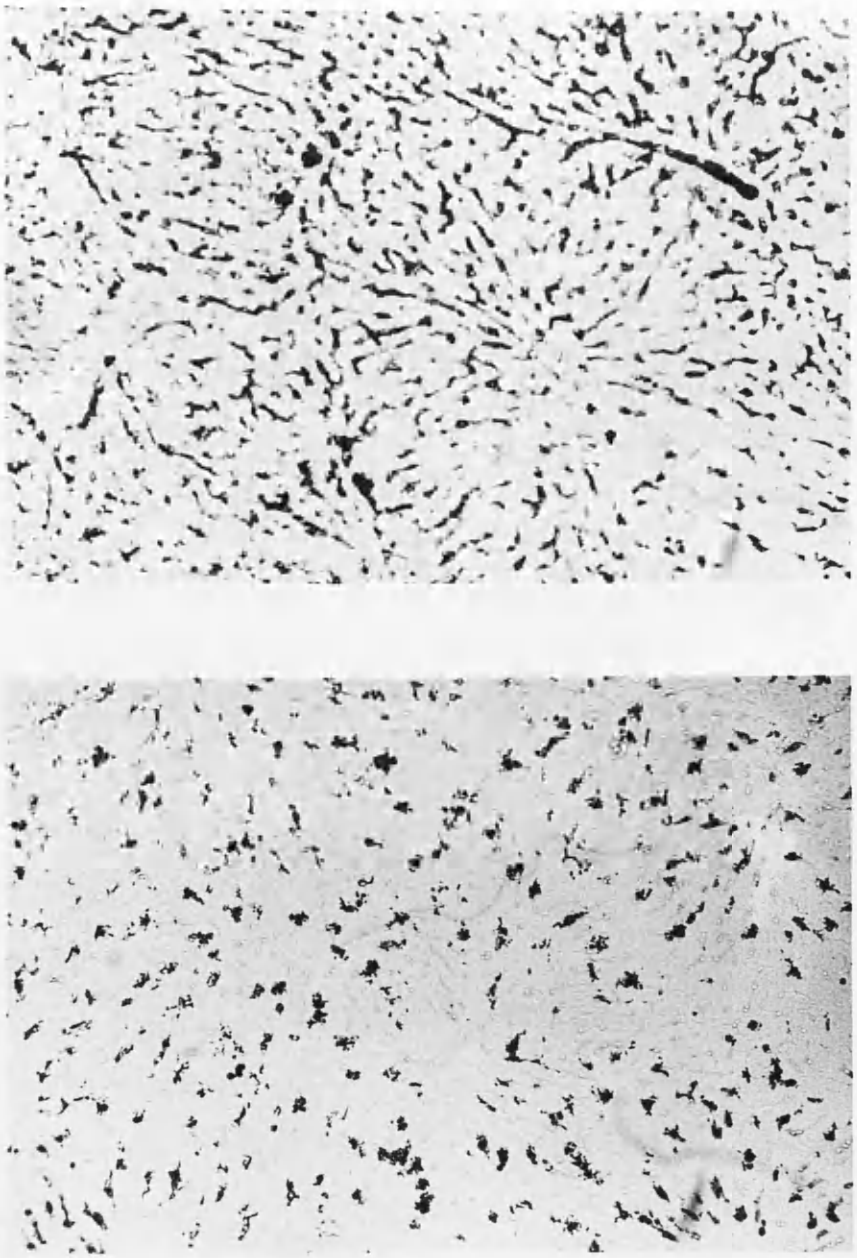


Figure 27

Effect of adrenaline on the distribution of carbon particles after perfusion of the isolated rat liver with Indian ink, 10% (v/v) in the perfusion medium. A typical result. Upper plate, sections from deep central and, lower plate, from peripheral regions of the lobes were taken from livers first perfused with adrenaline, 6 µM and then, at the peak of the pressure response, with adrenaline, 6 µM and Indian ink, 10% (v/v) in the perfusion medium. Magnification 100x.

Perfusion with adrenaline, 6 μM caused marked changes in the pattern of distribution of carbon granules. In sections from the centre of the lobes (Fig. 27) the distribution of granules was much denser than in corresponding control sections. In contrast, in those sections taken from the edge of the lobes, the distribution of granules was much less dense than in either sections from the centre of the lobes or in corresponding control sections. The lobular structure of the liver in these sections was not as evident as in the other sections and in addition most of the carbon granules appeared concentrated in the lumen of larger vessels. This corresponded to the macroscopic appearance of livers perfused with adrenaline and Indian ink, the surface of which became mottled within a few minutes, as if small areas of the liver were not being perfused with carbon granules, particularly at the edges of the lobes.

High power microscopy of all sections revealed that the granules from the Indian ink were exclusively located within the lumen of blood vessels, none of the particles having been taken up by reticulo-endothelial cells in the short time in which they were exposed to the ink. Indian ink, at the concentrations employed, was totally devoid of any pressor activity in the perfused liver.

It was concluded from this study that adrenaline could cause a change in the distribution of blood flow within the liver, resulting mainly in a shunting of perfusate away from peripheral areas to more central areas.

5.4 Drug metabolism in liver slices

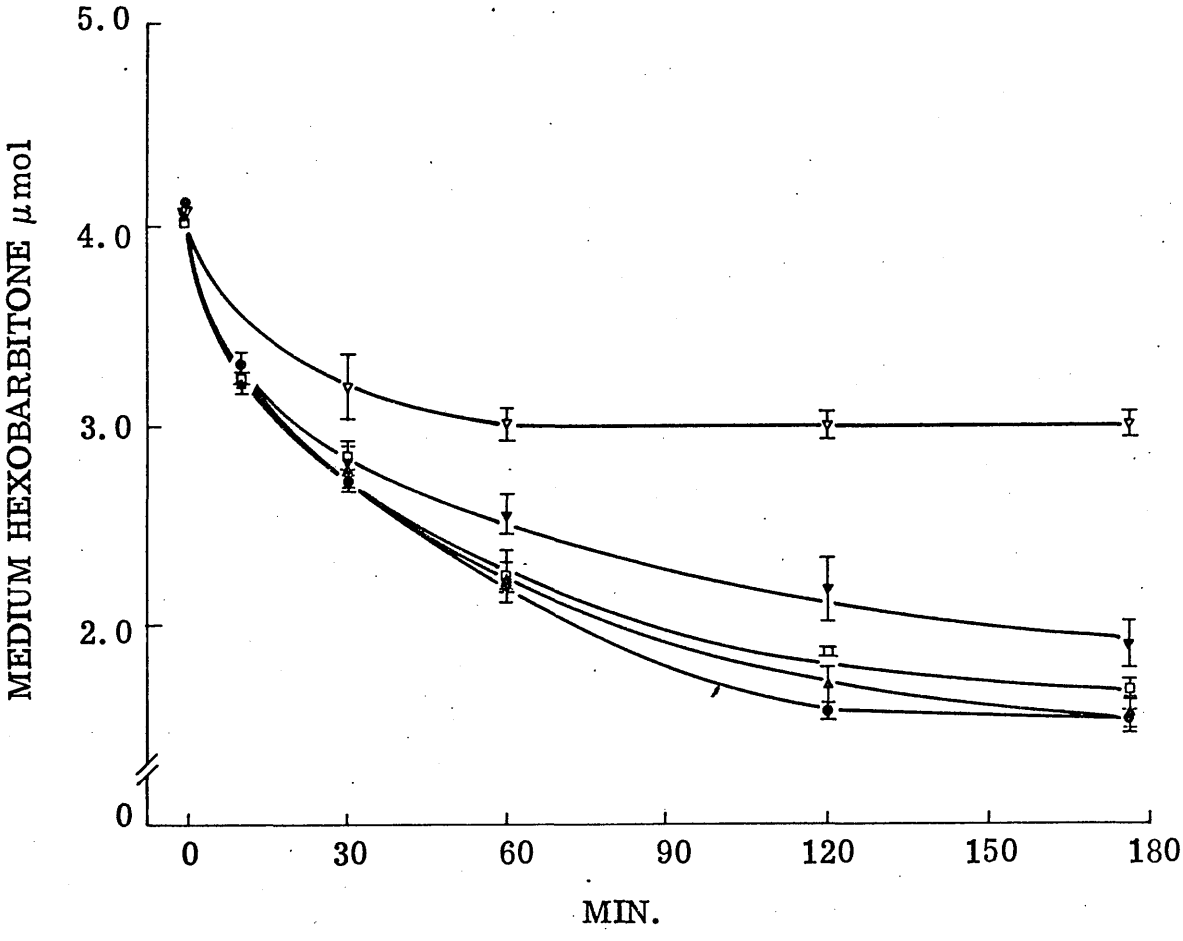


Figure 28

Hexobarbitone metabolism in liver slices. Liver slices were incubated with substrate alone (o), with adrenaline 10^{-3}M (▲), noradrenaline 10^{-3}M (□), dibutyryl cyclic 3'; 5' AMP 10^{-3}M (▼) or SKF 525-A, 10^{-3}M (▽). Values are mean (± S.E.M.) of three determinations.

TABLE 6

Hexobarbitone and aniline metabolism by liver slices

Liver slices were incubated with the compound shown. Figures are mean half-lives ± S.E.M. of substrates shown.

*P < 0.05 **P < 0.01 compared to control liver slices.

	Half-life, min			
	Hexobarbitone		Aniline	
	n		n	
Control	3	81.7 ± 5.6	6	84.4 ± 10.6
Adrenaline 10^{-3}M	4	91.3 ± 7.4	6	77.5 ± 7.0
Noradrenaline 10^{-3}M	3	99.6 ± 18.1	5	88.2 ± 5.6
Dibutyryl cyclic AMP 10^{-3}M	3	131.7 ± 13.8*	-	-
SKF 525-A 10^{-3}M	3	362.8 ± 7.5**	-	-

Since catecholamines were shown to cause a redistribution of blood flow in the perfused liver this was thought a possible mechanism for their inhibition of hexobarbitone metabolism in this preparation. To test this hypothesis further, the effect of catecholamines on drug metabolism in liver slices was investigated. Liver slices provide a whole cell system which, by its nature, is free from the complications of a blood supply since oxygenation is achieved by diffusion from the incubation medium into the slices.

Both hexobarbitone (Fig. 28) and aniline were removed exponentially by a first order reaction in liver slices. Substrate metabolism has thus been expressed in terms of half-life over 2 h as shown in Table 6. SKF 525-A, a potent inhibitor of drug metabolism (Cooper et al, 1954), was included to test the integrity of the system. It cuased a pronounced inhibition of hexobarbitone metabolism (Fig. 28), the half-life increasing by 344% ($P < 0.01$).

In agreement with the findings of Weiner et al (1972b) dibutyryl cyclic AMP inhibited the metabolism of hexobarbitone by liver slices, the half-life increasing by 51% ($P < 0.05$).

Neither adrenaline nor noradrenaline, 10^{-3}M produced any significant alteration in the metabolism of either hexobarbitone or aniline. It thus appears that catecholamines, even at high concentrations, do not release sufficient cyclic AMP to inhibit drug metabolism in intact cells.

TABLE 7

Influence of origin of NADPH on drug metabolism

NADPH was supplied directly or by a generating system. Figures are mean ± S.E.M. of six determinations.

	Aniline metabolism mμmol/mg protein/30 min	Aminopyrine metabolism mμmol/mg protein/30 min
NADPH	13.4 ± 1.5	87.6 ± 10.1
Generating system, glucose-6-phosphate dehydrogenase	6.7 ± 0.3	56.9 ± 7.8
Generating system, DL-isocitrate dehydrogenase	6.6 ± 0.7	67.3 ± 7.3

5.5 Effect of catecholamines on drug metabolism by the microsomal subcellular fraction

5.5.1 Co-factor requirements

Before proceeding with this aspect of the investigation drug-metabolising activity was compared in the different NADPH-generating systems employed. The effect of varying the concentrations of the different constituents of the incubation system was also investigated to determine the optimum conditions for drug metabolism. When NADPH was generated by either glucose-6-phosphate dehydrogenase or DL-isocitrate dehydrogenase there was no significant difference in the microsomal metabolism of either aniline or aminopyrine (Table 7). However when NADPH was added direct the rate of metabolism of both aniline ($P < 0.01$) and aminopyrine ($P < 0.05$) was significantly higher.

When NADP, isocitrate dehydrogenase or the substrate in the NADPH-generating system was omitted there was an almost total loss of metabolising activity, showing that the microsomal fraction contained none of the normal endogenous components of the NADPH-generating system. When the concentration of any of the co-factors was halved, or doubled, there was no significant change in microsomal drug-metabolising activity with either aniline or aminopyrine as substrate. Thus optimum concentrations were being employed for a microsomal concentration of 4 mg/ml. Similar results were obtained with the glucose-6-phosphate dehydrogenase system.

Optimum concentrations of Mg^{2+} and Tris buffer were determined and used in subsequent incubations. The concentration of nicotinamide employed was slightly inhibitory but was included as a precaution against NADP degradation. The rate of drug metabolism increased as the concentration of microsomal protein decreased. A fixed concentration of 4 mg/ml microsomal protein was therefore used throughout the study.

5.5.2 Effect of adrenaline on the assay of p-aminophenol

When adrenaline, 10^{-4}M was included in a standard solution of p-aminophenol, 0.05 mM, the magnitude of the change in absorbance produced for the indo-phenol complex was reduced by 26% and the time taken for maximum colour development was increased by 138%. It was thus considered undesirable to measure p-aminophenol formation by this assay as an index of aniline metabolism in the presence of catecholamines.

For this reason, in these studies, aniline metabolism was determined by assaying unmetabolised substrate by the method of Bratton and Marshall (1939) and calculating the amount metabolised. In the presence of catecholamines high blanks were obtained, possibly due to auto-oxidation, so that multiple blanks of standard solutions had to be run to ensure accurate correction.

5.5.3 Effect of catecholamines on microsomal drug metabolism

TABLE 8

Catecholamines and hexobarbitone, aniline and aminopyrine metabolism by microsomal subcellular fraction

The microsomal fraction was incubated with the compound shown at the concentration indicated. Figures are mean ± S.E.M. of six determinations. *P < 0.05 compared to control microsomes.

	Hexobarbitone metabolism mμmol/mg protein/30 min	Aniline metabolism mμmol/mg protein/30 min	Aminopyrine metabolism mμmol/mg protein/30 min
Control	49.6 ± 2.6	5.9 ± 0.5	85.9 ± 1.9
Adrenaline 10^{-3}M	54.6 ± 3.7	7.1 ± 1.2	88.5 ± 0.6
Adrenaline 10^{-4}M	51.3 ± 1.1	5.6 ± 0.3	78.7 ± 4.1
Adrenaline 10^{-5}M	44.0 ± 2.5	5.4 ± 0.7	85.8 ± 1.0
Adrenaline 10^{-6}M	48.8 ± 0.9	6.1 ± 0.2	84.2 ± 0.7
Noradrenaline 10^{-3}M	58.3 ± 6.2	6.9 ± 0.6	86.8 ± 0.6
Noradrenaline 10^{-4}M	46.1 ± 0.4	6.2 ± 0.2	85.7 ± 2.2
Noradrenaline 10^{-5}M	46.3 ± 1.5	5.4 ± 0.4	86.1 ± 1.1
Noradrenaline 10^{-6}M	48.8 ± 1.0	5.5 ± 0.3	87.7 ± 1.8
Cyclic AMP 10^{-3}M	51.0 ± 3.0	-	-
Cyclic AMP 10^{-4}M	48.2 ± 0.7	-	-
Cyclic AMP 10^{-5}M	45.9 ± 2.1	-	-
Cyclic AMP 10^{-6}M	48.9 ± 3.2	-	-
Dibutyryl cyclic AMP 10^{-3}M	49.7 ± 1.8	-	-
Dibutyryl cyclic AMP 10^{-4}M	53.9 ± 5.7	-	-
Dibutyryl cyclic AMP 10^{-5}M	51.7 ± 2.1	-	-
Dibutyryl cyclic AMP 10^{-6}M	52.1 ± 1.9	-	-

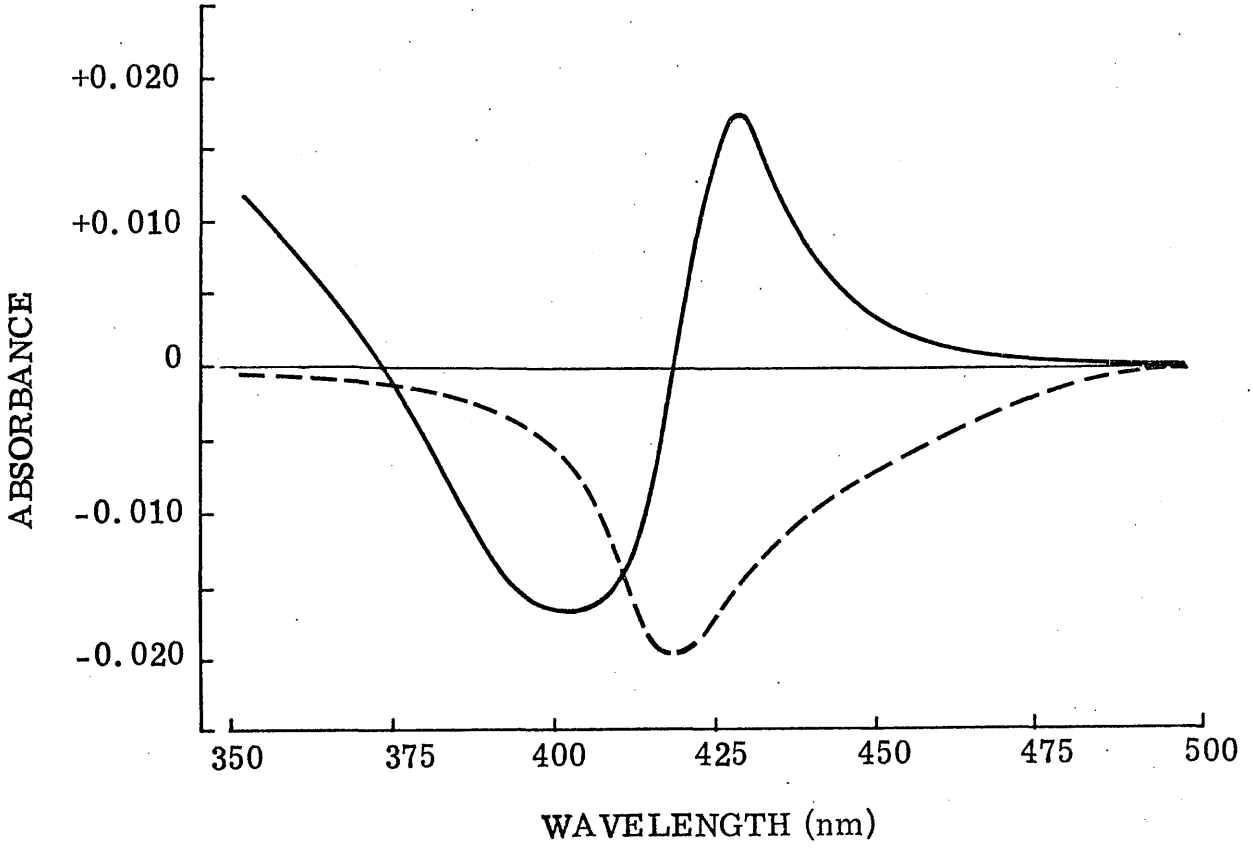


Figure 29

Microsomal difference spectra of catecholamines. Difference spectra were obtained with adrenaline 5 mM (broken line) and noradrenaline 5 mM (solid line) in microsomal suspensions from male rats.

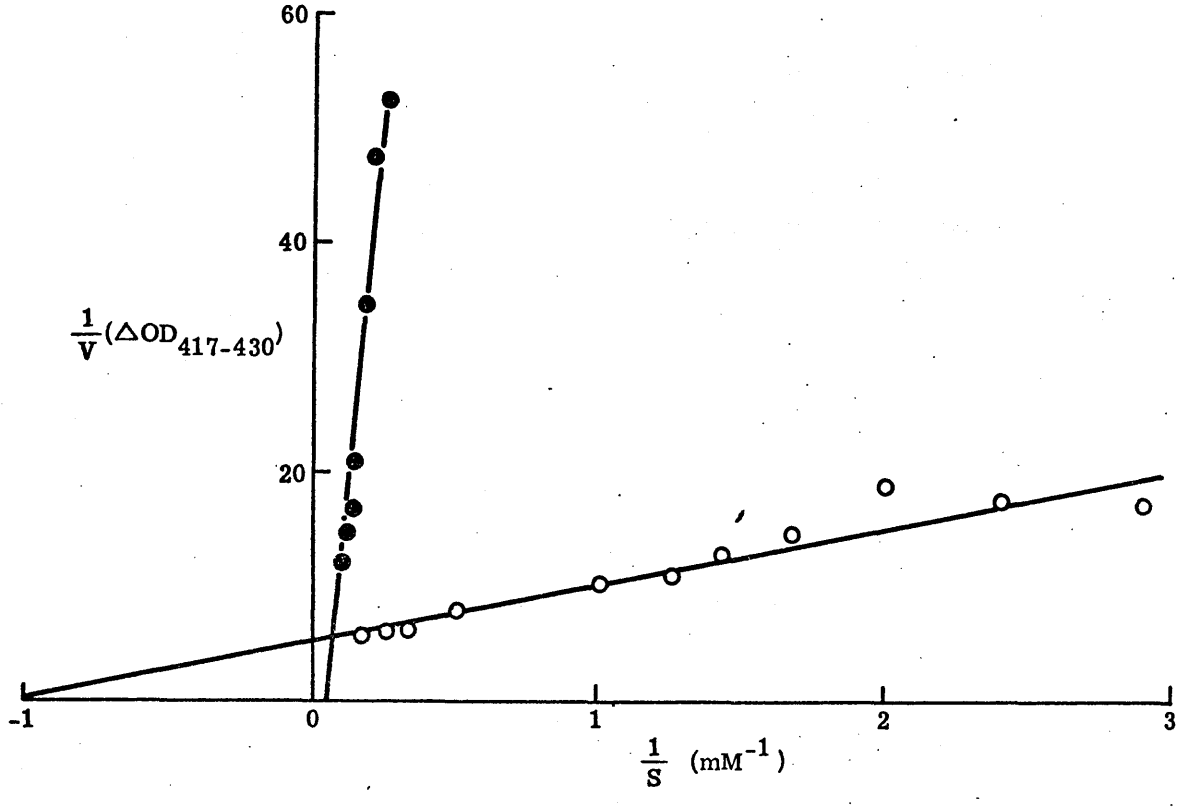


Figure 30

Lineweaver-Burke plot of the spectral changes caused by catecholamines. The reciprocal of the absorbance difference between 417 mμ and 430 mμ ($\frac{1}{V}$) is plotted against the reciprocal of the concentration ($\frac{1}{S}$) of adrenaline (●) and noradrenaline (o) producing the change.

The results are shown in Table 8. Neither cyclic AMP nor dibutyryl cyclic AMP, in concentrations from 10^{-6}M to 10^{-3}M, had any significant effect upon the metabolism of hexobarbitone. Similar findings were recently reported by Weiner et al (1972a).

The catecholamines adrenaline and noradrenaline, in concentrations up to 10^{-3}M, had no significant effect upon the metabolism of the three substrates investigated, aniline, hexobarbitone and aminopyrine.

5.5.4 Difference spectra of catecholamines

Both adrenaline and noradrenaline produced changes in the microsomal difference spectrum as shown in Fig. 29. The difference spectrum caused by adrenaline resembles that of a type I compound with λ_{min} at 418 mμ but with no real λ_{max}. Noradrenaline produced a typical type II difference spectrum with λ_{min} 405 mμ and λ_{max} at 428 mμ. Neither cyclic AMP nor dibutyryl cyclic AMP produced any difference spectrum in the microsomal fraction.

When a Lineweaver-Burke plot was constructed using the changes in absorption at varying concentrations of catecholamines (Fig. 30) noradrenaline was found to have a K_s of 1 mM. However adrenaline produced a line that had a positive intercept on the X-axis which would result in a negative K_s. Since this is not possible in Michaelis-Menton kinetics it may be that the spectral difference caused by adrenaline is due to non-specific binding, possibly of a product of auto-

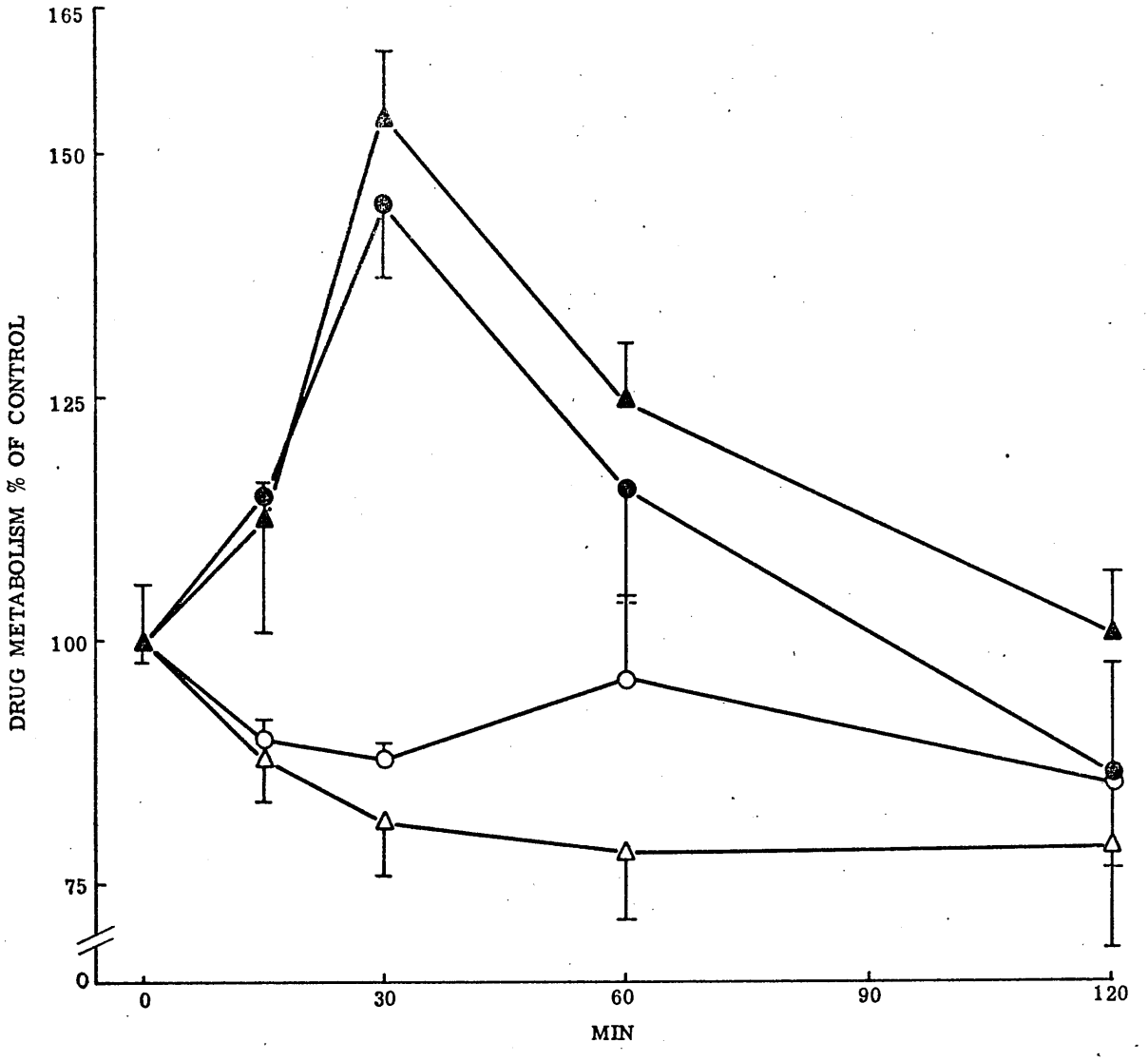


Figure 31

Hepatic microsomal drug metabolism after pre-treatment of rats with catecholamines. Male rats were pre-treated with adrenaline 1 mg/Kg (open symbols) or noradrenaline 1 mg/Kg (closed symbols) and microsomes prepared at various times thereafter. Metabolism of aniline (Δ,▲) and aminopyrine (o,●) was determined. Values are mean (± S.E.M.) of ten animals.

oxidation.

5.5.5 Effect of catecholamines on the difference spectra produced by other compounds

The difference spectra produced by the type I compound hexobarbitone and the type II compound aniline were not affected by cyclic AMP, dibutyryl cyclic AMP, or adrenaline. However noradrenaline at 1 mM was an inhibitor of aniline binding and at even higher concentrations of 4 mM, of hexobarbitone binding.

5.6 Effect of pre-treatment of rats with catecholamines on microsomal drug metabolism

5.6.1 Effect on microsomal metabolism of aniline and aminopyrine

Pre-treatment of male rats with noradrenaline, 1 mg/Kg caused an increase in the metabolism of both aniline and aminopyrine by the hepatic microsomal fraction maximal at 30 min after injection (Fig. 31). Aminopyrine metabolism was increased by 45 ± 8% ($P < 0.001$) and aniline metabolism by 54 ± 7% ($P < 0.001$). The metabolism of both substrates was not significantly different from control values after 2 h.

In contrast pre-treatment of rats with adrenaline, 1 mg/Kg, caused an inhibition in the microsomal metabolism of both substrates, apparent within 15 min after the injection (Fig. 31). Aniline metabolism was inhibited by 18.5 ± 5.5% ($P < 0.05$)

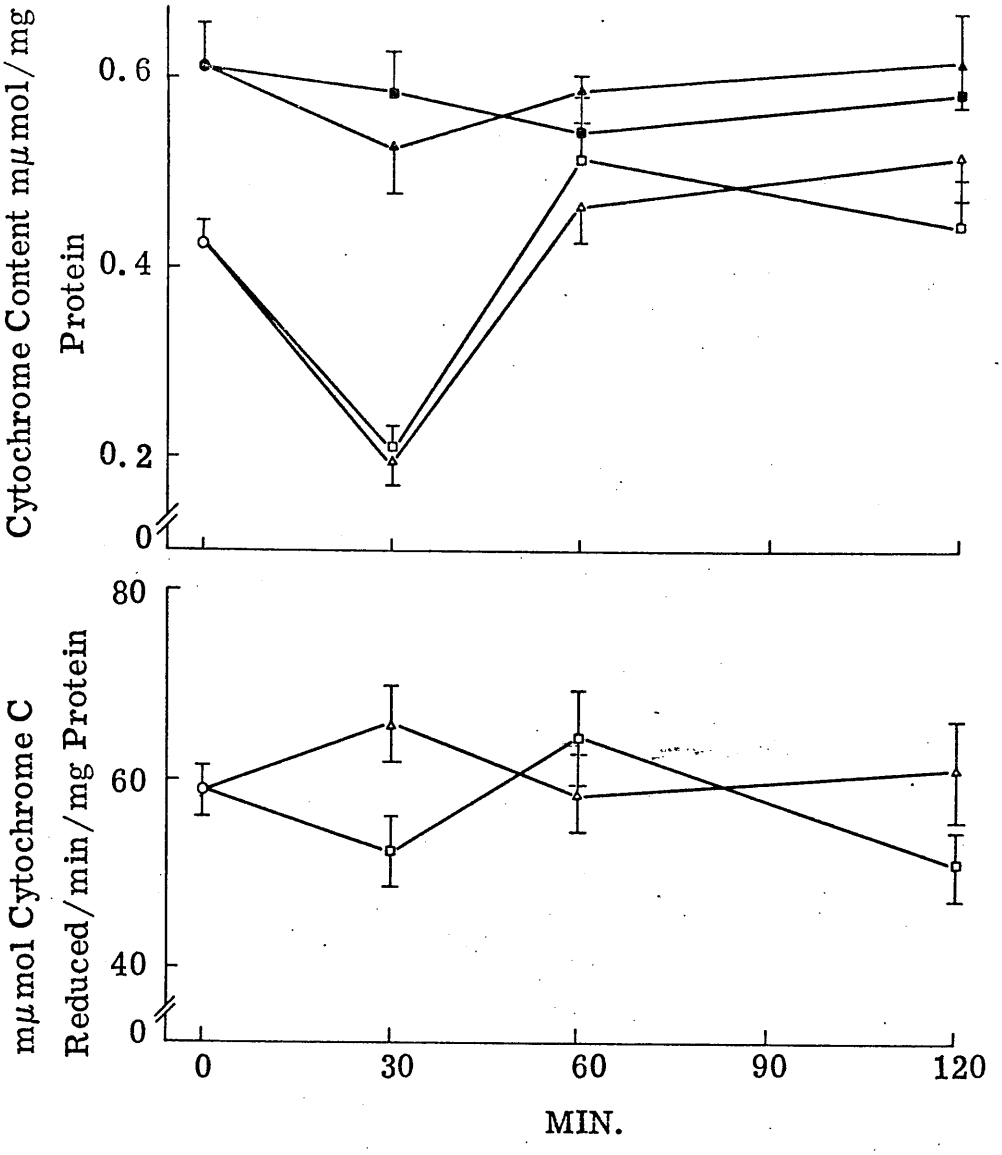


Figure 32.

Hepatic microsomal cytochrome content and NADPH-cytochrome c reductase activity after pre-treatment of rats with catecholamines. Male rats were pre-treated with adrenaline 1 mg/Kg (Δ,▲) or noradrenaline 1 mg/Kg (□,■). The upper panel shows the cytochrome content. Cytochrome b_5 content (open symbols) and cytochrome P-450 content (closed symbols). The lower panel shows NADPH-cytochrome c reductase activity. Values are mean (± S.E.M.) of six animals.

and aminopyrine metabolism by 12.0 ± 1.7% ($P < 0.005$) at 30 min. The metabolism of aminopyrine had returned to control values within 60 min whereas that of aniline was still inhibited after 2 h, although not significantly different from control values at this time.

5.6.2 Effect on the components of the microsomal electron transport chain

The possibility that catecholamine pre-treatment was causing changes in the microsomal drug-metabolising activity by affecting some component of the microsomal electron transport system was also investigated.

Pre-treatment with adrenaline or noradrenaline did not produce any significant alteration in the microsomal content of cytochrome P-450, but caused a significant decrease in cytochrome b_5 within 30 min, of 54 ± 5% ($P < 0.01$) and of 50 ± 5% ($P < 0.01$) respectively (Fig. 32). In both cases cytochrome b_5 levels had returned to control values within 60 min and remained constant thereafter. Pre-treatment with either adrenaline or noradrenaline produced no significant alteration in the activity of NADPH-cytochrome c reductase (Fig. 32).

5.6.3 Distribution of 3H-noradrenaline in the rat after injection

Although pre-treatment of rats with catecholamines had no significant effect upon the main components of the electron

TABLE 9

Distribution of label after injection of 3H-noradrenaline into rats

Rats were injected with 3H-noradrenaline, 1 mg/Kg, at time 0 and groups of animals sacrificed at the times indicated. Figures are concentrations of label assuming no metabolism and are mean ± S.E.M. of four animals.

	Plasma conc. ng/ml	Liver conc. ng/g of liver	Microsomal conc. pg/mg protein
0 min	0	0	0
15 min	203 ± 17	2383 ± 104	14.4 ± 0.7
30 min	267 ± 23	1171 ± 87	14.3 ± 0.6
60 min	514 ± 27	3583 ± 236	7.2 ± 1.1
120 min	395 ± 19	1926 ± 188	4.6 ± 0.3

transport chain it was considered possible that adrenaline and noradrenaline, or their metabolites, might be bound to the microsomal fraction and remain after isolation of this fraction. This was tested by injecting rats with 3H-labelled noradrenaline, 1 mg/Kg and measuring the label present in various fractions at different time intervals (Table 9).

Within 15 min after the injection label was detectable in the plasma at a concentration of 203 ng/ml and in the liver at a concentration of 2383 ng/g of tissue. However since no distinction was made between noradrenaline and its metabolites it is possible that a large fraction of this concentration was made up of metabolites, especially in the liver which is the primary site of metabolism of circulating catecholamines (Lightman and Hems, 1973). Only small amounts of label could be detected in the microsomal fraction and it appeared that noradrenaline was not bound to any significant extent to the microsomal fraction during its isolation.

5.7 Further studies on microsomal drug metabolism

5.7.1 Effect of washing microsomes with catecholamines on drug metabolism

In view of the high concentrations of label found in the liver soon after an injection of noradrenaline it was thought possible that catecholamines might have been exerting their effects upon the microsomes during the homogenisation procedure. This was investigated by re-suspending the microsomal fraction, from female rat liver prepared in the normal

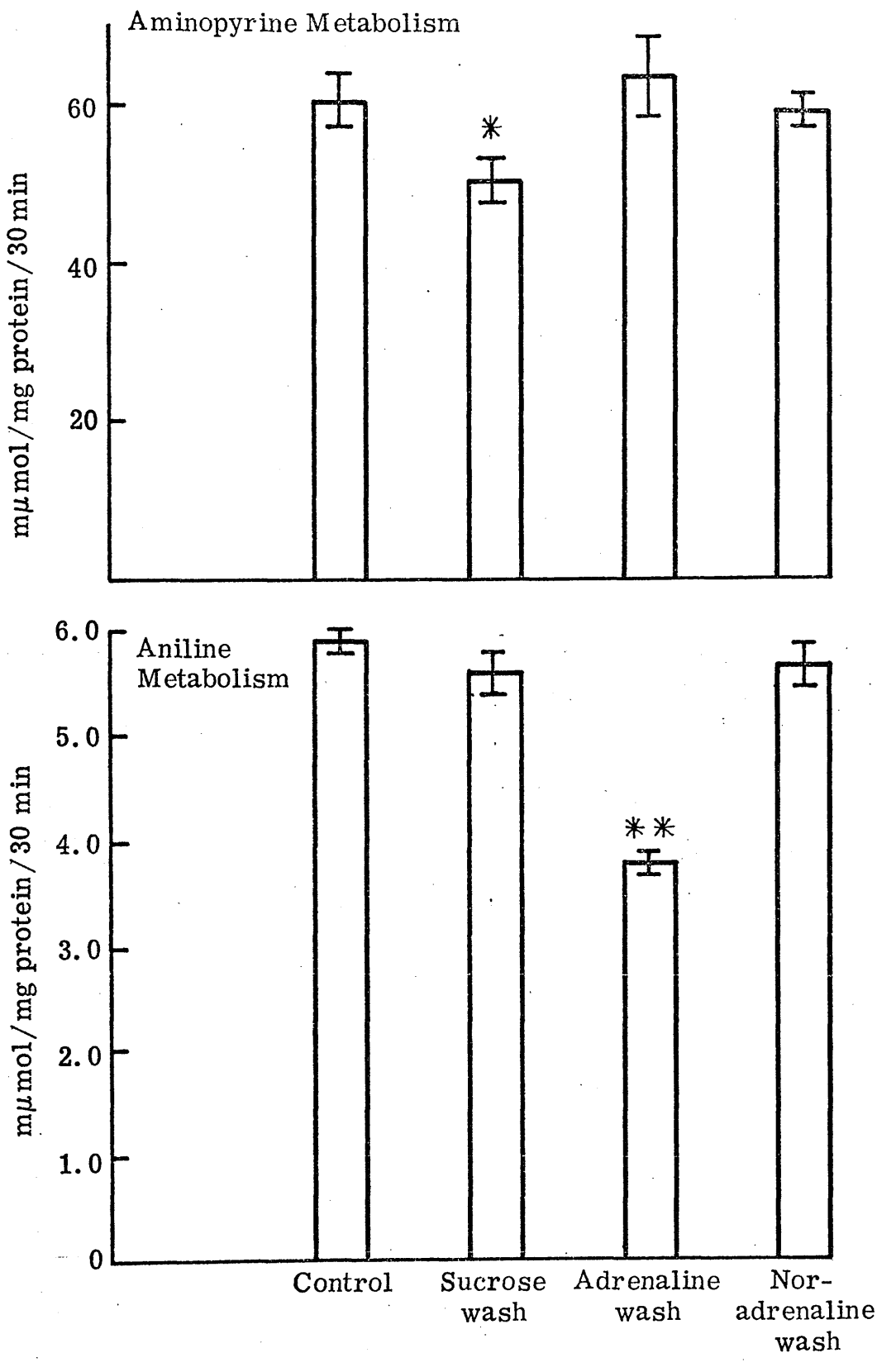


Figure 33

Effect of washing microsomes in catecholamines on drug metabolism. Microsomes were prepared as normal (control), washed in sucrose (sucrose wash), adrenaline, 10^{-3}M (adrenaline wash) or in noradrenaline, 10^{-3}M (noradrenaline wash). The upper panel shows aminopyrine metabolism and the lower panel shows aniline metabolism. NADPH was generated by isocitrate dehydrogenase. Values are mean (± S.E.M.) of six determinations.

*$P < 0.05$ **$P < 0.01$ compared with control microsomes.

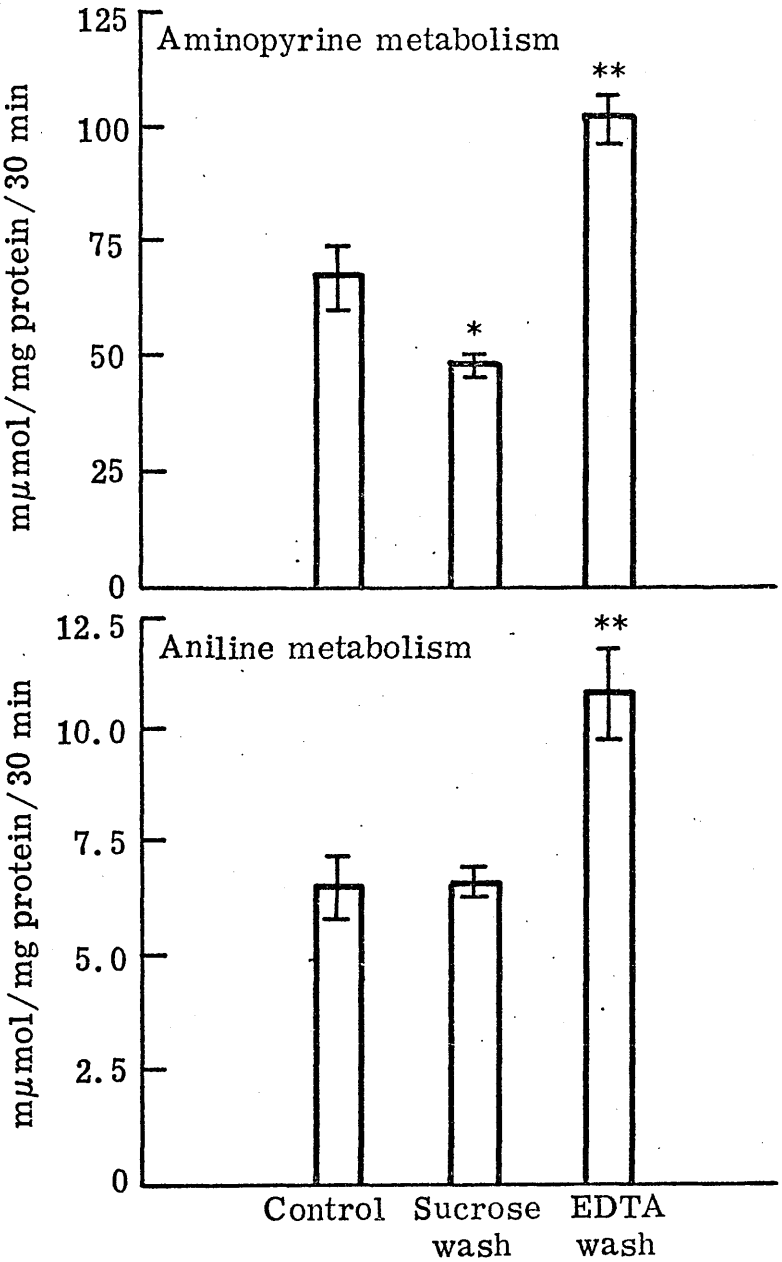


Figure 34

Effect of washing microsomes in sucrose or EDTA on drug metabolism. Microsomes were prepared as normal (control), washed in sucrose (sucrose wash) or washed in EDTA 10^{-3}M (EDTA wash). The upper panel shows aminopyrine metabolism and the lower panel shows aniline metabolism. NADPH was generated by isocitrate dehydrogenase. Values are mean (± S.E.M.) of six determinations.

*P < 0.05 **P < 0.01 compared with control microsomes.

manner, in sucrose containing catecholamine and then subjecting it to a second high speed spin.

The results obtained are shown in Fig. 33. Washing the microsomal fraction in sucrose caused no significant alteration of aniline metabolism but a 17% inhibition of aminopyrine metabolism ($P < 0.05$). Noradrenaline, 10^{-3}M, prevented any loss of aniline or aminopyrine metabolising activity whereas adrenaline, although preventing the loss of aminopyrine metabolising activity caused a loss of aniline metabolising activity of 35% ($P < 0.01$).

5.7.2 Effect of washing microsomes on drug metabolism

The loss of activity observed on washing microsomes in sucrose was studied in more detail and the effect of other possible protective compounds investigated.

Aniline metabolism was unaffected after washing the microsomes in sucrose but aminopyrine metabolism was reduced by 28% ($P < 0.05$) (Fig. 34). This is almost twice as great an effect as observed in the initial experiments (Section 5.7.1). When microsomes were washed in sucrose containing EDTA, 10^{-3}M both aniline and aminopyrine metabolism were increased by about 60% over the control values ($P < 0.01$). Similar changes were also observed when NADPH was added directly to the incubation instead of employing a generating system. This indicated that none of the observed effects could be ascribed to changes in activity of the NADPH-generating system.

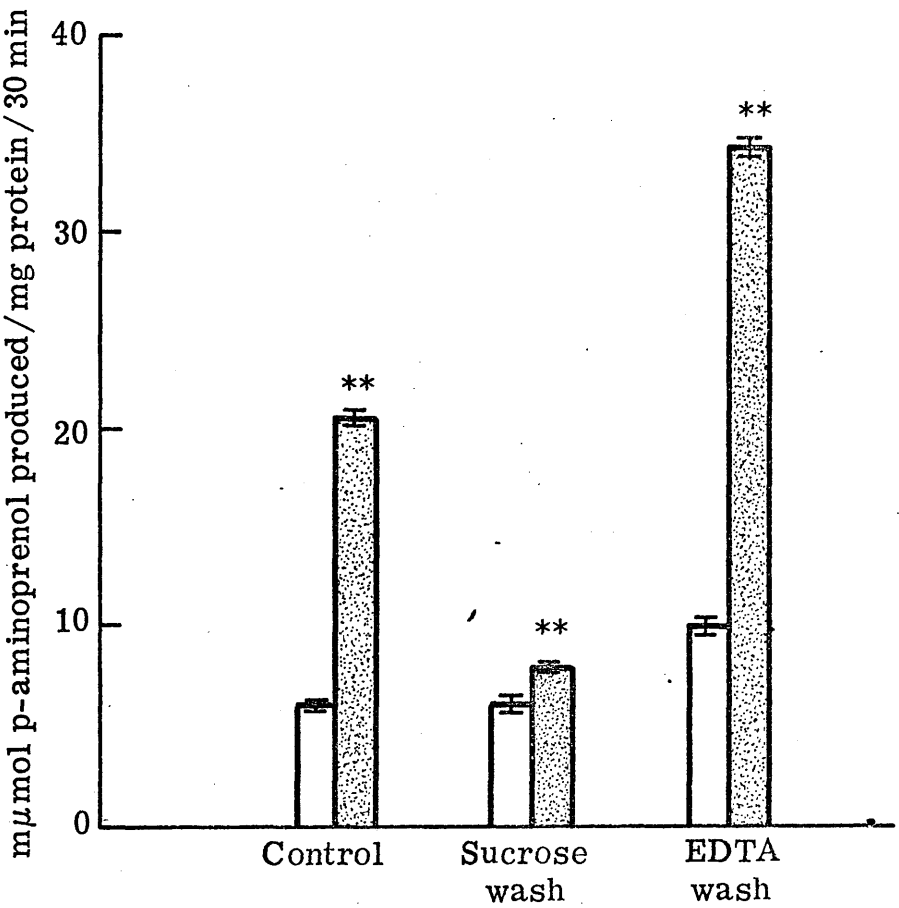


Figure 35

Acetone enhancement of aniline metabolism in washed microsomes. Microsomes were prepared as normal (control), washed in sucrose (sucrose wash) or washed in EDTA 10^{-3}M (EDTA wash). Open columns show aniline metabolism with no acetone and filled columns aniline metabolism in the presence of acetone, 0.5 M. NADPH was generated by isocitrate dehydrogenase. Figures are mean (± S.E.M.) of six determinations.

*P < 0.05 **P < 0.01 compared with metabolism in the absence of acetone.

One finding that proved of interest was that the enhancement of aniline metabolism by acetone (Anders, 1968) was almost abolished by washing the microsomes. Peak enhancement of aniline metabolism occurred between 0.5 M and 0.75 M acetone. The percentage increase in aniline metabolism was 240 ± 9% at 0.5 M acetone. Inhibition of aminopyrine metabolism was also observed, as already reported by Anders (1968), 23% inhibition with acetone, 0.5 M.

When microsomes were washed in sucrose, acetone enhancement of aniline metabolism was only 21 ± 3% (Fig. 35). However when EDTA, 10^{-3}M was added to the washing sucrose there was complete protection against any relative loss of acetone enhancing ability, even though the absolute value of aniline metabolising activity was increased compared to the control incubations.

Similar changes in acetone enhancement were observed after washing in sucrose or EDTA when NADPH was added directly to the incubation medium, thus showing that the effects were other than at the level of the NADPH-generating system. This finding was used to check whether acetone enhancement was reversible. It was found that microsomes exposed to 0.5 M acetone then washed by re-centrifuging to remove the acetone no longer responded to acetone. However when EDTA, 10^{-3}M was added to the acetone to which the microsomes were exposed it was found that they retained full susceptibility to acetone enhancement. Thus acetone enhancement is a reversible phenomenon.

TABLE 10

Effect of washing with activators and inhibitors of lipid peroxidation on microsomal drug metabolism

Microsomes were washed in a solution of the compound in sucrose buffer. BHT and vit E were first dissolved in 0.1 ml absolute ethanol which had no effect on drug-metabolising activity itself. NADPH was generated by isocitrate dehydrogenase. Acetone enhancement of aniline metabolism was determined in the presence of acetone 0.5M and the figures in parentheses show the percentage increase in aniline metabolism compared with the corresponding control. Values are mean ± S.E.M. of six determinations. *P < 0.05 **P < 0.01 compared to control microsomes. (Comparisons not shown for acetone column).

	Aniline metabolism mμmol/mg protein/30 min		Aminopyrine metabolism mμmol/mg protein/30 min
	No acetone	Acetone 0.5M	
Control	6.2 ± 0.2	14.0 ± 0.2 (+125)	67.3 ± 4.7
Washed microsomes			
Sucrose	5.8 ± 0.2	8.1 ± 0.1 (+ 39)	53.8 ± 2.7*
EDTA 10^{-3}M	9.2 ± 0.4**	21.0 ± 0.3 (+130)	95.6 ± 3.4**
BHT 10^{-3}M	5.5 ± 0.1**	7.9 ± 0.2 (+ 43)	57.2 ± 3.9
Vitamin E 34×10^{-3}M	5.1 ± 0.1**	6.6 ± 0.3 (+ 29)	52.5 ± 2.0**
EGTA 10^{-3}M	5.8 ± 0.2	8.9 ± 0.1 (+ 53)	65.3 ± 2.7
Mn^{2+} 10^{-3}M	6.9 ± 0.3*	10.4 ± 0.1 (+ 50)	70.0 ± 2.0
Vitamin C 10^{-3}M	5.4 ± 0.1**	7.1 ± 0.2 (+ 32)	-
Fe^{2+} 10^{-3}M	6.5 ± 0.2	10.3 ± 0.2 (+ 58)	45.3 ± 2.0**

TABLE 11

Effect of second post-microsomal supernatant on drug-metabolising activity

Supernatant obtained from washing microsomes in sucrose was added to the incubation medium where indicated. Controls were carried out by adding sucrose to the incubation. Figures in parentheses show percentage enhancement of aniline metabolism by acetone, 0.5M compared with values obtained with no acetone. NADPH was added directly to the incubations. Figures are mean ± S.E.M. of six determinations.

*P < 0.05 **P < 0.01 compared with aniline metabolism with no acetone present.

Microsomes	Additions to incubation	Aniline Metabolism mμmol/mg protein/30 min	
		No acetone	Acetone 0.5M
Control	None	13.4 ± 0.4	29.9 ± 0.5** (+123)
Control	Sucrose	5.3 ± 0.2	12.4 ± 0.2** (+134)
Control	Supernatant	3.3 ± 0.1	10.9 ± 0.2** (+227)
Sucrose washed	Sucrose	5.0 ± 0.3	6.1 ± 0.3* (+ 22)
Sucrose washed	Supernatant	4.4 ± 0.5	5.7 ± 0.1* (+ 30)

5.7.3 Effect of washing the microsomal fraction in anti-oxidants

Since EDTA is an inhibitor of lipid peroxidation the effect of other drugs, affecting lipid peroxidation, upon washed microsomes was investigated. The results are shown in Table 10. EDTA, EGTA, BHT and Mn^{2+} prevented any significant reduction of aminopyrine metabolising activity after washing, but only EDTA provided much protection against the loss of acetone enhancement of aniline metabolism that normally occurred after washing the microsomes. Vitamin E, either in the natural form or as the acid succinate or acetate salts had no protective effect on drug metabolism.

Vitamic C and Fe^{2+} which stimulate lipid peroxidation failed to significantly potentiate the changes occurring on washing.

5.7.4 Effect of washing the microsomal fraction in post-microsomal supernatant

When control microsomes were incubated during the assay with sucrose there was over 60% reduction in aniline metabolism but the percentage increase in aniline metabolism due to acetone enhancement was unaffected (Table 11). When either control microsomes or those washed in sucrose were incubated with the supernatant from this wash aniline metabolism was even further reduced than with sucrose alone. This may be an effect on the detection of p-aminophenol rather than its formation possibly due to an interaction with haemoglobin in

TABLE 12

Drug metabolism in microsomes washed in first post-microsomal supernatant

Microsomes were washed in the supernatant, obtained from the first isolation of the microsomes, where indicated. Figures in parentheses show percentage enhancement of aniline metabolism by acetone 0.5M compared with activity with no acetone present. NADPH was generated by isocitrate dehydrogenase. Figures are mean ± S.E.M. of six determinations.

*P < 0.05 **P < 0.01 compared with control microsomes.

	Aniline metabolism mμmol/mg protein/30 min		Aminopyrine metabolism mμmol/mg protein/30 min
	No acetone	Acetone 0.5M	
Control	7.1 ± 0.2	18.3 ± 0.9 (+130)	74.5 ± 3.1
Washed microsomes			
Sucrose	6.9 ± 0.4	10.3 ± 0.5** (+ 27)	56.4 ± 4.3**
Supernatant	7.1 ± 0.3	22.3 ± 0.7** (+205)	81.9 ± 2.3*
Heated supernatant	5.5 ± 0.2**	10.2 ± 0.4** (+ 85)	-

the supernatant. Acetone enhancement in control microsomes was unaffected by this post-microsomal supernatant but there was no reversal of the loss of activity that occurred after washing in sucrose. Thus it was unlikely that during washing of the microsomes some component of the microsomal fraction was lost into the supernatant which could be added back to restore activity.

Another possibility was that during the normal isolation of the microsomal fraction some soluble endogenous compound prevented the loss of activity that occurred on washing microsomes in the absence of post-microsomal supernatant. Thus microsomes were washed in the supernatant obtained after their first isolation. This was found to prevent any loss of aminopyrine metabolising activity, and even to increase it slightly (Table 12). Similarly the acetone enhancement of aniline metabolism was even greater than before washing, in contrast to the loss of activity that occurred after washing in sucrose alone. These results are very similar to those observed with EDTA.

When the post-microsomal supernatant was boiled for 10 min and filtered to remove the precipitate it provided only some protection against the loss of acetone enhancement.

5.7.5 Effect of storing microsmes on drug-metabolising activity

A loss of aminopyrine metabolising activity with very little loss of aniline metabolising activity has been

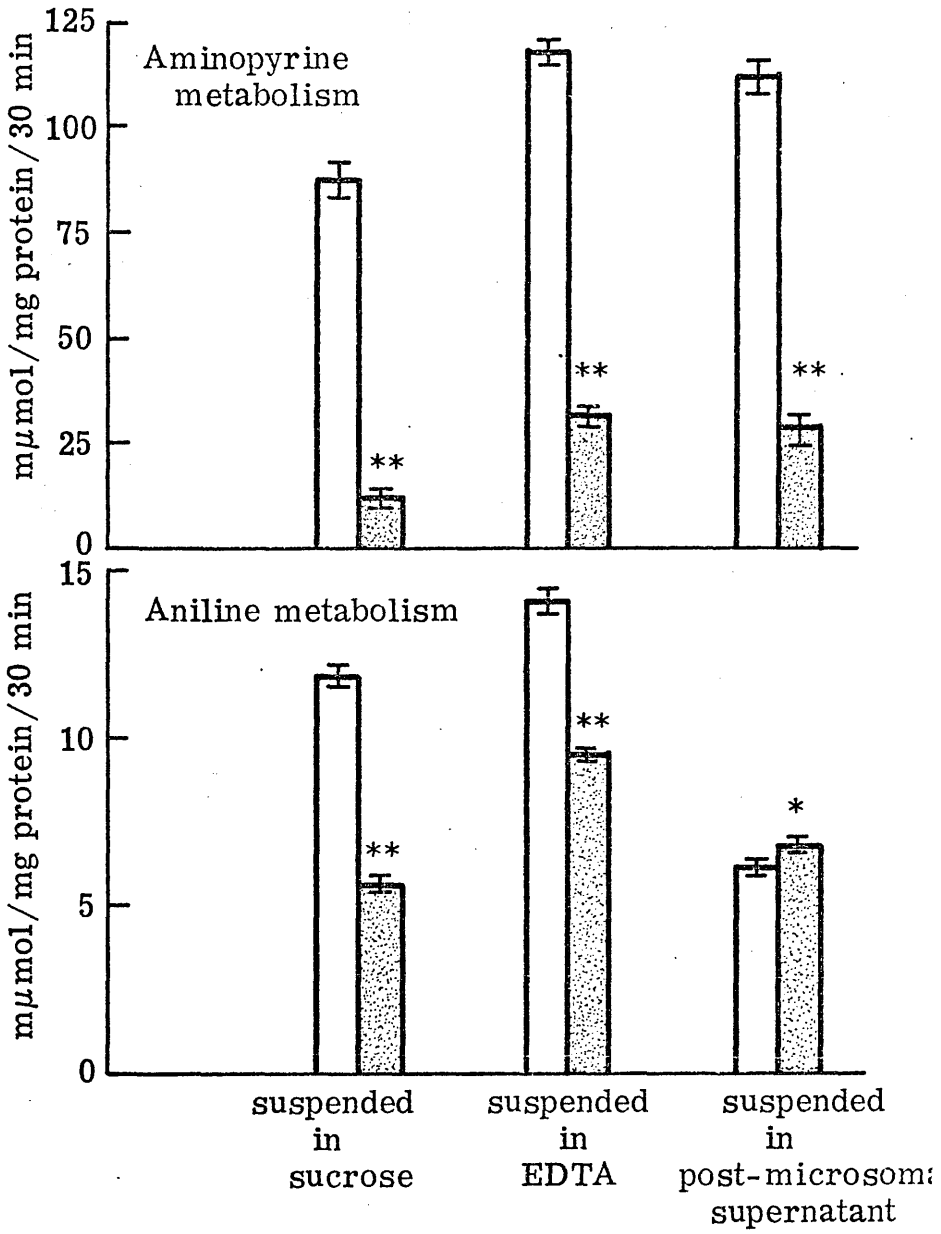


Figure 36

Effect of storage of microsomes on drug metabolism. Microsomes were suspended in sucrose buffer, EDTA 10^{-3}M or the post-microsomal supernatant from the first spin, and assayed for aminopyrine metabolism (upper panel) and aniline metabolism (lower panel) on the day of preparation (open columns). The microsomes were also assayed after storage at 4°C for 90 h (stippled columns). NADPH was added directly to the incubation. Figures are mean (± S.E.M.) of four determinations.

*$P < 0.05$ **$P < 0.01$ compared with metabolism before storage.

reported to occur in the microsomal fraction when stored for more than a few hours at 4°C (Leadbeater and Davies, 1964; Hewick and Fouts, 1970). Because of the similarities in effects produced by this treatment and washing of the microsomes, the effect of storing microsomes in EDTA and the post-microsomal supernatant were investigated. To avoid possible complications of EDTA interacting with the NADPH-generating system reduced NADPH was added directly to the incubation medium.

Storing microsomal fraction at 4°C suspended in sucrose resulted in a much greater loss of aminopyrine metabolism, reduced by 86.4%, compared with aniline metabolism which was reduced by 52.1% (Fig. 36). The enhancement of aniline metabolism by acetone was almost completely abolished by storage (results not shown).

Suspension of microsomal fraction in sucrose containing EDTA 10^{-3}M caused a significant increase in both aniline and aminopyrine metabolism, 18.5% and 34.8% respectively. After storage at 4°C these microsomes showed less reduction of drug-metabolising activity when compared with microsomes stored in sucrose, both in absolute terms and in percentage changes. Aminopyrine metabolism was reduced by 73.1% and aniline metabolism by only 32.6%.

Microsomes suspended in post-microsomal supernatant obtained after the first spin exhibited a 27.6% increase in aminopyrine metabolism but aniline metabolism was reduced by

TABLE 13

Microsomal metabolism in presence of EDTA

Drug metabolism was determined in microsomes in the presence and absence of EDTA 2.5×10^{-5}M. Microsomes were treated as shown. Drug-metabolising activity is expressed as mμmol/mg protein/30 min. NADPH was generated by glucose-6-phosphate dehydrogenase. Figures are mean ± S.E.M. of six determinations.

*P < 0.05 **P < 0.01 compared with values in absence of EDTA.

	Aniline metabolism mμmol/mg protein/30 min				Aminopyrine metabolism mμmol/mg protein/30 min	
	No acetone		Acetone 0.5M			
	No EDTA	EDTA	No EDTA	EDTA	No EDTA	EDTA
Control	7.1 ± 0.2	7.2 ± 0.1	19.7 ± 0.3	22.9 ± 0.5**	89.9 ± 2.5	98.4 ± 1.6**
Washed microsomes						
Sucrose	5.2 ± 0.3	5.2 ± 0.2	7.2 ± 0.4	8.0 ± 0.3	79.7 ± 1.3	85.6 ± 2.1*
EDTA 10^{-3}M	9.8 ± 0.4	10.2 ± 0.3	29.4 ± 0.3	32.8 ± 0.7**	117.1 ± 5.2	133.9 ± 3.3*

47.1%, probably due to interference in the assay of p-aminophenol. When these microsomes were stored at $4^{o}C$ some protection of aminopyrine metabolism was evident, the loss of activity being 74.3%. Aniline metabolism was also protected when compared to the appropriate control, but this was complicated by the inhibitory effect of post-microsomal supernatant itself.

It was concluded that many parallels existed between the effects of storage of microsomes and of washing.

5.7.6 Effect of EDTA on drug metabolism

As reported previously EDTA at $10^{-3}M$ appeared to increase the metabolism of both aniline and aminopyrine when included in the incubation medium. This was further investigated at a lower concentration of EDTA, and the results are shown in Table 13. At $2.5 \times 10^{-5}M$ EDTA increased aminopyrine metabolism, by 10% in control microsomes, and by 7.5% and by 14% in sucrose washed and EDTA washed microsomes respectively. However at this concentration of EDTA there was no significant effect upon aniline metabolism although in the presence of 0.5 M acetone EDTA did cause a light increase in aniline metabolism over and above that caused by acetone enhancement ($P < 0.01$).

This effect of EDTA was insufficient to account for its protective effect against loss of acetone enhancement or aminopyrine metabolism occurring after washing in EDTA, if

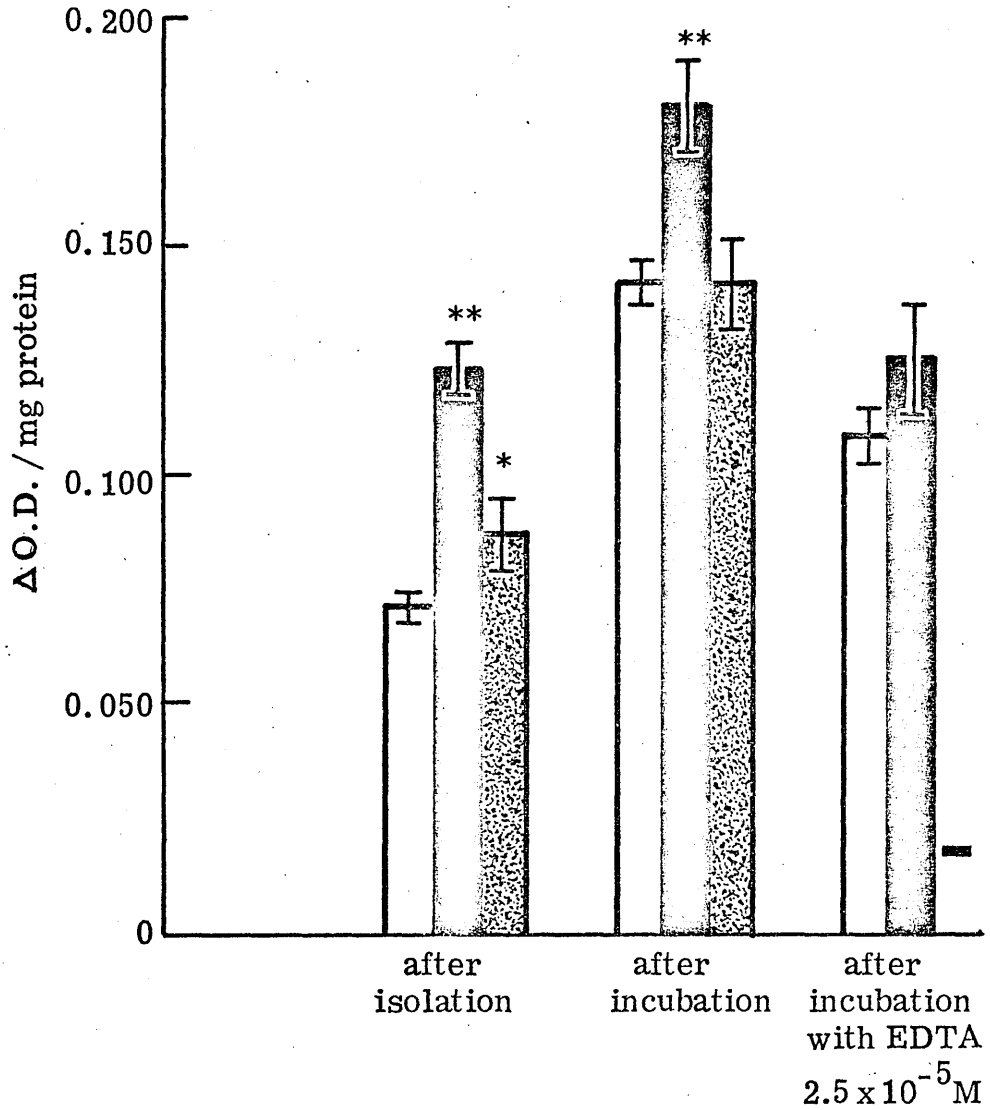


Figure 37

Lipid peroxidation in washed microsomes. Lipid peroxidation was measured in control microsomes (open columns), microsomes washed in sucrose (filled columns) and microsomes washed in EDTA 10^{-3}M (stippled columns). Measurements were performed after isolating the microsomes, after incubation for 30 min with NADPH generated by glucose-6-phosphate dehydrogenase, and after incubation as before but in the presence of EDTA 2.5×10^{-5}M. Values are mean (± S.E.M.) of six determinations. ▬ No measurement taken.
*$P < 0.05$ **$P < 0.01$ compared with control microsomes.

this was only due to a carry-over of EDTA into the microsomal pellet. This was confirmed by incubating sucrose washed microsomes with varying concentrations of EDTA in the presence of 0.5 M acetone. In the absence of EDTA, acetone increased aniline metabolism slightly to 10.7 mμmol/mg/30 min. EDTA, at most, increased this to 13.0 mμmol/mg/30 min at 10^{-2}M. This was insufficient to account for the observed enhancement after washing in EDTA, 10^{-3}M in which aniline metabolism was increased to 29.0 mμmol/mg/30 min.

5.7.7 Effect of washing microsomes on lipid peroxidation

The effects of washing microsomes in sucrose could be prevented by adding certain inhibitors of lipid peroxidation. It was therefore possible that these effects of washing were due to lipid peroxidation occurring during washing. Malonaldehyde formation was used as a measure of lipid peroxidation (Bidlack et al, 1973).

After washing microsomes in sucrose there was significantly more malonaldehyde present than in control microsomes (Fig. 37). When EDTA, 10^{-3}M was included in the sucrose this increase in malonaldehyde was largely prevented. Similarly, after incubation of the washed microsomes for 30 min, more lipid peroxidation occurred in washed microsomes than in control microsomes and this increase did not occur in microsomes washed in 10^{-3}M EDTA. Although the inclusion of 2.5×10^{-5}M EDTA in the incubation medium of either control or sucrose washed microsomes resulted in a reduction of lipid peroxidation there were still significant amounts of malonaldehyde formed.

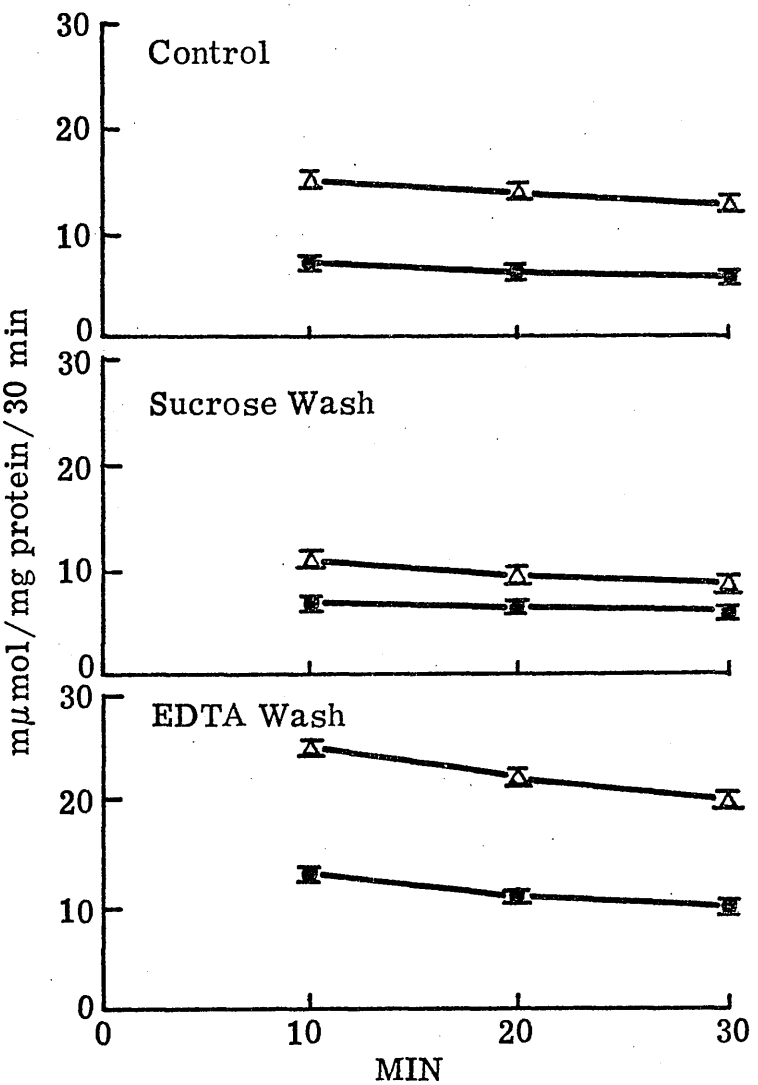


Figure 38

Effect of the duration of incubation on drug metabolism by washed microsomes. Aniline metabolism was determined in the absence of acetone (●) and in the presence of acetone, 0.5 M (Δ). NADPH was generated by isocitrate dehydrogenase. Microsomes were incubated after their first isolation (control) or washed in sucrose (sucrose wash) or EDTA 10^{-3} M (EDTA wash). Aniline metabolism is expressed as the rate of formation of p-aminophenol per 30 min. Values are mean (± S.E.M.) of six determinations.

The effect of incubating EDTA washed microsomes was not tested since the experiment was designed to detemine if EDTA, carried over from washing the microsomes, could inhibit lipid peroxidation.

5.7.8 Effect of the duration of incubation of washed microsomes on drug metabolism

The possibility that the loss of drug-metabolising activity that was apparent after washing the microsomes in sucrose was due to a loss of activity during the incubation at $4^{o}C$ rather than during the washing procedure was investigated.

When the period of washing, before isolating the microsomes, was increased from 30 min to 2 h or reduced to 5 min no significant change in the loss of aminopyrine metabolism or of acetone enhancement was evident, when compared with that occurring after 30 min. Similarly the protection afforded by EDTA, $10^{-3}M$ was not significantly affected by altering the time between spins. It was also found that increasing the temperature of the incubation from $4^{o}C$ to $37^{o}C$ had no significant effect upon the extent of the activity lost after washing in sucrose or upon the protective effect of EDTA.

Thus it appeared that the changes that were evident after washing in sucrose occurred either during the second spin or after it. Fig. 38 shows the results obtained from experiments designed to judge the latter possibility.

In control microsomes the rate of aniline metabolism fell slightly, by 15% between 10 and 30 min of incubation. However

TABLE 14

Effect of washing microsomes on components of electron transport chain

Microsomes were washed as shown. Cytochrome b_5 and P-450 content are expressed as mμmol/mg protein, NADPH-cytochrome c reductase is expressed as mμmol cytochrome c reduced/min/mg protein and NADPH-cytochrome P-450 reductase is expressed as mμmol cytochrome P-450 reduced/mg protein/min. Figures are mean ± S.E.M. of four determinations.

*P < 0.05 **P < 0.01 compared to control microsomes.

	Cytochrome b_5	Cytochrome P-450	NADPH-cytochrome c reductase	NADPH-cytochrome P-450 reductase
Control	0.463 ± 0.030	0.799 ± 0.020	0.245 ± 0.024	0.286 ± 0.057
Washed microsomes				
Sucrose	0.408 ± 0.035	0.744 ± 0.039	0.205 ± 0.020	0.400 ± 0.086
EDTA 10^{-3}M	0.551 ± 0.028*	1.144 ± 0.022**	0.180 ± 0.033	0.343 ± 0.029

TABLE 15

Difference spectra after washing microsomes in sucrose

Microsomes were treated as shown. Difference spectra were obtained as described in the text. Results are ΔOD (Peak-trough)/mg protein and are expressed as a percentage of the values obtained with control microsomes. Symbols in parentheses indicate the type of spectral change produced. Figures are mean ± S.E.M. of six determinations.

*P < 0.05 **P < 0.01 compared with control microsomes.

	Substrate		
	SKF 525-A (I)	Aniline (II)	Acetone (II)
Control	100 ± 2	100 ± 4	100 ± 9
Washed microsomes			
Sucrose	73 ± 3**	98 ± 2	0 ± 3**
EDTA 10^{-3}M	140 ± 5**	121 ± 8*	72 ±11*

over this period the percentage enhancement of aniline metabolism caused by acetone did not change. Similar results were obtained with microsomes previously washed in EDTA, 10^{-3}M.

Incubation of microsomes washed in sucrose caused a fall in aniline metabolising activity of only 8% between 10 and 30 min, while over the same period percentage acetone enhancement was reduced from 57% to 40%. These changes were insufficient to completely account for the effect of washing micrsomes in sucrose.

5.7.9 Effect of washing microsomes on the components of the electron transport chain

Washing the microsomal fraction in sucrose produced no significant change in the microsomal content of cytochrome b_5 or cytochrome P-450 nor was there any significant alteration in the activity of NADPH-cytochrome c reductase or NADPH-cytochrome P-450 reductase (Table 14). Microsomes that were washed in sucrose containing EDTA, 10^{-3}M had a significant increase in both cytochrome b_5 levels and in cytochrome P-450 levels. The activity of NADPH-cytochrome c reductase and NADPH-cytochrome P-450 reductase was not significantly affected in these microsomes.

Microsomes washed in sucrose displayed a 17% reduction in cytochrome P-450 binding of the type I substrate, SKF 525-A but did not have significantly altered binding of type II substrates such as aniline (Table 15). In contrast microsomes washed in EDTA showed significant increases in the binding of

TABLE 16

Microsomal protein yield after washing in sucrose

Microsomes were treated as shown. Protein was determined in both the microsomal pellet and the supernatant after washing. Figures are mean ± S.E.M. of twenty determinations.

	Protein in pellet mg	Protein in supernatant mg	Total
Control	30.5 ± 1.0	-	30.5 ± 1.0
Washed microsomes			
Sucrose	24.0 ± 0.8	6.7 ± 0.2	30.7 ± 1.0
EDTA 10^{-3}M	21.1 ± 0.4	9.2 ± 0.3	30.3 ± 0.7

both type I substrates, increased by 40%, and of type II substrates, increased by 21%.

In agreement with an observation by Vainio and Hänninen (1972) acetone was found to produce a typical type II spectral change in microsomal suspensions, though the magnitude of this change was small. Microsomes washed in sucrose showed no binding of acetone (Table 15).

5.7.10 Effect of washing on microsomal protein yield

All the washing procedures were found to cause a reduction in the amount of protein recovered from the microsomal pellet. Microsomes washed in sucrose yielded only 79 ± 2% of the protein obtained from control microsomes and washing in EDTA reduced the yield further, to 71 ± 3% of the control (Table 16). The protein lost from the pellet could be recovered from the supernatant, both after washing in sucrose and in EDTA.

CHAPTER 6

Discussion

TABLE 17

Values obtained for various tests of function of the isolated perfused rat liver

The values obtained in the present study are compared with those found by other workers and with the values found *in vivo* in the rat. - indicates no value quoted.

Source	Initial glucose production mg/g/h	L:P ratio	pH change pH units/h	K^+ release µmol/g/h	Haemo-lysis %/h	Peak bile flow ml/h
This work	3.3	8.0	0.00	10	0.2	0.7
In vitro						
Woods & Krebs, 1971	5.4	8.5	-	-	-	0.7
Schimassek, 1963a	4.9	10.0	-	-	-	-
Mortimore, 1961	8.0	-	-	17	-	-
Brauer *et al*, 1951	-	-	-	-	-	0.38
Craig, 1966	2.4	-	-0.08	7	2.0	-
Ostashever *et al*, 1960	-	-	0.00	-	0.4	0.45
Northrop & Parks, 1964	7.0	-	-	-	-	-
Hems *et al*, 1966	2.4	-	-	-	-	0.40
Exton & Park, 1967	9.0	10.0	0.00	10	0.0	0.30
Ryoo & Tarver, 1968	-	-	-	-	-	0.40
Kalser *et al*, 1965	2.0	-	-	-	-	0.60
Penhos *et al*, 1966	1.4	-	-	-	-	-
Fisher & Kerly, 1964	1.5	-	-0.03	19	-	0.50
Levine *et al*, 1964	0.6	-	-	-	-	0.75
Flock & Owen, 1965	-	-	-	-	-	0.50
John & Miller, 1969	-	-	-	-	-	0.65
Herz *et al*, 1973	-	7.7	-	4	2.6	-
In vivo						
Ross, 1972		10.0	0.00	0	0.0	0.75

6.1 Viability of the perfused liver

The operative procedure used in isolating the liver was designed to be quick, and to avoid any period of anoxia to the liver, so characteristic of many previous perfusion experiments (see review by Ross, 1972). The viability of the liver was assessed for periods of up to six hours of perfusion.

Within a few minutes of the transfer of the liver to the perfusion chamber its appearance corresponded closely with that normally observed *in vivo* and in successful perfusions a similar appearance was still evident at the end of the experiment. Electron microscopy of the liver after 4 h perfusion revealed none of the characteristic signs of cell damage or necrosis or even of the reversible changes occurring during hypoxia (Bock *et al*, 1972). There were none of the changes caused by the increased lysosomal activity associated with high oxygen tensions (Abraham *et al*, 1968).

A comparison of the values obtained for the various tests of liver function in the present study with those reported by other workers is shown in Table 17. It is only by comparison with the *in vivo* values and with those reported for successful perfusions that an assessment of the viability of the liver can be made.

Glucose production is expressed over the initial period of perfusion during which glycogenolysis occurs due to operative trauma (Bartosek *et al*, 1972b). The values are well within the range of values reported by others for the

successfully functioning perfused liver. Changes in the perfusate pH, extent of haemolysis, and K^+ efflux from the liver correlate well with the results of others. The lactate: pyruvate ratio, which has been described as one of the most sensitive tests of liver function (Ross, 1972) was maintained between 8 and 10 after the first 60 min of perfusion for periods of up to 5 h. This is similar to the ratio measured *in vivo* (Schimassek, 1963a) and in the functioning perfused liver (Schimassek, 1963a; Woods and Krebs, 1971). In anoxia (Schimassek, 1963a; Faupel, Seitz, Tarnowski, Thiemann and Weiss, 1972) or inadequate perfusion (Woods and Krebs, 1971) the ratio increases rapidly to values above 25.

Although bile flow is not regarded as a good test of liver function (Ostashever *et al*, 1960) it was found that flow rates agreed with most reported values and the initial rate was similar to that observed *in vivo*. The bile flow was initially high but then declined over the duration of perfusion. This is probably a reflection of the loss of bile salts from the system which, *in vivo*, would enter an entero-hepatic circulation and stimulate bile production (O'Maille, Richards and Short, 1966). Addition of bile salts to the perfused liver has been found to increase bile production to near *in vivo* rates (Kendler, Bassan and Zimmerman, 1971; Liersch, Barth, Hackenschmidt, Ullmann and Decker, 1973).

Thus the isolated liver could be maintained in a viable condition for periods of up to 6 h as judged by various tests of morphology, and biochemical and physiological function.

Little evidence of bacterial contamination was found making it unnecessary to add antibiotics. A period of 60 min was allowed for equilibration after transfer of the liver to the perfusion chamber during which time the lactate:pyruvate ratio attained a steady value and most of the other parameters tested reached a steady state.

6.2 Drug metabolism in the perfused liver

6.2.1 Hexobarbitone metabolism

The metabolism of hexobarbitone by the isolated perfused rat liver was found to be a first order reaction, which confirms the findings of Cumming and Mannering (1970) and Bock *et al* (1972).

The half-life of hexobarbitone during the first pulse of substrate in the perfused liver was 29.7 ± 2.0 min. This correlates well with values for the half-life of hexobarbitone *in vivo* which has been variously reported as 36 min (Anders, 1966), 28 min (Axelrod, Reichenthal and Brodie, 1954) and 30 min (Bush and Weller, 1972). It has been shown by von Bahr *et al* (1970) that the half-life of those drugs with a small volume of distribution *in vivo* closely resembles the half-life in the perfused liver. The volume of distribution of hexobarbitone in the rat is relatively small (Cumming and Mannering, 1970; Bush and Weller, 1972). It is thus not unreasonable that the half-life of this substrate in the perfused liver closely resembles the half-life *in vivo*.

The half-life of hexobarbitone in the perfused liver reported by other workers (Stitzel et al, 1968; Weiner et al, 1972a) is lower than the values found in the present study. However this is possibly due to the abnormally high oxygen tensions used by these workers. Hexobarbitone metabolism is very sensitive to changes in oxygen tension (Cumming and Mannering, 1970).

The half-life of hexobarbitone during the second pulse was significantly greater than during the first pulse. Stitzel et al (1968) also found that there was a small but significant increase in the half-life of hexobarbitone from the first to the second pulse. The reason for this decrease in drug-metabolising activity is unknown. It may be due to the depletion of some essential co-factor or the destruction of some essential component of the drug-metabolising system. The levels of the components of the microsomal electron transport chain cytochrome P-450, cytochrome b_5 and NADPH-cytochrome c reductase have, however, been reported to remain stable over 4 h perfusion (Bock et al, 1972; Bartosek et al, 1974).

It has recently been reported that the metabolism of the type I substrate aminopyrine by the 9,000 x G fraction isolated from perfused rat liver was reduced by 50% whereas that of aniline, a type II substrate, was reduced by less than 20% after 2 h perfusion (Bartosek et al, 1974). The loss in aminopyrine metabolising activity could be partially prevented by the infusion of nicotinic acid, a precursor of NADP, during perfusion. A loss of NADP(H) may explain the loss of

hexobarbitone metabolising activity observed in the perfused liver.

6.2.2 Aniline metabolism

It has been known for some time that the metabolism of aniline by the microsomal fraction is slower than the metabolism of hexobarbitone (Dixon *et al*, 1964). This has been explained in terms of the inhibition and activation of the rate of reduction of cytochrome P-450 by the two substrates respectively (Gigon *et al*, 1968, 1969). It has also been found that *in vivo* the metabolism of hexobarbitone (Bush and Weller, 1972) is much faster than the metabolism of aniline (Parke, 1960). The metabolism of aniline by the perfused liver has now been shown to be much slower than that of hexobarbitone. Three hours after the addition of aniline to the perfused liver 20% of it remained unmetabolised in the perfusion medium whereas almost all the hexobarbitone had been metabolised within 90 min.

The phase I metabolism of aniline *in vivo* is largely to p-aminophenol (Parke, 1960). In the present study although p-aminophenol could be liberated from the perfusion medium by strong acid hydrolysis no free p-aminophenol could be detected. Separate studies with red cell suspensions revealed that free p-aminophenol was bound to some component of the erythrocytes, most probably haemoglobin, so that it could not be detected. Further studies with 3H-aniline showed that over 7% of the label could be detected bound to haemoglobin. This may represent free p-aminophenol that has reacted with haemoglobin. Kiese and Pekis (1964) have shown that p-aminophenol reacts

with haemoglobin to convert it to hemiglobin while there is a coupled oxidation of the p-aminophenol itself, and the hemiglobin, by molecular oxygen. It then no longer forms an indo-phenol complex.

The main metabolites of aniline produced by the perfused liver are an acid-labile conjugate of aniline, accounting for 33% of the aniline added, and conjugates of p-aminophenol, accounting for 13% of the aniline added. If the haemoglobin-bound form of p-aminophenol is taken into account 20% of the aniline is metabolised to p-aminophenol.

Smith and Williams (1949) found that rabbits fed aniline excreted almost 50% of the dose as an acid-labile conjugate which it was suggested could have been a glucuronide. In contrast Parke (1960) reported that rabbits fed aniline excreted over 50% of a dose as p-aminophenol or its conjugates and only 5.5% could be detected as the aniline-N-glucuronide. It was observed however that large quantities of free glucuronic acid were excreted in the urine of these animals (Parke, 1960).

In the present study, mild acid hydrolysis liberated free aniline from the perfusion medium, possibly from an acid-labile conjugate, which may be aniline-N-glucuronide. Although aniline-N-glucuronide can be produced as a result of enzymatic activity it can also be formed spontaneously (Parke, 1960). In the present study SKF 525-A, which has been reported to inhibit the enzymes of glucuronide formation (Anders, 1971), reduced the formation of the acid-labile aniline conjugate by 62%.

After 3 h, 7% of the aniline administered was present in the perfusion medium in an unidentified form. Since only certain of the possible metabolites were being assayed it is probable that this fraction represents one or more of these other metabolites known to be formed. Traces of acetanilide and m-aminophenol are produced by rabbits fed aniline (Parke, 1966) and o-aminophenol is produced in the rat in the ratio of 1:6 to p-aminophenol (Parke, 1960). o-aminophenol could thus account for 3 to 4% of the aniline, remaining in the perfusate, after 3 h perfusion. Phenylsulphamic acid accounted for 5.5% of a dose of aniline fed to rabbits (Parke, 1960) and so may form part of the unidentified metabolites in the perfused liver of the rat.

Over 7% of the aniline was excreted in the bile produced by the perfused rat liver over 3 h. Almost half of this was as an acid-labile conjugate of aniline. Hirom, Millburn, Smith and Williams (1972) have shown that the threshold for active excretion of compounds into the bile of the rat is 325 ± 50. Thus aniline, with a molecular weight of 93, is well below the threshold and most free aniline in the bile probably results from passive diffusion into the cannuliculi. The molecular weight of aniline-N-glucuronide is 269 and is thus on the threshold for active excretion. Compounds can enter the bile both from the hepatocytes and from the blood (Back and Calvey, 1972). Thus the presence of a trace of free p-aminophenol in the bile may derive from compound that has diffused from the hepatocyte, and supports the hypothesis that most free p-aminophenol is either conjugated in the cell

or binds with haemoglobin in the perfusion medium.

After 3 h perfusion 10% of the 3H-aniline added to the perfused liver had been lost by non-metabolic processes, and was not recoverable from the perfusion medium in any form. Krieglstein, Krieglstein and Urban (1972) have shown that lipophilic drugs are readily adsorbed on to many types of plastic, particularly silicone rubber, and as much as 50% is adsorbed on to polyethylene in the case of very lipophilic compounds. When the polyethylene and silicone rubber of the perfusion circuit were extracted after perfusion with 3H-aniline a total of less than 1% of that added could be recovered. Thus neither aniline nor its metabolites are adsorbed to any appreciable extent by the material of the perfusion circuit. Over half of the aniline lost by non-metabolic processes was lost by transexudation across the liver capsule into the liquid paraffin in the perfusion chamber. This loss may represent free aniline or a metabolite, its identity was not investigated. It has been suggested that transexudation across the liver may occur in man after perfusion of actinonycin D directly into the liver (Mulcare, Solis and Fortner, 1973). The rest of the aniline lost in this non-metabolic fraction could be quantitatively accounted for as residue remaining in the sampling pipettes, glassware and perfusion apparatus. This was assessed by soaking all contaminated material, after perfusion with 3H-aniline, in a large volume of detergent and determining the activity in the extract.

As already discussed in the Results (Section 5.2.2) the

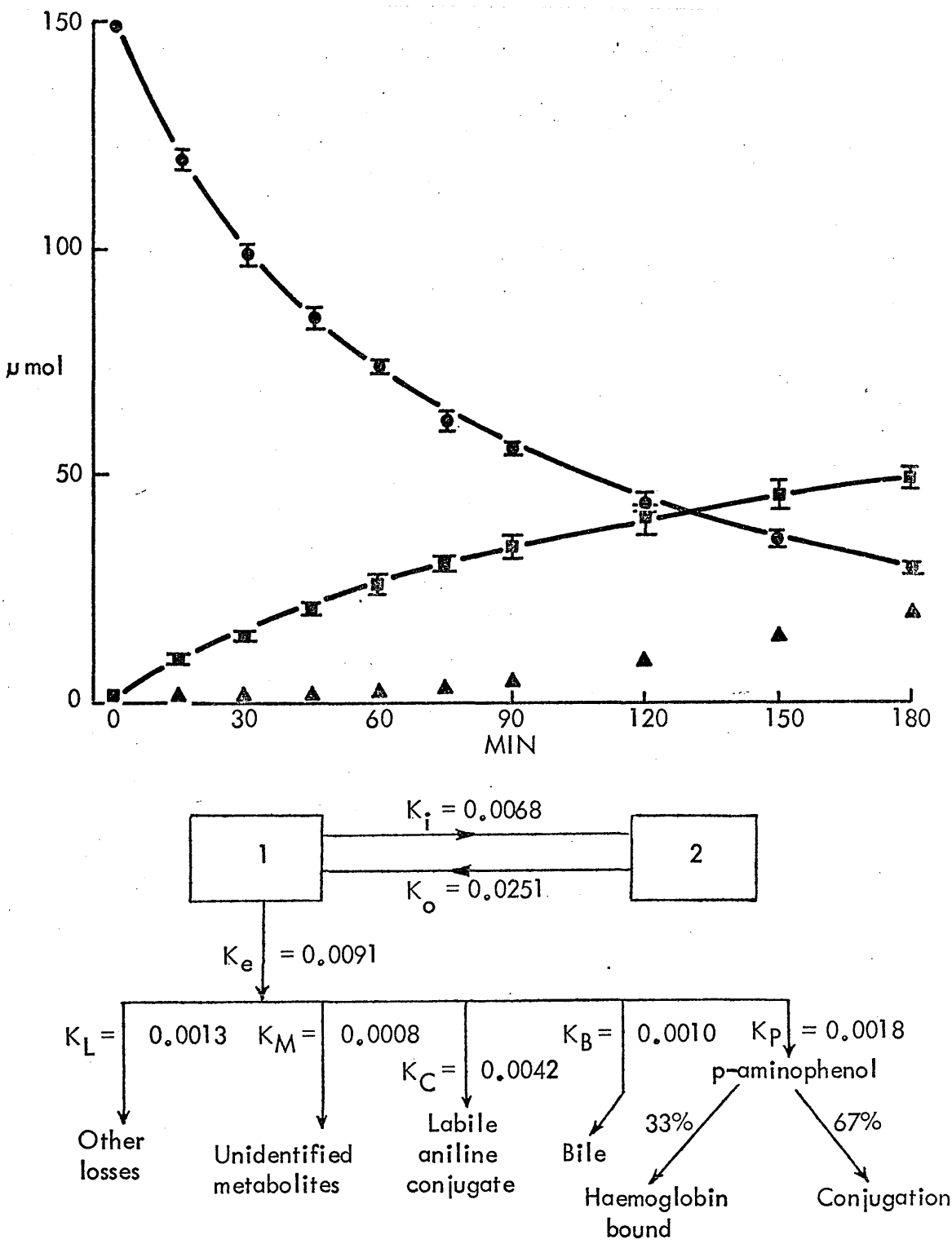


Figure 39

A suggested model to describe the distribution and metabolism of aniline in the isolated perfused rat liver. The points in the upper panel show the experimental data on which the model was constructed as described in Appendix I. Aniline removal (●), acid-labile conjugate appearance (■) and the appearance of p-aminophenol conjugates (▲), bars represent S.E.M. of four perfusions. The lines are the theoretical predictions based on the model shown in the lower panel. The rate constants, K, are in min^{-1}. No predicted curve could be obtained for p-aminophenol.

removal of aniline from the perfusion medium falls into two phases. Kinetic analysis of the data enabled a model to be derived as described in Appendix I. This model is shown in Fig. 39. The experimental data for both the removal of aniline and the formation of its metabolites were in close accord with the predicted values, as also shown in Fig. 39. All of the processes of metabolism and excretion of aniline appeared to occur from compartment 1. Thus compartment 1 probably corresponds to the whole system, the intracellular water being in rapid equilibrium with the perfusion medium, so that it does not represent a separate compartment. A similar situation has been shown to exist for propranolol in the perfused liver (Evans et al, 1973). Thus access of substrate to the microsomal enzymes does not appear to be rate limiting in this system.

This leaves compartment 2 unaccounted for. It seems most probable that it represents some form of binding of free aniline. Non-specific binding to hepatic tissue proteins has been reported for propranolol (Evans et al, 1973; Shand, Branch, Evans, Sies and Wilkinson, 1973). It has been shown that aniline does not bind to serum albumin (Bickel and Bovet, 1962). Aniline is a type II substrate (Schenkman, 1967) and so will bind to cytochrome P-450 at the non-metabolic type II site. This site could represent compartment 2 for aniline in the perfused liver although this remains highly speculative.

SKF 525-A inhibits many drug-metabolising enzymes including glucuronyl transferase (Anders, 1971). In the perfused liver it inhibits p-aminophenol formation completely,

i.e. $K_p = 0$. Kinetic analysis showed that the rate constant, K_c, for the formation of the acid-labile aniline conjugate was reduced to 0.0016 min^{-1}. Using these new values of K_c and K_p in the equations of Appendix I it was found that K_o was reduced to 0.0170 min^{-1} and K_i was increased to 0.0086 min^{-1}. Thus SKF 525-A had inhibited the two main pathways of aniline metabolism in the perfused liver and increased the size of compartment 2 by almost 100%. Schenkman (1970) has suggested that at increasing concentrations of aniline, binding of this substrate to the non-metabolic type II site may be facilitated, possibly due to a solvent effect. SKF 525-A, by inhibiting aniline metabolism, would increase the concentration of aniline present and might thus increase type II binding. It has also been shown that compounds such as SKF 525-A, that produce a type I difference spectrum, can increase the binding of type II compounds, probably by preventing the small degree of binding to type I sites that normally occurs with these substrates (Schenkman, 1970; Gorrod and Temple, 1973). Although the type I component of the difference spectrum of type II substrates is not very great, it has been shown by these authors that blocking it results in a large increase in type II binding. This could thus possibly account for the increase in the size of compartment 2 caused by SKF 525-A.

6.3 Effect of catecholamines on drug metabolism

6.3.1 The isolated perfused rat liver

The effect of catecholamines on drug metabolism in the

perfused liver has not previously been reported. Both adrenaline and noradrenaline inhibit the metabolism of hexobarbitone but have no effect on the metabolism of aniline. The known differences in the binding of these two substrates to cytochrome P-450 and the effect this has on their respective metabolism (Anders, 1971) might explain the different effects of catecholamines on the metabolism of the two substrates. However the mechanism whereby catecholamines might produce this effect is not known.

Most of the metabolic effects of catecholamines are mediated by cyclic AMP and it has recently been shown that cyclic AMP inhibits hexobarbitone metabolism in the perfused liver (Weiner et al, 1972a). Catecholamines could thus be acting by the liberation of cyclic AMP.

Although no significant direct effect of catecholamines on drug metabolism by the microsomal fraction was found (Dixon et al, 1964) Cooper and Rosenthal (1962a) found that noradrenaline would increase steroid hydroxylation by a microsomal oxidase of adrenal homogenates. Catecholamines may therefore be acting by a direct interaction with the enzymes of drug metabolism.

Long term pre-treatment of rats, for 24 h or more, with adrenaline (Fouts, 1962) or noradrenaline (Dixon et al, 1964) inhibits the metabolism of both aniline, aminopyrine and hexobarbitone by the hepatic microsomal fraction although Kato and Gillette (1965) found that adrenaline inhibited the microsomal metabolism of only type I substrates whilst the

metabolism of type II substrates was unaffected in male rats.

Although the effects of catecholamines on drug metabolism have not previously been described several workers have investigated the influence of catecholamines on the clearance of a number of substances by the liver. Both adrenaline and noradrenaline were found to reduce the clearance of bromsulphthalein in the isolated dog liver (Andrews, Maegraith and Richards, 1956) and it was later shown that the extraction of $CrPO_4$ was reduced by catecholamines in the perfused rat liver (Brauer, Leong, McElroy and Holloway, 1956). A similar effect of catecholamines upon the hepatic extraction of indocyanine green has recently been reported in the anaesthetised cat (Krarup, 1973). Catecholamines may be acting on drug metabolism by a similar mechanism, either by reducing substrate uptake or by causing vasoconstriction and thus reducing the supply of substrate.

Thus it was concluded from these known effects of catecholamines that their effect on hexobarbitone metabolism in the isolated perfused rat liver could be attributed to one or more of the following effects.

a) Direct inhibition of hexobarbitone binding to cytochrome P-450 or some component of the microsomal electron transport chain.

b) Liberation of cyclic AMP which could then inhibit drug metabolism.

c) Alteration in uptake of substrate at the site of metabolism.

d) Alteration in supply of substrate or other co-factor through an effect on blood flow.

These possibilities will be considered individually.

6.3.2 Direct effect of catecholamines on drug metabolism

Although Dixon et al (1964) reported that catecholamines caused a slight and non-significant increase in the rate of microsomal drug metabolism when added directly, the current work has shown that even at concentrations as high as 10^{-3}M catecholamines have no direct effect upon the metabolism of either aniline or hexobarbitone by the microsomal fraction. Hepatic drug metabolism thus differs from adrenal steroid hydroxylation which is stimulated by noradrenaline (Cooper and Rosenthal, 1962a).

It was found that high concentrations of noradrenaline could inhibit the binding of type II substrates to cytochrome P-450 in microsomal suspensions. Even higher concentrations of noradrenaline were necessary to inhibit the binding of hexobarbitone, and it may be that at these concentrations the effect was non-specific. Inhibition of the binding of type II substrates rarely affects their metabolism (Jefcoate et al, 1969). Noradrenaline itself produced a type II difference spectrum with a K_s of 1 mM and Liebman, Hildebrandt and Estabrook (1969) have shown that type II substrates produce competitive inhibition of the binding of type I substrates, although they never produce competitive inhibition of the binding of type II substrates rather a non-competitive inhibition. However it was clearly shown that catecholamines

did not inhibit the microsomal metabolism of hexobarbitone, either directly or by inhibition of the enzymes of the microsomal electron transport chain.

6.3.3 Possible effect through the release of cyclic AMP

The effects of cyclic AMP on hepatic drug metabolism have been reviewed in Section 2.5. Many of the metabolic effects of catecholamines are mediated through the release of cyclic AMP (Sutherland and Robison, 1966). Therefore it could be that catecholamines were inhibiting drug metabolism through this mechanism. Cyclic AMP is thought to act at the level of the intact cell membrane to liberate some inhibitory substance, possibly free fatty acids, and this then inhibits the enzymes of drug metabolism (Weiner, 1973). In agreement with the findings of Weiner et al (1972b) it was shown in the present study that although cyclic AMP does not inhibit microsomal drug metabolism directly, even at high concentrations, it does inhibit metabolism by liver slices. However in the present study catecholamines had no effect on the metabolism of either type I or type II substrates by this system. Weiner and his associates (Weiner et al, 1970, 1972a, b) investigated the effects of cyclic AMP on the metabolism of type I substrates only, p-chloromethylaniline which they used as a type II substrate is probably a type I substrate in rat microsomes (Ross and Oppelt, 1973) despite findings to the contrary with guinea pig liver (Kupfer and Orrenius, 1970). However it has been clearly shown that cyclic AMP administered to male rats inhibits the metabolism of both type I and type II substrates (Ross and Oppelt, 1971, 1973; Ross et al, 1973). In contrast,

in the present study, with the perfused liver, catecholamines inhibit the metabolism of type I substrates only.

Papaverine is a potent inhibitor of phosphodiesterase (Goren and Rosen, 1972). It should therefore potentiate the effects of any cyclic AMP released by catecholamines in the perfused liver by preventing its metabolism. However in the presence of high concentrations of papaverine adrenaline failed to have any further inhibitory effect on hexobarbitone metabolism, and it did not develop any inhibitory effect on aniline metabolism. If adrenaline was inhibiting hexobarbitone metabolism by the release of cyclic AMP, then blocking the breakdown of cyclic AMP should potentiate the action of adrenaline. This has been observed when glucagon, which also liberates cyclic AMP (Sutherland and Robison, 1966) was added to the perfused liver in the presence of the phosphodiesterase inhibitor theophylline (Weiner et al, 1972a). Glucagon alone produced a slight inhibition of hexobarbitone metabolism but was much more potent in the presence of theophylline (Weiner et al, 1972a).

How can the difference on drug metabolism be explained between adrenaline and glucagon, both of which liberate cyclic AMP? Weiner et al (1972a) found that a relatively high threshold concentration of cyclic AMP was necessary to produce inhibition of drug metabolism. A perfusate concentration of 3×10^{-7}M glucagon could only produce a 20% reduction in the metabolism of hexobarbitone by the perfused liver and the concentration of dibutyryl cyclic AMP necessary to produce a 50% inhibition was 10^{-4}M. In the isolated perfused rat liver

glucagon, at concentrations as low as 3 x 10^{-9}M, will produce a thirty-fold increase in cyclic AMP levels (Kuster, Zapf and Jakob, 1973). Exton, Robison, Sutherland and Park (1971) found that maximum cyclic AMP production occurred at a glucagon concentration of 2 x 10^{-8}M, with an 85-fold increase in cyclic AMP levels (Kuster et al, 1973). In contrast, a maximum two-fold increase in cyclic AMP levels was produced by adrenaline, 5 x 10^{-7}M, (Exton et al, 1971). This increase was transient, lasting only five minutes unlike the glucagon effect, which lasted for over 30 min (Exton et al, 1971; Kuster et al, 1973). Cyclic AMP levels could not be elevated further even by the addition of theophylline (Exton et al, 1971). In the perfused liver noradrenaline was slightly less potent than adrenaline at releasing cyclic AMP (Exton et al, 1971).

Thus although catecholamines release cyclic AMP in the perfused liver, the amount of nucleotide released is very low. To produce inhibition of drug metabolism levels of cyclic AMP about 25-fold above normal are necessary. All the evidence thus seems to preclude the possibility that the mode of action of catecholamines on inhibition of drug metabolism in the perfused liver is through the release of cyclic AMP.

6.3.4 Alteration of substrate uptake by hepatocytes

Among several possibilities suggested by Andrews et al (1956) to explain the decrease in bromsulphthalein clearance observed in the perfused liver after the addition of catecholamines was that catecholamines could be altering the uptake

of bromsulphthalein into the hepatocytes. Such a mechanism could explain some of the results observed in this study if different uptake mechanisms exist for aniline and hexobarbitone, or alternatively if uptake is rate limiting for hexobarbitone metabolism but not for aniline metabolism. However in the studies of Cooper et al (1954) there was no evidence of an uptake process for hexobarbitone across the plasma membrane in liver slices. In the present study catecholamines had no effect on the rate of disappearance of aniline or hexobarbitone in liver slices. Thus the effects of catecholamines on drug metabolism cannot be due to the reduction in uptake of substrate into the hepatocyte, even if such a process exists.

6.3.5 Effect on blood flow

It has been found that Ca^{2+} is necessary for the contraction of smooth muscle (Bohr, 1964). When Ca^{2+} was omitted from the perfusion medium the pressor effects of catecholamines were almost completely abolished. Similarly there was a loss of the inhibitory effects of catecholamines upon hexobarbitone metabolism. It was considered possible therefore, that this effect on drug metabolism might be related to the vascular effects of catecholamines in the perfused liver.

In almost all previous studies in which the effects of sympathomimetics on hepatic blood flow have been studied in the perfused liver, a constant pressure system has been employed (Brauer, 1963; Greenway and Stark, 1971) and those studies carried out in vivo have involved anaesthetised

animals in which vasomotor reflexes are abolished (Ho, 1972). Catecholamines cause a pronounced vasoconstriction of the hepatic vasculature, and in a constant pressure system this will result in a considerable reduction in total blood flow through the liver (Northrop and Parks, 1964). However when catecholamines are administered in vivo there is a reflex change in blood flow to the liver so that the total blood flow through the organ is unchanged or even increased, despite a splanchnic vasoconstriction (Andrews, 1957). In his review Brauer (1963) likened the liver in vivo to the organ in a perfusion system in which the total blood flow through the organ is determined externally. Circulatory regulation within such a system would then depend on modifications of blood flow distribution within the organ. Thus the liver perfused at a constant flow rate provides a system that more closely resembles the situation in vivo than perfusion at a constant pressure. It also enables the effects of catecholamines on blood flow distribution to be investigated, rather than just measuring changes in the total blood flow through the organ.

Considerable controversy exists as to whether catecholamines can cause a redistribution of blood flow within the liver. Several workers have reported that such a phenomenon does occur (Daniel and Prichard, 1951c; McCuskey, 1966) whereas others claim that there is no such phenomenon (Greenway and Oshiro, 1972b). The evidence available to date has been reviewed in Appendix II.

The possibility that catecholamines were causing a

redistribution of hepatic blood flow has been investigated in the present study. X-radiography of the liver perfused with adrenaline revealed that adrenaline does, indeed, cause a change in the distribution of perfusate flow within the liver, and perfusion of the liver with Indian ink and adrenaline followed by histology showed that this effect is manifest as a redistribution of perfusate away from peripheral areas of the lobes to more central regions, with many small blood vessels in the periphery no longer being perfused.

How can a redistribution of blood flow by catecholamines explain their selective inhibitory effect on hexobarbitone metabolism in the perfused liver? There are several possible explanations. The perfusion medium in the isolated liver is responsible for the transport of both substrates and molecular oxygen to the hepatocyte. Molecular oxygen is necessary for the oxidative metabolism of both hexobarbitone and aniline (Estabrook, 1971).

If a reduced supply of substrate to peripheral hepatocytes is responsible for the inhibitory effects of catecholamines it might be expected that the metabolism of both aniline and hexobarbitone would be inhibited. However compounds that are rapidly cleared by the perfused liver are susceptible to changes in blood flow whereas for those compounds whose clearance is slow changes in blood flow are offset by increases in clearance (Branch, Shand and Nies, 1973a, b; Nies, Evans and Shand, 1973; Branch, Nies and Shand, 1973). Hexobarbitone is metabolised at more than three times the rate of aniline in the perfused liver so that it is possible that during the

restricted circulation caused by catecholamines aniline metabolism by the central hepatocytes increases to compensate for decreased metabolism by peripheral cells whereas hexobarbitone metabolism could already be maximal and so would not increase further to offset decreased metabolism in the periphery. It would be fortuitous however if the two effects were to exactly balance out, as they would have to, since no net change in aniline metabolism occurs.

Another possibility is that the enzymes responsible for aniline and hexobarbitone metabolism are distributed unevenly throughout the lobes. If the enzymes of aniline metabolism are located in the centre of the lobes and those of hexobarbitone metabolism in the periphery the redistribution of blood flow caused by catecholamines would result in a selective inhibition of hexobarbitone metabolism. However a search of the literature failed to reveal any evidence that any microsomal drug-metabolising enzymes are unevenly distributed throughout the lobes, although there is good evidence that at least some of the enzymes of drug metabolism are unevenly distributed throughout the liver lobule (Wattenburg and Leong, 1962; Rappaport, 1973).

The third possibility to be considered is that a peripheral hypoxia caused by a redistribution of blood flow selectively inhibits hexobarbitone metabolism. Exposure of rats to hypoxia causes a subsequent decrease in the metabolism of type I substrates by the microsomal fraction whereas the metabolism of type II substrates is unaffected (Kato and Gillette, 1965). Incubation of the microsomal fraction in graded oxygen concentrations has shown that aniline metabolism

is relatively resistant to the effects of hypoxia, even at very low P_{O_2} values, whereas the metabolism of type I compounds is inhibited at P_{O_2} values even slightly below normal (Kampffmeyer and Kiese, 1964). The metabolism of hexobarbitone is also reduced in the perfused liver by hypoxia (Cumming and Mannering, 1970; Bock et al, 1972). However no studies have been carried out on the effects of hypoxia on the metabolism of a type II substrate by the perfused liver.

The liver in vivo receives blood with a low oxygen content so that it may normally be close to hypoxia (Cumming and Mannering, 1970). The total oxygen content of arterial blood, measured by the Lex-O_2-Con total oxygen analyser, is between 10 and 12 vol% and in venous blood between 5 and 7 vol% (Valeri, Zaroulis, Marchionni and Patti, 1972). The hepatic artery accounts for less than 20% of blood flow to the liver (Fischer, 1963), thus the mean oxygen content of blood reaching the liver is no more than 6.8 vol%. In the present study the total oxygen content of the perfusion medium measured in the same way is 8.6 vol%. Since the liver is perfused at a flow rate similar to that in vivo (Ross, 1972) the level of oxygenation of the liver must closely resemble that in vivo since, in vivo, blood entering the sinusoids is almost entirely mixed venous blood (Greenway and Stark, 1971).

Thus catecholamines, by causing a redistribution of blood flow within the liver, may cause hypoxia of peripheral hepatocytes, which would result in a selective inhibition of hexobarbitone metabolism since the metabolism of hexobarbitone is very susceptible to hypoxia and that of aniline is not. In

liver slices, which by their nature are not dependent on blood flow, oxygen being supplied by diffusion, the metabolism of neither substrate was affected by the catecholamines adrenaline and noradrenaline.

It has been suggested that drug metabolism in the perfused liver may compete with intermediary metabolism for the available molecular oxygen (Cumming and Mannering, 1970; Bock et al, 1972) and evidence to this effect was provided by Scholz, Hansen and Thurman (1973) who found that the metabolism of type I substrates in the perfused liver reduced gluconeogenesis by competing for molecular oxygen. Although the reverse effect has not been reported, at high rates of intermediary metabolism oxygen utilisation in the perfused liver can increase three-fold (Woods and Krebs, 1972). Both adrenaline and noradrenaline stimulate intermediary metabolism (Sutherland and Robison, 1966) and increase oxygen utilisation in the liver (Andrews and Glockling, 1956) adrenaline being the more potent compound. Since the liver is normally close to hypoxia such an overall increase in oxygen utilisation would enhance an hypoxia caused by the blood flow redistribution caused by catecholamines and would explain why adrenaline causes a greater inhibition of hexobarbitone metabolism in the perfused liver than noradrenaline, despite the similarity in the magnitude of the pressor response caused by the two catecholamines.

One possible criticism of the present investigation is that removal of Ca^{2+} from the perfusion medium would affect the metabolic effects of catecholamines as well as the vascular effects. There is evidence that the mechanism of stimulation

of adenyl cyclase by glucagon in the liver differs from that for adrenaline, the glucagon system not requiring Ca^{2+} unlike that for adrenaline (Bitensky, Russell and Robertson, 1968; Marinetti, Ray and Tomasi, 1969). However more recent evidence in other tissues suggests that stimulation of adenyl cyclase by catecholamines does not depend upon Ca^{2+}, and that Ca^{2+} may in fact inhibit the enzyme (Andersson, 1972; Matsuzaki and Dumont, 1972). In the isolated perfused rat liver the metabolic effects of catecholamines were not affected by omitting Ca^{2+} from the perfusion medium (Exton et al, 1971). It was concluded from this that if Ca^{2+} was necessary for the metabolic effects of catecholamines there was sufficient in the tissue and the removal of Ca^{2+} from the perfusion medium would only affect the vascular effects of these compounds.

The vessels of the portal tree have been shown to be sensitive to sympathomimetic compounds, which cause a redistribution of blood flow. In addition many of the vessels of the hepatic portal tree have a sympathetic innervation (Brauer, 1963) and the possibility that this innervation plays a role in regulating blood flow distribution within the liver in vivo is discussed in Appendix II. It seems reasonable to suggest that a mechanism for the redistribution of blood flow could exist in vivo which would be sensitive to catecholamines released from the adrenals, sympathetic nerve endings, or both. This would either directly or indirectly modify the activity of the drug-metabolising system. It has recently been found that hypoxic hypoxia decreases drug metabolism to a greater extent than CO-hypoxia and it was suggested that this could be due to alterations in blood flow within the liver (Montgomery and Rubin, 1973a, b). During enzyme induction by phenobarbitone

part of the increase in *in vivo* drug metabolism has been attributed to an increased blood flow through the liver (Ohnhaus, Thorgeirsson, Davies and Breckenridge, 1971) which may be caused by relaxation of sphincteric tone in the liver blood vessels.

An interesting finding by Vainio (1973) is that in endotoxin shock the metabolism of type I substrates by the microsomal fraction was immediately inhibited whereas that of type II substrates was initially elevated. The author suggested that this decreased metabolism of drugs could also decrease the metabolism of naturally occurring steroids which have a therapeutic effect in this syndrome. It may be that there is a reduction in drug metabolism as part of the body's defence mechanism to maintain elevated levels of circulating steroids. In stress, as well as a release of steroids, there is an increase in circulating catecholamines released from the adrenals (Irvine, Cullen, Stewart, Ewart and Baird, 1968) and it may be that one of the roles of the catecholamines is to cause redistribution of blood flow within the liver and thereby limit the destruction of these steroids.

6.3.6 Pre-treatment of rats with catecholamines

When rats were pre-treated with noradrenaline the metabolism of both aniline and aminopyrine by the microsomal fraction was increased within 30 min, whereas pre-treatment with adrenaline caused a slight inhibition in the metabolism of both substrates. It has been found previously that pre-treatment of rats with adrenaline (Fouts, 1962) or noradrenaline (Dixon *et al*, 1964) increased the microsomal metabolism

of type I substrates within 2 h. The metabolism of type II substrates was not investigated at time intervals less than 2 h. These findings were not pursued as the authors were concerned with the longer term effects of catecholamine pre-treatment on drug metabolism.

Although Gielen and Nebert (1972) found that noradrenaline induced benzpyrene hydroxylase in cultured foetal rat liver cells the short time course of events in the present study, maximum effect within 30 min, almost certainly precludes enzyme induction as the mechanism of action (Conney, 1967). Thus catecholamines must be acting by either a direct effect on some component of the microsomal drug-metabolising system to cause an alteration in activity, or affecting the release of some endogenous compound which in turn is responsible for modifying the activity of some portion of the microsomal fraction.

Since drug-metabolising activity was studied in an isolated microsomal fraction, to which was added optimum concentrations of all the necessary co-factors for drug metabolism, catecholamines could not be acting by altering the endogenous levels of some co-factor. Examination of the components of the microsomal electron transport chain revealed that catecholamine pre-treatment does not affect cytochrome P-450 levels or the activity of NADPH-cytochrome c reductase. Cytochrome b_5 levels are reduced 30 min after pre-treatment with either adrenaline or noradrenaline. The significance of this finding to the effects of catecholamines on drug metabolism is unknown. The role of cytochrome b_5 in drug metabolism is however debatable

and has been discussed in Section 2.2.4. It appears unlikely that catecholamines are affecting drug metabolism by directly modifying some component of the drug-metabolising system.

An alternative mechanism is that catecholamines were altering the levels of some endogenous modifier of drug metabolism. This is quite possible. For example, corticosterone inhibits drug metabolism (Radzialowsky and Bousquet, 1968) and noradrenaline inhibits the secretion of ACTH in the rat, thereby reducing the plasma levels of corticosterone (Scapagnini, Van Loon, Moberg, Preziosi and Ganong, 1972). Thus noradrenaline, acting by reducing the levels of an inhibitor, might appear to stimulate drug metabolism. However adrenalectomy did not alter the effects of pre-treatment with either adrenaline or noradrenaline (Boobis and Powis, 1973) so that adrenal steroids do not appear to be involved. Catecholamines may act, however, by altering the levels of some other modifier but how this would act without affecting the components of the microsomal drug-metabolising system is not clear.

Experiments with 3H-noradrenaline revealed that high concentrations of the catecholamine or its metabolites were present in the liver at the time of isolation of the microsomal fraction, although very little of the label was bound to the microsomes themselves. It was therefore unlikely that catecholamines were exerting their effects on drug metabolism by direct interaction with the microsomal enzymes after preparation of the microsomes. This was further supported by incubating the microsomal fraction with catecholamines. There

was no significant alteration in the metabolism of either aniline or aminopyrine.

The presence of catecholamines in the liver homogenate during preparation may have some effect on the activity of the microsomal fraction. This was investigated by washing the microsomal fraction in sucrose containing catecholamine. This caused changes in drug-metabolising activity similar to those observed after pre-treatment of rats with catecholamines. Washing microsomes in sucrose alone resulted in a reduction in drug-metabolising activity, which was prevented by noradrenaline. Adrenaline however increased the loss of activity that occurred on washing the microsomes. The effects of catecholamines on drug metabolism during washing of the microsomes could not be properly evaluated until the effects of washing itself had been studied in more detail. The remaining part of the study was concerned with an investigation into this aspect of drug metabolism.

6.4 Effect of washing microsomes

6.4.1 Metabolism of type I and type II substrates

Washing microsomes in sucrose buffer causes a reduction in the metabolism of type I substrates but very little change in the metabolism of type II substrates. Catecholamines largely prevent this loss of activity. Catecholamines are known inhibitors of lipid peroxidation (Privett, 1962) so the effects of another inhibitor of lipid peroxidation, EDTA (Chatterjee and McKee, 1965) were investigated. When micro-

somes were washed in EDTA no loss of drug metabolism occurred and the metabolism of both type I and type II substrates was even increased compared to control values. It may be that EDTA, either itself or by some chelating action, alters the configuration of the type I binding site to increase the binding of substrates, and in fact increased binding of type I substrates was observed in microsomes washed in EDTA. When other inhibitors of lipid peroxidation were tested Mn^{2+}, BHT and EGTA had some protective effect but this was less than that of EDTA. However vitamin E and its salts and BHT are not water soluble so that there may have been insufficient compound in solution to have much of an effect. Mn^{2+}, although inhibiting enzymatic lipid peroxidation, has little effect on non-enzymatic lipid peroxidation (Kitabchi, McCay, Carpenter, Trucco and Caputto, 1960).

Several reports in the past have shown that there is competition between some hepatic microsomal mixed function oxidase reactions and enzymatic lipid peroxidation (Orrenius et al, 1964; Gram and Fouts, 1966; Lewis et al, 1967; Carpenter, 1972). However in most instances, although drug metabolism was shown to inhibit lipid peroxidation, an inhibition of the lipid peroxidation did not stimulate drug metabolism as might have been expected (Gram and Fouts, 1966; Carpenter, 1972). More recently Kamataki and Kitagawa (1973) have shown that inhibition of lipid peroxidation can stimulate the microsomal metabolism of foreign compounds. Lewis et al (1967) had already shown that microsomal epoxidation could be increased by inhibitors of lipid peroxidation but that if microsomes were pre-incubated with NADPH the loss of epoxidation activity could not be completely reversed by inhibitors

of lipid peroxidation. It appears that there may thus be two different components of inhibition, one a simple competition between enzymatic lipid peroxidation and drug metabolism, for NADPH or oxygen, and the second a structural alteration caused by either enzymatic or non-enzymatic peroxidation of the lipid of the microsomal membrane that results in loss of activity.

Although it was found that inhibitors of lipid peroxidation did cause some increase in drug metabolism during 30 min incubation at 37°C, and would inhibit the formation of malonaldehyde, a product of lipid peroxidation, this effect was insufficient to account for the effect of washing the microsomes. Peroxidation of the microsomal lipid increased during the washing of microsomes in sucrose, and was inhibited by EDTA. Because of the absence of NADPH during the washing process and because it occurred at 2°C, it is probable that the process was non-enzymatic. A similar process occurs when microsomes from vitamin E deficient rats are incubated with the post-microsomal supernatant (Kitabchi et al, 1960). In this instance material in the supernatant stimulated lipid peroxidation by the microsomes, and the process was non-enzymatic. It was suggested that some ion in the supernatant, possibly Fe^{2+} is an activator. In the present investigation it was found that none of the components of the microsomal electron transport chain were affected by washing. Hochstein and Ernster (1964) found that NADPH-cytochrome c reductase was not affected by lipid peroxidation but both cytochrome b_5 (Tappel and Zalkin, 1960) and cytochrome P-450 (Levin, Lu, Jacobson, Kuntzman, Poyer and McCay, 1973) have

been shown to exhibit lability to prolonged incubation during which lipid peroxidation occurs.

In the present study the relatively specific loss of the metabolism of type I substrates, the lack of effect on the components of the electron transport chain and, in addition, the changes observed in the binding of substrates by cytochrome P-450 led to the conclusion that perhaps lipid peroxidation and the binding of substrates by cytochrome P-450 were connected. Phospholipids have recently been shown to be involved in the type I binding site of cytochrome P-450 and the phospholipid involved was identified as phosphatidylcholine (Chaplin and Mannering, 1970). Lipid peroxidation may therefore cause degradation of phosphatidylcholine with loss of type I binding, and if lipid peroxidation is prevented type I binding is protected.

Although type I binding is not obligatory for metabolism, it is facilitative (Chaplin and Mannering, 1970) so that a reduction in type I binding will cause a reduction in the metabolism of type I substrates. Washing microsomes increases non-enzymatic lipid peroxidation, perhaps because some endogenous inhibitor in the post-microsomal supernatant is lost, and this causes decreased binding of type I substrates resulting in a decreased metabolism. EDTA, by inhibiting lipid peroxidation, perhaps by chelation of Fe^{2+}, prevents these effects.

This raises the question of whether there is a similar loss of activity during the original isolation of the micro-

somal fraction. It was found that the post-microsomal supernatant had similar protective effects to EDTA during the washing process. There thus appears to be an endogenous inhibitor of lipid peroxidation present in the post-microsomal supernatant. Grimwade, Lawson and Boyd (1971) found a heat-stable, thiol-containing, inhibitor of enzymatic lipid peroxidation in rat liver supernatant and Grinna and Barber (1973) have found a heat-labile inhibitor of lipid peroxidation, possibly protein, in rat hepatic post-microsomal supernatant. It may be that several different compounds can cause inhibition of lipid peroxidation, all acting by non-specific binding of ions normally necessary for peroxidation. It may be, however, that specific modes of action are involved. Although there are inhibitors present it is probable that some non-enzymatic lipid peroxidation does occur during the original isolation of microsomal fraction. If this were inhibited by catecholamines or their metabolites, which are present in the liver supernatant in high concentrations after pre-treating rats with catecholamines, then there would be an apparent increase in drug-metabolising activity. If adrenaline has such an effect it appears to be masked by an additional inhibitory effect present during preparation of the microsomes, which is not observed when microsomes are incubated with adrenaline. This mode of action of catecholamines remains a possibility in view of the observed results and the known effects of the compounds, but it is as yet unproven, and some other mechanism may be, in part or totally, responsible for the observed effects of catecholamine pre-treatment on drug metabolism. Inhibition of lipid peroxidation by catecholamines has been suggested as their mode of action in stimulating steroid hydroxylation

in adrenal microsomes (Lewis et al, 1967).

The similarity between the effects of washing the microsomes and storage of the microsomes at 0°C leads to speculation that a common mechanism is involved in the loss of drug-metabolising activity. Both processes lead to a decreased metabolism of type I substrates and loss of type I binding. Similar effects of storage have been found by other workers (Shoeman et al, 1969; Hewick and Fouts, 1970). Chan and Terriere (1969) have shown that storage of microsomes at 4°C causes increased lipid peroxidation and that the decreased drug-metabolising activity in stored microsomes was due to degradation of lipid rather than an inhibitory effect of accumulated product of the reaction. In the present study EDTA and post-microsomal supernatant, both of which inhibit lipid peroxidation, prevented some of the loss of type I drug-metabolising activity after storage. It is thus possible that during storage lipid peroxidation occurs and the reaction causes degradation of phosphatidylcholine, necessary for the integrity of the type I site, and thereby causing a reduction in the metabolism of type I compounds.

6.4.2 Acetone enhancement of aniline metabolism

In the past few years considerable interest has centred on a group of compounds with the ability to enhance drug metabolism. These compounds will increase the specific activity of the microsomal fraction in metabolising type II compounds such as aniline (Anders, 1971). The mechanism of action of most enhancers is not known. One of the most

widely investigated enhancers is acetone, and since this compound is the only enhancing agent found endogenously, it can reach concentrations of 10^{-2}M in untreated diabetes (Solway, Trotter, Trotter and Malins, 1971), it was thought earlier in the investigation that the effects of catecholamine pre-treatment of rats on hepatic microsomal drug-metabolising activity could be due to enhancement in a manner analogous to that of acetone. However subsequent work soon revealed that catecholamines had no direct effect on drug metabolism. It is still possible that some endogenous compound liberated by catecholamines and present in liver homogenates can enhance drug metabolism.

Because of these early lines of thought the effect of washing on the acetone enhancement of aniline metabolism was investigated. Rather surprisingly acetone enhancement was affected in a manner similar to the metabolism of type I compounds. Washing the microsomes almost completely abolished acetone enhancement, and EDTA or post-microsomal supernatant prevented this loss of activity. Storage of the microsomal fraction, while not causing a great reduction in aniline metabolism, completely abolished acetone enhancement.

Recently it has been shown that acetone promotes the type I binding of type II substrates and that degradation of phosphatidylcholine, which causes loss of type I binding, also inhibits acetone enhancement (Vainio and Hänninen, 1972). Thus the increase in aniline metabolism caused by acetone more closely resembles the metabolism of type I compounds in properties than it does the metabolism of type II substrates.

It is therefore probable that acetone causes enhancement of type II substrates by promoting their binding to type I sites, and since type I binding stimulates the reduction of the cytochrome P-450 - substrate complex (Gigon et al, 1969) this will cause increased metabolism.

Although acetone was reported not to cause a spectral change (McLean, 1967; Anders, 1972) in the present investigation it was found to produce a type II spectral change, an observation that was subsequently substantiated in a paper by Vainio and Hänninen (1972). Since type II substrates inhibit the reduction of the cytochrome P-450 - substrate complex (Gigon et al, 1969) it is possible that acetone inhibits the metabolism of type I compounds by this mechanism. The inhibitory effect of acetone on aniline metabolism could be more than overcome by the enhancing effect of type I binding of a substrate that was previously inhibiting its own metabolism.

APPENDIX I

Curve stripping and model fitting

This appendix describes the derivation of a model for the removal of aniline by the isolated perfused liver.

I.1 Symbols used

The symbols used in the equations that follow are:

- t time, min
- n number of compartments in the system
- q_j amount of compound in compartment j, μmol
- K rate constant for transport from a compartment, min^{-1}
- $q_j(0)$ amount of compound in compartment j at t = 0, μmol
- X_ι coefficient of the ιth exponential term
- λ_ι constant of the ιth exponential term
- K_i rate constant for transport into compartment 2, min^{-1}
- K_o rate constant for transport out of compartment 2, min^{-1}
- K_e rate constant for transport leading to loss, min^{-1}

The other symbols used are explained in the text.

I.2 Curve stripping

In a multi-compartment system, the mathematical model for the distribution of a compound can be expressed (Atkins, 1969)

$$q_\iota = \Sigma X_j e^{-\lambda_j t} \quad (j = 1, \ldots, n)$$

As t tends to a large value, so

$$\Sigma X_j e^{-\lambda_j t} \rightarrow X_n e^{-\lambda_n t}$$

i.e.

$$q_\iota \simeq X_n e^{-\lambda_n t}$$

Taking $\log_{10}$

$$\log q_1 = \log X_n - 0.4343\lambda_n t \qquad (1)$$

If the data are plotted semi-logarithmically against t then a straight line through the last few points has an intercept X_n at t = 0 and a gradient λ_n. If the term $X_n e^{-\lambda_n t}$ is subtracted from the remaining data points not fitted to the line this gives the curve

$$q_i' = \Sigma X_j e^{-\lambda_j t} \quad (j = 1, \ldots, n-1)$$

The points are re-plotted and the process is repeated until all the terms have been resolved. The equation of the curve is now

$$q = X_1 e^{-\lambda_1 t} + \ldots + X_n e^{-\lambda_n t}$$

I.3 Model fitting

Graphical analysis of the data by curve stripping revealed that aniline disappearance was biphasic, and followed the equation

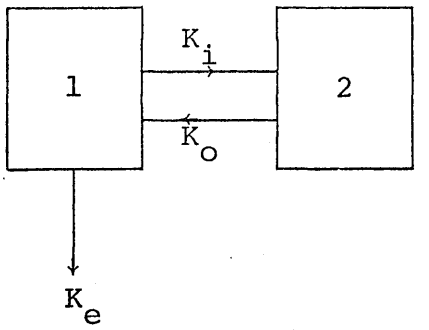
$$q = 50e^{-0.0343t} + 100e^{-0.0067t}$$

with q(0) = 150 μmol

50 and 100 were the intercepts of two exponentials on the Y-axis and 0.0343 and 0.0067 were the gradients of the two exponentials calculated from the expression

$$m = \frac{2.303(\log Y(0) - \log Y(t))}{t}$$

This means that aniline is probably distributed throughout a two compartment system. The simplest model that can be postulated, allowing for a loss of aniline from the system by metabolism, is represented thus, with rate constants as shown.



Let the amount of aniline in compartment 1 = q_1

and the amount of aniline in compartment 2 = q_2

The rate of change of substrate may be represented $\frac{d(q_j)}{dt}$ then the differential equations for the amount of aniline in each compartment at steady state can be represented

$$\frac{d(q_1)}{dt} = K_o q_2 - (K_i + K_e) q_1$$

$$\frac{d(q_2)}{dt} = K_i q_1 - K_o q_2$$

Since q_j is a function of time it may be represented by the function $f_j(t)$. Rewriting the expressions above

with $q_1 = f_1(t)$

and $q_2 = f_2(t)$, then

$$\frac{df_1(t)}{dt} = K_o f_2(t) - K_i f_1(t) - K_e f_1(t)$$

$$\frac{df_2(t)}{dt} = K_i f_1(t) - K_o f_2(t)$$

Now use the Laplace transformation

$F(s)$ for $f(t)$

and $s.F(s) - f(0)$ for $\frac{df(t)}{dt}$

and substitute, then

$$s.F_1(s) - f_1(0) = K_o F_2(s) - K_i F_1(s) - K_e F_1(s)$$

$$s.F_2(s) - f_2(0) = K_i F_1(s) - K_o F_2(s)$$

At t = 0 aniline will only be present in compartment 1 so

$$f_1(0) = q_1(0) \qquad \text{and} \qquad f_2(0) = 0$$

Substitute to give

$$s.F_1(s) - q_1(0) = K_o F_2(s) - K_i F_1(s) - K_e F_1(s)$$

$$s.F_2(s) = K_i F_1(s) - K_o F_2(s)$$

Rearrange

$$(s + K_i + K_e).F_1(s) - K_o F_2(s) = q_1(0)$$

$$- K_i F_1(s) + (s + K_o).F_2(s) = 0$$

Solution of these equations gives

$$F_1(s) = \frac{q_1(0).(s + K_o)}{(s + K_i + K_e)(s + K_o) - K_i K_o}$$

and

$$F_2(s) = \frac{q_1(0).K_i}{(s + K_i + K_e)(s + K_o) - K_i K_o}$$

These equations can be resolved into partial fractions which gives the result

$$F_1(s) = \frac{q_1(0).(\lambda_1 - K_o)}{(\lambda_1 - \lambda_2)(s + \lambda_1)} + \frac{q_1(0).(K_o - \lambda_2)}{(\lambda_1 - \lambda_2)(s + \lambda_2)}$$

$$F_2(s) = \frac{q_1(0)K_i}{(\lambda_1 - \lambda_2)(s + \lambda_1)} - \frac{q_1(0)K_i}{(\lambda_1 - \lambda_2)(s + \lambda_2)}$$

where

$$\lambda_1 = \frac{-(K_o + K_i + K_e) - \sqrt{(K_o + K_i + K_e)^2 + 4K_e K_o}}{2}$$

$$\lambda_2 = \frac{-(K_o + K_i + K_e) + \sqrt{(K_o + K_i + K_e)^2 + 4K_e K_o}}{2}$$

Now performing the inverse Laplace transformation

$$q_1 = \frac{q_1(0)}{(\lambda_1 - \lambda_2)} \cdot \{(\lambda_1 - K_o)e^{-\lambda_1 t} + (K_o - \lambda_2)e^{-\lambda_2 t}\}$$

$$q_2 = \frac{q_1(0)K_i}{(\lambda_1 - \lambda_2)} \cdot \{e^{-\lambda_1 t} - e^{-\lambda_2 t}\}$$

These may be reduced to the form

$$q_1 = X_1 e^{-\lambda_1 t} + X_2 e^{-\lambda_2 t} \qquad (2)$$

$$q_2 = X_3 e^{-\lambda_1 t} - X_3 e^{-\lambda_2 t} \qquad (3)$$

where

$$X_1 = \frac{q_1(0)(\lambda_1 - K_o)}{(\lambda_1 - \lambda_2)} \qquad (4)$$

$$X_2 = \frac{q_1(0)(K_o - \lambda_2)}{(\lambda_1 - \lambda_2)} \qquad (5)$$

$$X_3 = \frac{q_1(0)K_i}{(\lambda_1 - \lambda_2)} \qquad (6)$$

In addition the equations for λ_1 and λ_2 can be reduced so that

$$\lambda_1\lambda_2 = K_e K_o \qquad (7)$$

$$\text{and} \quad \lambda_1 + \lambda_2 = K_i + K_o + K_e \qquad (8)$$

I.4 Experimental results

$$q_1 = 50e^{-0.0343t} + 100e^{-0.0067t}$$

So that from (2)

$$X_1 = 50 \text{ µmol}$$

$$X_2 = 100 \text{ µmol}$$

$$\lambda_1 = 0.0343 \text{ min}^{-1}$$

$$\lambda_2 = 0.0067 \text{ min}^{-1}$$

$$\text{and} \quad q(0) = 150 \text{ µmol}$$

From (4) $$\frac{150 \times (0.0343 - K_o)}{(0.0343 - 0.0067)} = 50$$

Hence $K_o = 0.0251 \text{ min}^{-1}$

From (7) $0.0343 \times 0.0067 = K_e \times 0.0251$

So $K_e = 0.0091 \text{ min}^{-1}$

From (8) $0.0343 + 0.0067 = 0.0251 + 0.0091 + K_i$

So $K_i = 0.0068 \text{ min}^{-1}$

By substituting these values for the rate constants back into the equations for λ_1 and λ_2 it is possible to obtain the predicted values of these constants.

Using equations (4), (5) and (6) to obtain predicted values of X_1, X_2 and X_3 it is possible to solve equations (2) and (3) to obtain a predicted curve for the amount of aniline in compartment 1 and compartment 2 at different times.

If the formation of acid-labile aniline conjugate, C is assumed to be first order the rate of formation is given by the expression

$$\frac{dq_C}{dt} = K_C q_1$$

q_C is known experimentally and q_1 can be obtained from the solution already described. Solving this expression by the type of analysis above yields

$$K_C = 0.0042 \text{ min}^{-1}$$

If it is initially assumed that K_e represents all the metabolic loss of aniline from the perfusion medium (compartment 1) then $K_e - K_C$ = other losses apart from the formation of acid-labile conjugate of anilne.

An estimate of the remaining rate constants for aniline loss by processes such as bile formation can be obtained from the general expression

$$\frac{\text{Amount of aniline lost by process a (A)}}{\text{Amount of aniline lost by process b (B)}} = \frac{K_a}{K_b}$$

A, B etc. are obtained from the values in the histogram shown in Fig. 14.

The results are

$K_B = 0.0010\ min^{-1}$ Aniline lost in bile

$K_L = 0.0013\ min^{-1}$ Aniline lost by transexudation and sampling errors

$K_M = 0.0008\ min^{-1}$ Aniline lost by metabolism to unidentified metabolites

$K_P = K_e - K_C - (K_B + K_L + K_M)$

Hence $K_P = 0.0018\ min^{-1}$ Aniline lost by metabolism to p-aminophenol

Para-aminophenol formation must be estimated by subtraction because the binding of free p-aminophenol to haemoglobin does not permit an experimental value for the total formed in 3 h to be determined

However from K_P and the amount of p-aminophenol detected in the medium it can be estimated that ∿33% of the p-aminophenol formed is bound to haemoglobin and the remaining 67% is detected as a conjugate. These two processes may be zero order since a first order reaction results in an appearance curve which begins to plateau after 3 h whereas the experimental data indicates an almost linear formation of p-aminophenol after 60 min (Fig. 14).

Since the experimental data correlates so closely with the predicted curves from the model (Fig. 39) it is apparent that the data is not inconsistant with the postulated model.

APPENDIX II

The Hepatic Circulation

II.1 Introduction

When it was realised that the effects of catecholamines on hexobarbitone metabolism in the perfused liver were possibly due to an effect of hepatic blood flow the literature was consulted to determine the known effects of catecholamines on blood flow in the liver. However it was soon found that a considerable controversy exists as to whether or not there is any regulatory mechanism of blood flow distribution within the liver, and from this it follows that there is little agreement on what effects catecholamines have on blood flow in the liver. This appendix has been designed to review the information available to date on this subject. A short review on the anatomy of the hepatic circulation is included, against which background the effects of sympathomimetics is discussed.

II.2 The anatomy of the hepatic circulation

The liver receives two supplies of blood, one from the portal vein and one from the hepatic artery. The former normally supplies 80% of the blood to the liver (Fischer, 1963). The portal venous tree branches considerably (Elias and Petty, 1952) and these branches can give off side branches of all the higher orders down to terminal vessels, along their entire path through the liver, unlike other veins which terminate in two or three branches of the next higher order (Elias and Petty, 1952; Elias and Gershbein, 1954; Knisely, Harding and Debacker, 1957). These offshoots of the portal vein finally supply blood to the sinusoid bed, which is also extremely complex, consisting of a series of interconnected blood spaces

(Elias, 1949, 1953). The sinusoids of one lobe of the mammalian liver are in direct communication with all other similar vessels of that lobe (Hyrtl, 1873).

Much controversy still exists as to the detailed anatomy of the hepatic arterial supply to the liver (Brauer, 1963; Greenway and Stark, 1971) and the role of the hepatic artery is still in question. Friedman, Milrod, Frank and Fine (1953) ligated the hepatic artery of a dog without any apparent adverse effects. However, more recently, the possible role of the hepatic artery in providing a functional reserve of oxygen has been put forward (Nakata and Kinosita, 1963; Fischer, 1963), although in small animals such as the rat this does not appear to be an important consideration and the liver of the rat has been shown to function normally in the absence of an arterial blood supply even during metabolic stimulation at which time oxygen uptake would be greatly increased (Hems et al, 1966; Powis, 1970). It has been established that the hepatic artery does not provide a significant supply of blood outside of the portal tracts (Olds and Stafford, 1930; Cameron and Mayes, 1930; Julian and De Ome, 1948; Andrews, Maegraith and Wenyon, 1949; Elias and Petty, 1953; Nakata and Kinosita, 1963). Studies on bromsulphthalein clearance (Brauer et al, 1959) Kr^{85} washout curves (Hollenberg and Dougherty, 1966) and later with tagged serum albumin and red cells (Cohn and Pinkerson, 1969) led to the conclusion that most of the arterial blood is mixed with portal venous blood before entering the sinusoids, though some direct arteriole pathways may supply unmixed arterial blood direct to the proximal end of a few sinusoids (Chenderovitch and Caroli, 1956; Greenway and Stark, 1971). In addition to admixing with portal blood and

supplying a few sinusoids directly the hepatic artery is thought to supply the capillary network round the bile duct which drains into the portal vein (Andrews et al, 1949; Elias and Petty, 1953; Elias and Sherrick, 1969).

II.3 The regulation of the intrahepatic distribution of blood flow

In his excellent review on liver circulation Brauer (1963) makes the important point that blood flow through the normal liver in vivo is not determined by intrahepatic factors, so that the liver behaves like an organ in a perfusion system, the system determining externally the rate of blood flow through the tissue, with other parameters stabilising at associated values. Circulatory regulation within such a system would depend on modifications of the blood flow distribution within the organ rather than changes in the total resistance to flow.

The existence of sphincteric mechanisms within the hepatic vasculature capable of causing a redistribution of blood flow is still in dispute (review by Greenway and Stark, 1971). In his prior review, Brauer (1963) cited considerable evidence that constriction of the portal vein prior to the major sinusoidal bed could occur as well as constriction of arterioles, selected portal and hepatic venules and possibly some parts of the sinusoids themselves. More recently Elias and Sherrick (1969) proposed that the weight of evidence suggested the presence of sphincters in the portal venous inlet branches, in the sinusoidal outlets, at the entrance of the central into sublobular vein and, in addition, at several different arteriolar

sites. It was further suggested that Kupfer cells in the walls of the sinusoids may act to regulate blood flow, a suggestion which has been repeated by Conway and Saba (1972) and Rappaport (1973).

Sphincters have been described at the ends of sinusoids by many groups (Irwin and MacDonald, 1953; Bloch, 1955; Knisely et al, 1957).

McCuskey (1966) used direct transillumination of the in vivo liver, coupled with video recording, to demonstrate the apparent presence of both pre- and post- sinusoidal sphincters and, in addition, sphincters at the junction of intrasinusoidal sinusoids. He claimed that the sphincters consisted of reticuloendothelial cells and that they may act by contraction of intracellular fibres. Reticular fibres had already been reported from electron microscopic studies of the sinusoid wall (Wasserman, 1958). In what was an extremely explicit demonstration, by a direct means, of apparent sinusoidal sphincters McCuskey (1966) attributed the failure of other workers to observe such structures in transilluminated livers to poor resolution caused by movement of the liver or low magnification (Seneviratne, 1949; Maegraith, 1958; Brauer, 1963). Although Ho and Ma (1972) have shown considerable evidence to the contrary they were unable to visualise the presence of sphincters at these sites. These authors concluded that this was due to an abolition of vasomotor tone by the anaesthetic.

There is a large pressure drop between the portal venule

and the hepatic venule (Nakata, Leong and Brauer, 1966). This they attributed to possible deviations of the sinusoids from Poiseuille assumptions, since it had been concluded that there were no sinusoidal contractile elements due to the failure to demonstrate the existence of such structures photographically both by themselves and by several other groups. However subsequent work has provided apparent photographic proof of the presence of sphincters in the hepatic sinusoids and this has led several workers to point out that, in the light of this evidence, the pressure drop across the sinusoids could be explained simply, in terms of such structures (McCuskey, 1966; Noguchi and Plaa, 1970).

It is thus apparent that at present the anatomical evidence for the existence of regulatory sphincters in the hepatic vasculature is in considerable dispute. Some groups accept that sufficient evidence exists to establish beyond doubt the presence of such a system for control of the distribution of blood flow whereas other groups deny this and cite the negative results of other papers to support their claim that no such system operates in the mammalian liver.

II.4 The effect of nerve stimulation and vasoactive compounds on the flow of blood and its distribution within the liver

Early work had indirectly shown that sympathetic nerve stimulation caused vessels of the portal tree to constrict (Bayliss and Starling, 1894; Francois-Franck and Hallion, 1897; Burton-Opitz, 1912a, b, 1914; Griffith and Emery, 1930; Eckardt, 1935). Adrenaline was reported to cause constriction of portal venules, sinusoids and efferent venules (Loeffler

and Nordmann, 1925), portal venules and sinusoids (Seneviratne, 1941) and sinusoids only (Wakim, 1944).

In the rat Daniel and Prichard (1951a, b) observed that sometimes the liver showed a restricted circulation. It was surmised that this could be due to sympathetic nerve activity. Daniel and Prichard, using X-radiography, (1951c) then showed that direct stimulation of the hepatic sympathetic nerves, or the portal venous administration of adrenaline, produced a restricted intrahepatic blood flow.

In his review, Brauer (1963) concluded that catecholamines caused active vasoconstriction of the portal venous tree thus causing a transition from a diffuse to a restricted intrahepatic circulation, so that the total number of hepatic vascular channels perfused is reduced. The total blood flow will be relatively unaffected. Andrews (1957) and Greenway and Stark (1971) have since again made the point that the administration of catecholamines to the general circulation results in either no change in the total blood flow to the liver or in an increase in flow. They demonstrated that this was the result of a reflex initiated by the aortic and carotid sinus receptors.

Direct transillumination of the rat liver revealed that catecholamines cause constriction of what were apparently sinusoidal sphincters (McCuskey, 1966). Later it was demonstrated in the in vivo transilluminated rat liver that the contractile elements of the portal venous circulation were under control of the sympathetic nervous system (Ho, 1972).

Recent work by Greenway and his colleauges (Greenway and

Lautt, 1970; Greenway and Stark, 1971; Greenway and Lautt, 1972; Greenway and Oshiro, 1972b) suggests that there is no pre-sinusoidal sphincter in the liver of the cat. Although it was concluded that catecholamines had not been shown to cause a marked intrahepatic redistribution of portal blood flow it was stressed that this had by no means been positively established. In addition it may be of some relevance that all of these studies were performed on either cats or dogs. It was also reported that in dogs histamine causes a redistribution of portal blood flow in the liver, the flow being decreased at the free ends of the lobes and redistributed to the hilar ends (Greenway and Oshiro, 1972a). This was ascribed to pressure changes at the hilar and free ends of the lobes. In a subsequent paper the same authors (Greenway and Oshiro, 1972b) concluded that sympathetic nerve stimulation did not cause a significant redistribution of blood flow in the liver of cats or dogs.

A survey of the literature reveals that catecholamines cause vasoconstriction of several elements of the portal venous system with a concomitant increase in portal venous pressure. In vivo, net blood flow to the liver is unaltered or reflexly increased. Considerable controversy exists as to whether vasoactive stimuli initiate a redistribution of portal blood flow within the liver. Evidence exists from transillumination studies that such a phenomenon does occur but in other studies, such as the use of radio-active microspheres (Greenway and Oshiro, 1972b), no such redistribution could be observed. Several groups have generalised their results from a study of one, or possibly two, species.

Whether or not species differences exist in the control of hepatic distribution of blood flow is still a matter of conjecture but if it did it would help to resolve some of the contradictions in the literature.

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